Springer Protocols

Rajib Deb · Ajay Kumar Yadav
Swaraj Rajkhowa · Yashpal Singh Malik
Editors

Protocols for the Diagnosis of Pig Viral Diseases

Humana Press

SPRINGER PROTOCOLS HANDBOOKS

For further volumes:
http://www.springer.com/series/8623

Springer Protocols Handbooks collects a diverse range of step-by-step laboratory methods and protocols from across the life and biomedical sciences. Each protocol is provided in the Springer Protocol format: readily-reproducible in a step-by-step fashion. Each protocol opens with an introductory overview, a list of the materials and reagents needed to complete the experiment, and is followed by a detailed procedure supported by a helpful notes section offering tips and tricks of the trade as well as troubleshooting advice. With a focus on large comprehensive protocol collections and an international authorship, Springer Protocols Handbooks are a valuable addition to the laboratory.

Protocols for the Diagnosis of Pig Viral Diseases

Edited by

Rajib Deb, Ajay Kumar Yadav and Swaraj Rajkhowa

National Research Centre on Pig, Indian Council of Agricultural Research, Guwahati, Assam, India

Yashpal Singh Malik

College of Animal Biotechnology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India

Humana Press

Editors
Rajib Deb
National Research Centre on Pig
Indian Council of Agricultural Research
Guwahati, Assam, India

Ajay Kumar Yadav
National Research Centre on Pig
Indian Council of Agricultural Research
Guwahati, Assam, India

Swaraj Rajkhowa
National Research Centre on Pig
Indian Council of Agricultural Research
Guwahati, Assam, India

Yashpal Singh Malik
College of Animal Biotechnology
Guru Angad Dev Veterinary and Animal Sciences University
Ludhiana, Punjab, India

ISSN 1949-2448 ISSN 1949-2456 (electronic)
Springer Protocols Handbooks
ISBN 978-1-0716-2042-7 ISBN 978-1-0716-2043-4 (eBook)
https://doi.org/10.1007/978-1-0716-2043-4

This Humana imprint is published by the registered company Springer Science+Business Media, LLC, part of Springer Nature.
The registered company address is: 1 New York Plaza, New York, NY 10004, U.S.A.

Preface

Pigs are an adaptable and rapidly growing species that may be attractive for small farms and beginning farmers seeking to incorporate livestock into their farm. The pig is very much a vulnerable species from the disease point of view. The species is not so hardy in different environmental conditions also. There are several diseases of pigs, which affect them in a very acute manner like African Swine Fever (ASF) or Porcine Reproductive and Respiratory Syndrome (PRRS). Pigs act as an amplifier host for several zoonotic diseases like Swine Influenza, Japanese Encephalitis, West Nile Virus, Nipah Virus, and Foot and Mouth Disease Virus. After the introduction of African Swine Fever in Asian countries, the sector became very vulnerable and at very high risk to survive in future. One of the contributing factors in favor of the sustainability of the piggery sector was prompt diagnosis of diseases and application of preventive measures. There are many advanced diagnostic assays that have been evolved in recent years. These assays include improved methods of nucleic acid extraction, polymerase chain reaction (PCR), droplet digital PCR (ddPCR), polymerase spiral reaction (PSR), cross-priming amplification (CPA), enzyme-linked immunosorbent assay (ELISA), as well as peptide nucleic acid (PNA) based tools, aptamer-based tools, and lateral flow assays and different immune assay-based diagnostics for porcine diseases.

The book is a compilation of 26 chapters written by renowned national and international veterinary academicians (researchers/young investigators). This book covers new molecular biological techniques for the detection of both antigens and antibodies of porcine diseases. Additionally, throughout the book, tables and figures portray the important diagnostic tools and recommendations, with specific references at the end for readers who want to obtain further details on each topic. This book aims to build capacity for researchers who can take advantages from the protocols mentioned in the book. The knowledge provided in this book will help in developing new technologies for the diagnosis of pig pathogens.

We believe that owing to the in-depth knowledge on important diagnostics tools, the present book will be an excellent source of information for scientists, researchers, and students from different fields.

We, the editors, would like to express our gratitude to all the authors who drafted the chapters in a very critical way and presented them in a simple form. The editors are appreciative of Springer Nature for accepting this book proposal, and we extend our special thanks to David C. Casey, Senior Editor, Springer Protocols, for providing all the editorial help and high cooperation while processing the manuscripts for its successful publishing.

Guwahati, Assam, India — *Rajib Deb*
Guwahati, Assam, India — *Ajay Kumar Yadav*
Guwahati, Assam, India — *Swaraj Rajkhowa*
Ludhiana, Punjab, India — *Yashpal Singh Malik*

Contents

About the Editors

Rajib Deb, PhD is currently employed as Scientist at the Indian Council of Agricultural Research-National Research Centre on Pig, Rani, Guwahati, Assam, India. He has served more than 9 years at Agricultural Research Service, India. He completed his postgraduation and PhD at Indian Veterinary Research Institute, Uttar Pradesh, India, in the subject of Animal Biotechnology. He is currently pursuing the National Post-Doctoral Fellowship program at National Dairy Research Institute, Karnal, Haryana, India. He has published more than 35 research papers in peer-reviewed journals. He is the recipient of several national and international awards including TWAS Fellowship, Italy; Associate of National Academy of Agricultural Sciences, India; a member of the National Academy of Sciences, India; and a member of Indian National Young Academy of Sciences, India. He has filed nine Indian patents and developed several technologies. He is currently working on the development of diagnostics and vaccines against porcine disease.

Ajay Kumar Yadav, PhD is a scientist in veterinary virology at the Indian Council of Agricultural Research-National Research Centre on Pig, Rani, Guwahati, Assam, India. He has completed graduation from Govind Ballabh Pant University of Agriculture and Technology, Uttarakhand, India. Later on, he completed his MVSc and PhD in veterinary virology from the premier veterinary institute named Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh, India. Dr. Yadav joined the Agricultural Research Services (ARS) in the discipline of veterinary microbiology in the year 2018. His research interests include disease diagnosis, cell culture, and virus isolation. He is a member of several societies and has also published more than 20 research articles in peer-reviewed international and national journals. He has more than 60 GenBank submissions.

Swaraj Rajkhowa, PhD joined Agricultural Research Service (ARS) as Scientist in the year 1999. He completed his PhD from Assam Agricultural University in the year 2005. He did his postdoctoral studies at internationally reputed institutes like Cornell University, New York, NY, USA, and the University of California, Irvine, CA, USA, where he learned advanced molecular tools like MLST, SLST, SNP, and DNA Microarray technology. His area of interest is molecular epidemiology and molecular diagnosis of infectious diseases of animal origin, including diseases of public health significance. He has been actively involved in the diagnosis and management of livestock diseases over the last 20 years and particularly on porcine diseases for the last 12 years. He has published more than 100 research papers in peer-reviewed national and international journals of repute, acted as a reviewer of several national and international journals, handled more than ten externally funded projects as Principal Investigator and several institute-funded projects, guided master's and doctoral degree students (including postdoctoral scholar), and acted as an external examiner of both MVSc and PhD students of SAUs.

Yashpal Singh Malik, PhD serves as Dean, College of Animal Biotechnology at Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, Punjab, India, and previously served as ICAR-National Fellow at Indian Veterinary Research Institute, Izatnagar, India. He works in viral disease epidemiology, virus-host interactions, microbial biodiversity, characterization, and diagnosis of pathogens. Prof.

Malik has acquired advanced training in Molecular Virology from the University of Minnesota, Minneapolis, MN, USA; Division of Virology, University of Ottawa, Ottawa, ON, Canada; and Wuhan Institute of Virology, Wuhan, China. He is a recipient of several prestigious national, state, and academy awards and honors, including the ICAR-Jawaharlal Nehru Award. He has supervised 5 PhD and 17 MVSc students. He has authored 5 books, 25 book chapters, and published 217 scientific research and review articles. Dr. Malik has been the Editor-in-Chief of the *Journal of Immunology and Immunopathology*. He has also edited special issues of the Springer Nature journal *VirusDisease* and Bentham's journal *Current Drug Metabolism* on emerging thematic areas.

Contributors

R. K. Avasthe • *ICAR Research Complex for NEH Region, Sikkim Centre, Gangtok, Sikkim, India*

Luit Barkalita • *Department of Animal Biotechnology, Faculty of Veterinary Science, Assam Agricultural University, Guwahati, Assam, India*

Himalaya Bhardwaj • *College of Animal Biotechnology, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, Punjab, India*

Rohini Bhat • *Department of Biotechnology, University of Agricultural Science, GKVK, Bengaluru, Karnataka, India*

Mukesh Bhatt • *ICAR-Indian Veterinary Research Institute (IVRI), Bareilly, Uttar Pradesh, India; Division of Biological Products, ICAR-IVRI, Bareilly, Uttar Pradesh, India; ICAR-National Organic Farming Research Institute, Tadong, Sikkim, India; ICAR Research Complex for NEH Region, Sikkim Centre, Gangtok, Sikkim, India*

Deepika Bisht • *Division of Virology, ICAR-Indian Veterinary Research Institute, Mukteshwar, Uttarakhand, India*

Vishal Chander • *ICAR-IVRI, Bareilly, Uttar Pradesh, India*

Manjisha Choudhury • *ICAR-National Research Centre on Pig, Guwahti, Assam, India*

Chayanika Das • *Centre for Animal Disease Research and Diagnosis (CADRAD), ICAR-Indian Veterinary Research Institute, Bareilly, Uttar Pradesh, India*

Ujjwal Kumar De • *Referral Veterinary Polyclinic & Teaching Veterinary Clinical Complex (RVP-TVCC), ICAR-Indian Veterinary Research Institute, Bareilly, Uttar Pradesh, India*

Rajib Deb • *ICAR-National Research Centre on Pig, Guwahati, Assam, India; Animal Biotechnology, ICAR-National Research Centre on Pig, Guwahati, Assam, India*

Sarmistha Debbarma • *ARDD, Govt. of Tripura, Agartala, Tripura, India*

Dipak Deka • *College of Veterinary Science, Assam Agricultural University (AAU), Guwahati, Assam, India*

Pankaj Kumar Dhaka • *Centre for One Health, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, Punjab, India*

Monuj Kumar Doley • *Krishi Vigyan Kendra, Karbi Anglong, Assam Agricultural University, Diphu, Assam, India*

Zunjar Dubbal • *Division of Veterinary Public Health & Epidemiology, ICAR-Indian Veterinary Research Institute, Bareilly, Uttar Pradesh, India*

Gorachand Dutta • *School of Medical Sciences and Technology, Indian Institute of Technology, Kharagpur, West Bengal, India*

Nirmita Dutta • *School of Medical Sciences and Technology, Indian Institute of Technology, Kharagpur, West Bengal, India*

Chris Einstein • *Division of Biological Products, ICAR-IVRI, Izatnagar, India*

Arfa Fayaz • *Division of Biological Products, ICAR-IVRI, Bareilly, Uttar Pradesh, India*

Siddharth Gautam • *Division of Temperate Animal Husbandry, ICAR-Indian Veterinary Research Institute, Mukteshwar, Uttarakhand, India*

Vikas Gupta • *CCS-National Institute of Animal Health (NIAH), Baghpat, Uttar Pradesh, India; National Institute of Animal Health, Baghpat, Uttar Pradesh, India*

Vivek Kumar Gupta • *ICAR-National Research Centre on Pig, Guwahati, Assam, India*

Ankita Gurao • *College of Animal Biotechnology, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, Punjab, India*

Ian M. Jones • *School of Biological Sciences, University of Reading, Reading, UK*

Vinay G. Joshi • *Department of Animal Biotechnology, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS), Hisar, Haryana, India*

Ramya Kalaivanan • *Faculty of Veterinary Microbiology, Veterinary College and Research Institute, Tamil Nadu Veterinary and Animal Sciences University, Namakkal, Tamil Nadu, India; Faculty of Veterinary Pharmacology and Toxicology, Veterinary College and Research Institute, Tamil Nadu Veterinary and Animal Sciences University, Namakkal, Tamil Nadu, India*

Monu Karki • *Division of Biological Products, ICAR-IVRI, Bareilly, Uttar Pradesh, India*

Geetika Kaur • *College of Animal Biotechnology, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, Punjab, India*

Victoria C. Khangembam • *ICAR-Directorate of Coldwater Fisheries Research, Bhimtal, Uttarakhand, India*

Nitish Singh Kharayat • *Division of Temperate Animal Husbandry, ICAR-Indian Veterinary Research Institute, Mukteshwar, Uttarakhand, India*

Kiran • *Division of Biological Products, ICAR-IVRI, Bareilly, Uttar Pradesh, India; ICAR-IVRI, Bareilly, Uttar Pradesh, India*

Akhil Kumar • *Department of Veterinary Microbiology and Immunology, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana, India*

Ashok Kumar • *ICAR-Indian Veterinary Research Institute (IVRI), Bareilly, Uttar Pradesh, India; Division of Biological Products, ICAR-IVRI, Bareilly, Uttar Pradesh, India*

B. T. Naveen Kumar • *College of Fisheries, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, Punjab, India*

B. V. Sunil Kumar • *College of Animal Biotechnology, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, Punjab, India*

Naveen Kumar • *ICAR-National Institute of High Security Animal Diseases, OIE Reference Laboratory for Avian Influenza, Bhopal, Madhya Pradesh, India*

Obli Rajendran Vinodh Kumar • *Division of Epidemiology, ICAR-Indian Veterinary Research Institute, Bareilly, Uttar Pradesh, India*

Rohit Kumar • *Department of Biological Sciences, Sam Higginbottom Institute of Agriculture Technology & Sciences, Allahabad, Uttar Pradesh, India*

Sachin Kumar • *Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, Assam, India*

Satish Kumar • *Retired Principal Scientist, Central Instrumentation Facility, Department of Veterinary Biotechnology, Indian Veterinary Research Institute, IVRI, Izatnagar, Bareilly, Uttar Pradesh, India*

Tarun Kumar • *Veterinary Clinical Complex, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS), Hisar, Haryana, India*

Anu Kumari • *Department of Animal Biotechnology, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS), Hisar, Haryana, India*

Sinéad Lyons • *School of Biological Sciences, University of Reading, Reading, UK*

Sushila Maan • *Department of Animal Biotechnology, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS), Hisar, Haryana, India*

Hemanta Kumar Maity • *Department of Avian Science, West Bengal University of Animal and Fishery Sciences, Kolkata, India*

Yashpal Singh Malik • *College of Veterinary Science, Assam Agricultural University (AAU), Guwahati, Assam, India; College of Animal Biotechnology, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, Punjab, India*

Prabhakar Maurya • *Consultancy for Environmental, Human Toxicology and Risk Assessment (India Office), New Delhi, India*

Nihar Nalini Mohanty • *CCS-National Institute of Animal Health (NIAH), Baghpat, Uttar Pradesh, India*

Soumen Naskar • *ICAR-Indian Institute of Agricultural Biotechnology, Ranchi, Jharkhand, India*

Sankar Palanisamy • *Faculty of Veterinary Microbiology, Veterinary College and Research Institute, Tamil Nadu Veterinary and Animal Sciences University, Namakkal, Tamil Nadu, India; Faculty of Veterinary Pharmacology and Toxicology, Veterinary College and Research Institute, Tamil Nadu Veterinary and Animal Sciences University, Namakkal, Tamil Nadu, India*

Souvik Paul • *ICAR-National Research Centre on Pig, Guwahati, Assam, India*

Seema Rani Pegu • *ICAR-National Research Centre on Pig, Guwahati, Assam, India*

Vishal Rai • *ICAR-Indian Veterinary Research Institute (IVRI), Bareilly, Uttar Pradesh, India; Division of Biological Products, ICAR-IVRI, Bareilly, Uttar Pradesh, India*

A. Raja • *Education Cell, Veterinary College and Research Institute, Tamil Nadu Veterinary and Animal Sciences University, Namakkal, Tamil Nadu, India*

Kaushal Kishor Rajak • *ICAR-Indian Veterinary Research Institute (IVRI), Bareilly, Uttar Pradesh, India; Division of Biological Products, ICAR-IVRI, Bareilly, Uttar Pradesh, India*

Swaraj Rajkhowa • *ICAR-National Research Centre on Pig, Guwahti, Assam, India*

Pramod W. Ramteke • *Department of Biological Sciences, Sam Higginbottom Institute of Agriculture Technology & Sciences, Allahabad, Uttar Pradesh, India*

Aditya Prasad Sahoo • *ICAR-Directorate of Foot and Mouth Disease, Nainital, Uttrakhand, India*

Laxmi Narayan Sarangi • *National Dairy Development Board, Research and Development Laboratory, Hyderabad, Telangana, India*

Shikha Saxena • *Division of Animal Genetics, ICAR-Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, India*

Gyanendra Singh Sengar • *ICAR-National Research Centre on Pig, Guwahati, Assam, India*

R. S. Sethi • *College of Animal Biotechnology, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, Punjab, India*

Anuradha Sharma • *College of Animal Biotechnology, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, Punjab, India*

Sathish B. Shivachandra • *ICAR-National Institute of Veterinary Epidemiology and Disease Informatics (ICAR-NIVEDI), Bengaluru, Karnataka, India*

Ajay Kumar Singh • *National Institute of Animal Biotechnology (NIAB), Hyderabad, Telangana, India*

Niraj Kumar Singh • *College of Animal Biotechnology, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, Punjab, India*
R. K. Singh • *ICAR-Indian Veterinary Research Institute (IVRI), Bareilly, Uttar Pradesh, India*
R. P. Singh • *ICAR-Indian Veterinary Research Institute (IVRI), Bareilly, Uttar Pradesh, India; Division of Biological Products, ICAR-IVRI, Izatnagar, India*
Vinod Kumar Singh • *Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, DUVASU, Mathura, Uttar Pradesh, India*
Shubhankar Sircar • *Amity Institute of Virology and Immunology, Amity University, Noida, Uttar Pradesh, India*
Jupi Talukdar • *Mahisah Perspicientia LLP, Guwahati, Assam, India*
Dimpal Thakuria • *ICAR-Directorate of Coldwater Fisheries Research, Bhimtal, Uttarakhand, India*
Yoya Vashi • *Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, Assam, India*
Ajay Kumar Yadav • *ICAR-National Research Centre on Pig, Guwahati, Assam, India; Division of Biological Products, ICAR-IVRI, Bareilly, Uttar Pradesh, India; ICAR-IVRI, Bareilly, Uttar Pradesh, India*

Chapter 1

Requirements and Preparedness for Attending a Viral Disease Outbreak in Pig Farms

Dipak Deka, Pankaj Kumar Dhaka, Ujjwal Kumar De, Obli Rajendran Vinodh Kumar, and Yashpal Singh Malik

Abstract

Animal husbandry and livestock rearing are important for rural livelihood and economic development of a country. The exponential growth of human population demands quality proteins for better health status. Meat is one of the important sources of high quality proteins, and faster growth and higher feed conversion efficiency make pig a better animal to bridge the gap of protein demand and population growth. Keeping in view swine farming is rapidly expanding, both in socio-economically weaker sections of the society for income source and also emerged as industry for its huge commercial potential. Good husbandry practices and hygiene are the main factors of animal health and to provide economic benefits through maximized production. Disease in animals causes negative economic impact in animal production system. Intensive system of animal rearing poses comparatively greater risk of disease problem than extensive system of rearing. Pig is a highly prolific animal with greater economic return; however, disease outbreaks are major point of concern on socio-economical perspective. In recent past swine industry has witnessed significant numbers of viral disease outbreaks. Proper biosecurity at swine farm level may help in the prevention of introduction of infectious agents into farm. In addition to proper biosecurity, vaccination of the pigs with suitable vaccine will keep the infectious diseases at bay. An outbreak investigation requires knowledge on methods of descriptive and analytical epidemiology. Proper investigation of disease outbreaks is paramount importance to identify the cause, mode of spread of the disease, and other factors involved in spreading the outbreak and to control and prevent the spread of devastating diseases. Efficient outbreak investigation involves proper planning and strategy to answer these important questions. In this chapter we narrated basics of requirement and preparedness for attending a viral disease outbreak in pig farms.

Key words Epidemic, Biosecurity, Epidemiology, Economics, Health

1 Introduction

Animal husbandry and livestock sectors are very critical for rural livelihood and economic development of a country. Keeping in view the rapid expansion of swine farming, presently pig farming finds not only an important place in socio-economically weaker sections of the society for income source but it has been also

Rajib Deb et al. (eds.), *Protocols for the Diagnosis of Pig Viral Diseases*,
Springer Protocols Handbooks, https://doi.org/10.1007/978-1-0716-2043-4_1,

emerged as industry for its huge commercial potential. Globally, the pigs contribute around 2.01% of the total livestock population. A recent survey reports of January 2020 revealed that there were about 677.6 million pigs worldwide, from which 310.41 million in China, 148.2 million in European union, 78.66 million in the USA, 37.85 million in Brazil, 13.88 million in Canada, 11.28 million in South Korea, 11.05 million in Mexico, and 9.06 million in Japan has been reported [1]. Among the livestock species, pig has immense potential to contribute for faster economic return because of certain inherent traits like better-feed conversion efficiency, high fecundity, early maturity, and short generation interval. It has enormous role to ensure nutritional and economic security of the society as it requires small investment on buildings and farm equipment. Furthermore, pork consumption being popular among select populations, the improved pig husbandry programs have found to be significantly contributed in the poverty alleviation strategies in many parts of the world. Keeping in view the huge commercial potential of swine farming, now a days, commercial pig farming has come up as great business idea, startups and income source for the people [2].

A sow can be bred as early as 8–9 months of age and can farrow twice in a year under optimal management conditions. They can produce 6–12 piglets in each farrowing [3]. It has immense potential to ensure nutritional and economic security for the weaker sections of the society. In spite of enormous economic potential and improved management practices, pig farmers regularly face huge economic losses due to various infectious diseases which lead to huge mortality of piglets and reproductive failure among sows [4]. The mortality of an adult pig or its litter influences the economic viability and overall profitability of the farm. Piglet mortality in neonatal age has emerged as major hurdle to make pig production as profitable enterprise. In India, piglet mortality was observed up to 25% in preweaning stage because of infectious diseases [5]. The important diseases related with pig husbandry have been listed in Table 1.

Among the various infectious causes, major viral disease outbreaks occur continuously and pose a major point of concern for its socio-economically devastating effect which causes huge economic loss to the pig industry. With the globalization of economies and abolition of trade barriers, the increase in national and international trade of animal and animal products pose multifaceted challenges to tackle the viral disease outbreaks in pig industry. In last decades, various countries across the globe experienced several outbreaks of porcine viral diseases (Table 2). Most recently in 2020, the outbreak of African swine fever has been recorded in India. In developing countries including India, unavailability of major vaccines except a few is also another reason of frequent outbreak of viral diseases, resulting in mortality of pigs.

Table 1
Important viral diseases of swine [6, 7]

S. No.	Name of the disease	Causative agent
1.	Classical swine fever	Classical swine fever virus, Pestivirus (family: *Togaviridae*)
2.	Foot-and-mouth disease	Foot-and-mouth disease virus (FMDV)-Aphthovirus; family: *Picornaviridae*
3.	PCV2-systemic disease (PCV2-SD) and PCV2-reproductive disease (PCV2-RD)	Porcine circovirus-2 (*family: Circoviridae*)
4.	Porcine reproductive and respiratory syndrome (PRRS)	Porcine reproductive and respiratory syndrome virus (PRRSV)-genus arterivirus
5.	Porcine epidemic diarrhea	Porcine epidemic diarrhea virus (PEDV)-coronavirus (family: *Coronaviridae*)
6.	African swine fever (ASF)	African swine fever virus (ASFV) Family: *Asfarviridae*
7.	Pseudorabies (Aujeszky's disease)	Suid herpesvirus 1; family: Herpesviridae
8.	Stillbirth, mummification, embryonic death, and infertility (SMEDI)	Porcine enteroviruses (*family: Picornaviridae*) and Porcine parvoviruses (family *Parvoviridae*)
9.	Blue eye disease	Porcine rubulavirus (family: *Paramyxoviridae*)
10.	Japanese B encephalitis	Japanese encephalitis virus-Flavivirus (*family: Flaviviridae*)
11.	Vesicular exanthema of swine	Vesicular exanthema of swine virus-Vesivirus (family: *Caliciviridae*)
12.	Nipah	Nipah virus- Henipavirus (family: *Paramyxoviridae*)
13.	Encephalomyocarditis	Encephalomyocarditis virus (EMCV) (Cardiovirus, *family: Picornaviridae*)
14.	Bovine viral diarrhea infection	Bovine viral diarrhea virus (Pestivirus) [8]

Some of these viral diseases can be effectively controlled while others become endemic and cause sporadic outbreaks in affected countries. The highly infectious viral diseases such as African swine fever, classical swine fever, foot-and-mouth disease, and reproductive failure due to Porcine Parvovirus, PRRS, Porcine circovirus, etc. can cause severe economic losses in pig production leading to shortage of supply and eventually affect global pork prices with increasing demand. To prevent and control the further spread of viral diseases during outbreak in effective manner, veterinary professionals often are engaged and play a key role in disease investigation. The important factors related with the disease outbreak (s) in pig farming are discussed in the following [31–33].

Table 2
Notable outbreaks of swine viral disease in Asian and other countries [7]

S. No.	Name of the disease	Country
1.	Classical swine fever	China [9]
2.	Foot-and-mouth disease	Taiwan (1997) ([10]; [11])
3.	PCV2-systemic disease (PCV2-SD) and PCV2-reproductive disease (PCV2-RD)	Kansas, North Carolina, and Iowa (2005); Canada (2004) ([12]; [13])
4.	Porcine reproductive and respiratory syndrome (PRRS)	China (2006, 2009–2010); Vietnam (2007); Philippines (2008); Laos (2010); Thailand (2010); Cambodia (2010); Myanmar (2011) ([14]; [15]; [16]; [7])
5.	Porcine epidemic diarrhea	Thailand (2007), Vietnam (2009), China (2010) and South Korea (2013) ([17]; [18]; [19])
6.	African swine fever (ASF)	China (2018), Mangolia (2019), Vietnam (2019), Cambodia (2019), Hong Kong (2019), North Korea (2019), Laos (2019), Myanmar (2019), Philippines (2019), South Korea (2019), Timor-Leste (2019), Indonesia (2019) and India (2020) ([20]; [7])
7.	Pseudorabies (Aujeszky's disease)	Italy (2019) [21]
8.	Blue eye disease	USA (2001) [22]
9.	Japanese B encephalitis	Australia (1998); China (2018) ([23]; [24])
10.	Vesicular exanthema of swine	China (2015) [25]
11.	Nipah	Malaysia (1998 to 1999) [26]
12.	Encephalomyocarditis	New South Wales (1970); [27]/Belgium (2007, 2016) ([28]; [29])
13.	Bovine viral diarrhea infection	England (1992), [30]

1. *Level of biosecurity practices*: Biosecurity is the implementation of measures that reduce the risk of the introduction and spread of disease agents; it requires the adoption of a set of attitudes and behaviors by people to reduce risk in all activities involving domestic, captive/exotic, and wild animals and their products ([34]). The biosecurity is implemented in two tier systems: national level and farm level. Development of strategies to prevent the spread of disease outbreaks, control or eradication of endemic diseases, and transboundary disease transmission are the key factors to contain the disease by biosecurity at national level. The valuable factors to strengthen the biosecurity at swine farm level are location of pig farm, size and design of farm, introduction of new pigs, replacement stock, pig buyers, entry of visitors, fencing, working personnel, quarantine method, vaccination, and management of farm. Small

(back-yard) to medium farms with poor biosecurity practices remain among the key factors involved in outbreaks. Most of the smallholders tend to feed the pigs with improperly treated/processed leftover food or food waste (swill feed) from various sources such as households, markets, factories, restaurants, which could contain infectious agents and contaminate the farm. Moreover, in many places the back-yard and big farms are frequently found in close proximity to each other which could be the major factor for facilitating the faster spread of infectious diseases under such scenarios. However, segregation, cleaning, and disinfection will be the key measures for effective biosecurity at farm level.

2. *Availability of vaccines*: Vaccinations are important tools for the control of viral disease in pigs like other animals. Few vaccines are available for major viral diseases of pigs such as FMD and CSF, but not ASF. Vaccination reduces the pressure of pathogens, shedding, and disease pressure in the region. However, the recommended vaccines must have been tested for efficiency with the existing standards for prophylactic measures against viral diseases (OIE) [34].
3. *Globalization and trade*: The rapid and huge trading of pigs and pork within and between neighboring countries can lead to the spread of infectious agents. The pork supply chains contain multiple stakeholders such as pig farmers, brokers, traders, slaughterhouses, retailers, and consumers. This could facilitate rapid and long-range disease transmission within countries via the movement of infected animals and contaminated vehicles [35].
4. *Epidemiological status of disease*: The risk of introduction of new viruses or viral strains in a region due to the increasing importation of the breeding pigs should also be considered. If these imported pigs are not carefully monitored, viruses of exotic origin and other pathogens can be introduced into the countries which may further lead to disease outbreaks. These viruses might get established and even become the dominant circulating viruses causing complicated problems [36]. Moreover, considering that there are already local viruses circulating in the given region, it could then act as a focus for viral recombination, especially for RNA viruses. Recombination between novel and local strains is not unexpected when co-circulated within the same farm. In fact, these phenomena have already been shown for some viruses in Asian countries, for example, NADC30-like PRRSVs in China [37]. However, the epidemiological points of view, continuous surveillance of disease, some determinants such as contagiousness, tenacity, and case fatality rate, and their impact on persistence and transmission of viruses are also important for future outbreak of diseases [31, 32].

5. *Consumer's behaviors*: Many consumers still prefer buying pork from wet animal markets. Poor biosecurity management in small-scale slaughterhouses and wet markets is responsible for contamination of pork and its products in the area with various swine pathogens. Moreover, consumption of wild boar as traditional delicacy also contributes to spill over of pathogens to humans and domestic pigs in a direct manner or through environment contamination.
6. *Wildlife as reservoir*: In many places, especially where extensive farming is practiced, the wild boars can play a major role in disease transmission. Cross-species disease transmission between wildlife and pigs is an increasing threat to outbreaks. The threats posed by diseases in wild pigs have been recognized in all pig raising countries. Wild pigs (*Sus scrofa*), that include feral domestic pigs (*Sus scrofa domestica*), Eurasian wild boar (*Sus scrofa linnaeus*), and hybrids between the two, are the most abundant free-ranging, exotic ungulates of increasing concern as a potential source for cross-species transmission. In some parts of the world, wild pigs have been identified as an important reservoir for disease epidemic such as classical swine fever virus, African swine fever virus, and foot-and-mouth disease. These diseases, often termed transboundary animal diseases, can cause high morbidity and mortality in pig populations through outbreaks [38].
7. *Lack of awareness*: During an infectious disease outbreak, it is vital to learn as much as possible about the concerns, knowledge, attitudes, and behavior of the public. Such information can be crucial to the improvement of communication efforts by veterinary disease investigation officers. High concern may not always translate into a higher compliance with precautionary recommendations, possibly due to the low or lack level of knowledge about the disease among the public. Frequent communication between veterinarians and the stakeholders is recommended to help dispel myths about the disease and to spread better information about the role that the public can play in limiting the spread of the disease. Lack of awareness regarding the diseases in farmers and livestock traders leads to the spread of diseases; as it is known that the movement of animals through livestock traders is often the key epidemiological factor in the spread of diseases. Therefore, collaborative efforts orchestrated by the animal health department are needed and should focus on public education and training through media resources [39].

2 Infectious Disease Outbreak and Its Spread

As discussed, the globalization, intensive swine production, increased trade and travel, and changing climate have increased the risk of catastrophic animal losses due to infectious diseases. Any outbreak originating from small farms due to the use of contaminated swill feeding, poor biosecurity, and lack of awareness could be a very important starting point of infectious viral outbreaks in the naïve areas. The routes of virus transmission into these farms could be varied and include swill feeding or contaminated fomites and vehicles. For larger farms with good biosecurity management, the risk of pathogen introduction is generally low. However, the risk will be increased when the viral load contaminating environment in the region increases due to outbreaks in neighboring farms, particularly back-yard farms. Therefore, farm standardization policy to eliminate the farms with poor biosecurity as well as compartmentalization or zoning is highly suggested in the affected regions. Thereby, effective surveillance is considered as prerequisite for the certification of the disease-free status of an area or country [7, 40].

3 Epidemiological "Know-How": An Important Pillar for Development of Surveillance System and Outbreak Preparedness

To substantiate the risk of any exotic disease(s) or status of endemic disease in a region for implementation of surveillance system, the epidemiological "know-how" about the infectious diseases in context of possible risk factors including its geographical distribution, viruses and its serotypes, vector distribution, host range, reservoirs, ongoing surveillance activities, climatic change scenario, disease and/or vector control programs, vaccination status of animal, and status of targeted disease/pathogens among trading partners, is essential. The surveillance objectives should depend on the outputs needed to support decision-making and thus be policy-driven [41]. The impact and epidemiology of various viral pig diseases are widely differing in different parts of the world; therefore, regions or countries should adapt the surveillance strategies as per local requirements.

Key considerations for use of surveys and diagnostic tools in surveillance system: The sampling strategies, sample size, design prevalence, and appropriate confidence interval for surveys should be justified based on prevailing epidemiological situations. On diagnostic front, proper validation of sensitivity and specificity of the diagnostic tests for targeted species should be carried out. Moreover, for the diseases where vaccination is available, it is important to differentiate "vaccinated *versus* infected" animals by

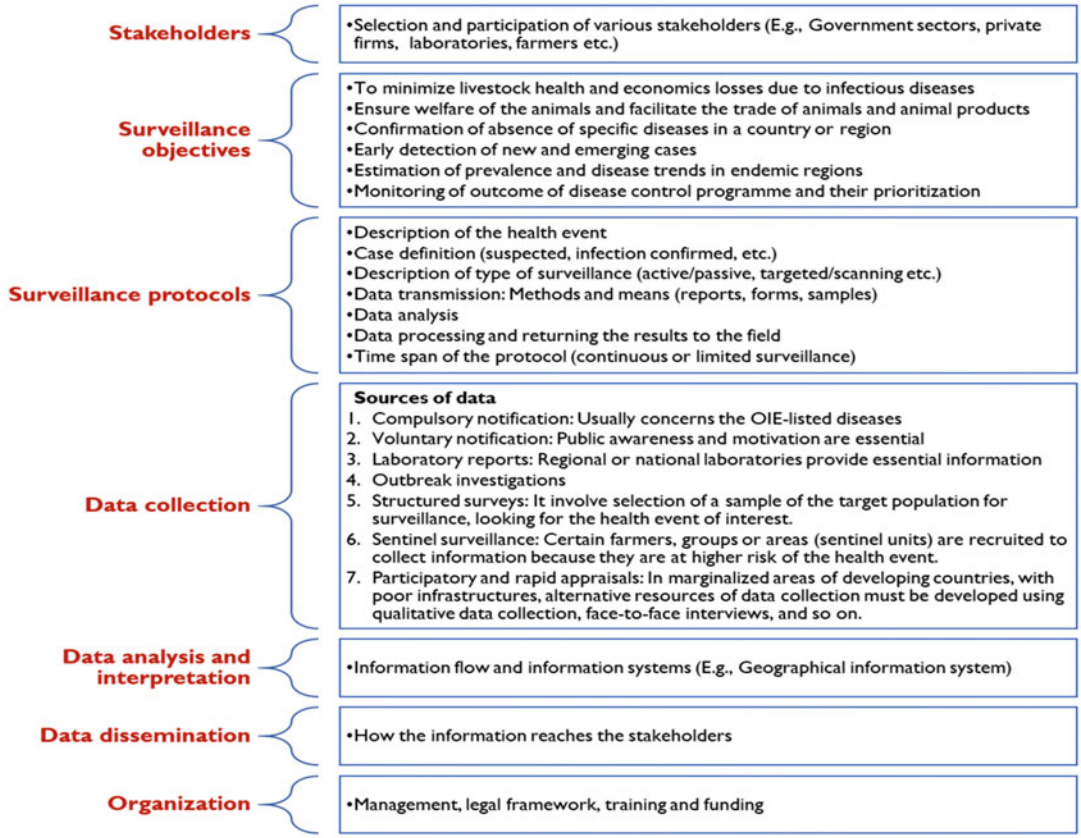


Fig. 1 An overview of the steps involved in designing of a surveillance system

diagnostic tests for unbiased interpretation of the surveillance data [42]. An overview of important steps for designing of the surveillance system has been provided in Fig. 1.

The important approaches to be planned for surveillance in order to estimate the prevalence or emergence of any infectious disease have been described in Table 3.

4 Investigation of Viral Disease Outbreak in Swine

It depends upon the clear-cut objective, availability of facilities, resources, and tolls of investigations such as specific diagnostic tests. The main objectives of viral disease outbreak investigations are: (a) to know the origin and identify the causal factors of disease and (b) mode of transmission.

4.1 Steps of Disease Outbreak Investigation and Management [43]

1. *Reporting of disease outbreak and collection of initial basic information*: The report of any suspected swine disease outbreak can be gathered from different sources or different

Table 3
Approaches for surveillance system with the objective of estimation of disease prevalence [43]

Approach	Description
Measure of disease/outbreak	Prevalence of the targeted disease/pathogen in the study population and associated reservoir and/or vector capacity
Coverage	Structured active surveillance to obtain the representative estimates about the disease/pathogen
Means of data acquisition	Mainly through active surveillance. The passive surveillance can also be used as complementary source of information, but it is often difficult to account for the possible bias
Frequency of sampling	It can be one-off (estimate prevalence) or repeated surveys (estimate changes in prevalence, e.g., to monitor the effect of control/intervention measures)
Testing method	Serological testing is preferred as it is more practical and often less costly as compared to pathogen detection
Design prevalence	According to the expected prevalence; a conservative estimate means choosing a design prevalence which tends towards 50% to maximize sample size and thus increase precision
Frequency of analysis	After each survey. The risk-based surveillance strategy may also be opted during high seasonal vector activity or in other risk associated scenario (import of infected animals, live vaccine related outbreaks, etc.)
For vaccination program	Serological and virological testing is required to characterize the circulating virus serotypes in given regions in order to ensure that all serotypes are included in vaccination program

peoples such as veterinary workers at village, pig farmers, local/public authorities, private veterinary practitioners, pork traders or others. Although reporting of disease outbreak is a very sensitive issue which can results in huge national and private-sector costs it should be encouraged by the animal health authorities otherwise it may discourage future reporting or the outbreak will be unnoticed resulting in uncontrollable disease situation. After receiving the report of disease outbreak, the basic information about the nature of the disease, location, and the extent and time frame of the outbreak should be recorded properly.

2. *Preparing to attend the field outbreak investigation*: It is important to obtain certain information such as the age group/breed of pigs involved, the time of the outbreak, the clinical signs, and the location of the outbreak prior to field visit which may help during the investigation process.. After that, a team of experts for disease investigation is to be prepared and the list of items/equipment's required for the investigation is to be decided by expert team. However, certain items such as disinfection equipment, recording accessories, post-mortem/collection of

biological and allied sample collection accessories, pig restraint equipment, paper and pens, and other necessary items which should always be included. However, this activity can effectively be executed using a standard checklist.

3. *Verification of actual problem*: To verify the outbreak, firstly, the probable cause of outbreak is to be identified. After that the extent of the outbreak is assessed and information is gathered for further investigation and adopting appropriate control measures. Accumulation of information like details of animals involved in the outbreak (species, breed, and age), place (geographical location, households, farms or villages), and time (the time of onset of disease) are very important. Apart from this, the morbidity, mortality, and case fatality rate as well as number of pig population at risk of disease are also to be recorded.
4. *Amalgamation of all the above information*: After gathering all the possible information, the disease is defined by considering the following steps.
   (a) Classical signs and symptoms of pig before death.
   (b) Recording the history and clinical examination of individual case of both affected and healthy pigs.
   (c) Collection of all types of biological/clinical samples from both healthy and affected pigs.
   (d) On-site post-mortem examination of carcasses by expert veterinary pathologists.
   (e) Collection of morbid samples during post-mortem examination.
   (f) Examination and collection of associated factors such as water, vectors, feeds, etc. to confirm their possible role in disease outbreak.
5. *Analysis*: After analysis of all the above-mentioned data, laboratory investigation and post-mortem examination of carcasses, a statistical association between the cause and possible diseases need to be established to determine the acceptance or rejection of hypothesis. In case of rejection, another possible hypothesis needs to be explored by re-examining the collected data.
6. *Preparation of report*: This section may comprise of two parts: (1) details of observations and analysis report and (2) appropriate recommendations to prevent mortality/new cases and future outbreak.
   (a) *Details of observations and analysis report*: It includes laboratory test, post-mortem examination finding, data analysis, photographs, and experimental findings, if any.

(b) *Appropriate recommendations*: The suitable recommendation after disease outbreak investigations is the utmost important to control the spread of disease quickly as possible. The key recommendation should be directed towards the following aspects:

- Immediate disinfection measures of the farm as it may vary from virus to virus (e.g., FMD virus is sensitive to pH).
- Strengthening the biosecurity measures of farm.
- Breaking/hindering of virus transmission pathway.
- Communication of the control measures to different stakeholder (local authorities, police, municipal officers, pork traders, pork-market owners, other farmers and the general public) groups who are responsible for the implementation.
- The line of treatment required for affected animals in an outbreak.
- Communication to concerned authorities (State and Central Government Animal Health Department) in case of notifiable disease outbreak.

7. *To-do-list for an effective outbreak investigation*: An outbreak of exotic animal disease would have widespread impact on animal farming, export potential, tourism, wildlife, and other sectors resulting in significant losses if such an outbreak was not dealt with in an effective and timely manner. In brief, the important facts to be observed before-, during-, and at the end of outbreak investigation are [44]:

8. *Before and early phase of an outbreak*:

(a) How vulnerable is the swine population to the disease, where will the pathogen arrive and where should surveillance be focused?

(b) Which surveillance method should be used?

(c) What is the ongoing outbreak size, and what is the present pathogen status?

(d) Where and how can interventions be introduced to eradicate the pathogen quickly?

(e) How the healthy animals can be saved from getting infection?

(f) Where and how the carcasses of the infected animals will be disposed of?

(g) Which data and resources are required to allow forecasting and control to be performed effectively?

9. *Once a major epidemic is ongoing, the following observations should be taken into account*:
   (a) How pathogen can transmit from one animal to another and by which route?
   (b) How many cases will there be reported in 24 h?
   (c) How effective are current prevention and control efforts?
   (d) Which interventions should be introduced, and how should they be adapted as the epidemic continues?
10. *Observations to be considered at the end of a major epidemic*:
   (a) Can the epidemic be declared over, or do hidden cases remain in the animal population (reservoir/carrier status)?

5 Outbreak Investigation Steps for Infectious Diseases

An outbreak investigation is a systematic procedure which is used to identify the source of cases of infection with a view to control and prevent possible future occurrence [45]. The outbreak investigation is important to ensure that appropriate preventive and control measures have been applied or not. The outbreak investigations help in the assessment of failures and successes of the intervention measures, and also in the identification of the changes in the disease agent, supporting environment or events that might be beyond the scope of a disease control program. Therefore, it is imperative to maintain the proper recording of outbreak investigations protocols. In zoonotic outbreaks, the outbreak investigation should be done in coordination with public health authorities [45].

5.1 Requirements for Outbreak Investigation

1. Laboratory Diagnostic capabilities: The rapid and accurate diagnosis can only be possible in well-equipped laboratories that have range of chemicals/reagents, experienced staff, and standardized instruments.
2. Collaboration with International laboratories and centers: There are a network of FAO and OIE reference laboratories and collaborating centers around the world which are available to provide advice and assistance to countries during disease outbreak. In India, Regional Disease Diagnostic Laboratories (RDDL) often collaborates with Central Disease Diagnostic Laboratory (CDDL) during the investigations of animal's disease outbreaks.
3. Preparation of rapid diagnostic tests (ELISA based, nucleic acid based, lateral flow assay or other) to obtain results in short time period (FAO animal production and health manual).

5.2 Descriptive Epidemiological Steps of Outbreak Investigation

Who?

- How many pigs affected from the outbreak?
- Which breed (including other parameters—age and sex, etc.) and human population [at-risk occupational and health groups (pregnancy, immunosuppression, children, elders, etc.)] are more sensitive?

When?

- Temporal relationship between the disease frequency and related events. Events that may be associated with infectious disease outbreaks are as follows.
  - Importation of animals from endemic areas.
  - Efficacy of used diagnostic and quarantine methods (if any).
  - Change in farm management and nutritional practices.
  - Seasonal changes and associated vector activity (especially ticks).

Where?

- The outbreak can be described in terms of spatial distribution to observe associated risk factor(s). The geographic information system (GIS) may be used to layout the spatial distribution parameters such as the following.
  - Climate zones and seasonal trends.
  - Geographical characteristics.
  - Vegetation index.

Why?

- Agent responsible for outbreak?
- Origin of infection?
- Wildlife reservoir?
- Mechanism of transmission?
- Risk factors that can predispose susceptible animals for infection.

The causal association between a factor and outcome can be established by using analytical and experimental studies. However, past epidemiological studies can be used to describe the outbreak characteristics.

5.3 Important Farm Related Risk Factors for Disease Outbreaks Are

In animal population, associated risk factors, such as farm size, stocking density, contact rate, production type, import of infected animals, quarantine procedures, nutritional status, infected feed, infected water, immunosuppressive disorders, and other farm management procedures, may play an important role at farm level outbreak.

Zoonoses in human population, associated risk factors, such as contact with animals, use of PPE, at-risk groups, culinary habits (e.g., uncooked meat), unhygienic habits and immunosuppressive disorders, may play an important role in outbreak.

5.4 Steps of Outbreak Investigation

The important steps of outbreak investigation along with the concerned details have been discussed in the following:

1. Establish the existence of outbreak:

   Are numbers of observed cases exceeding the expected levels?

   Example: In a farm free from any infectious disease, a single case may also be considered as an outbreak, whereas in endemic areas the number of cases above threshold level constitutes the outbreak.

2. Verify the diagnosis using clinical and laboratory diagnosis:

   During the suspected outbreak (e.g., high rate of abortions in pigs), the clinical samples (e.g., aborted material, vaginal mucus, serum samples, etc.) of the farm animals; associated human contacts and environmental samples (e.g., farm soil and sewage) can be collected for laboratory examination.

   Various laboratory examinations including conventional methods, serological diagnosis and molecular techniques can be employed for detection of the pathogens.

3. Define a case based on its standard elements (e.g., clinical information, time, place, and affected individuals) and varying degrees of certainty (with associated risk factors).
4. Identify additional cases linked by similarities to the case definition.
5. Perform descriptive epidemiology of the outbreak description of epidemic curve and spatial distribution of the disease.
6. Develop a hypothesis using descriptive epidemiology (e.g., animal herds/farms, place, and time with the clinical and laboratory findings) and test the new hypothesis using analytic epidemiology (e.g., cohort or case-control study).
7. Reconsider the hypothesis by "squaring" the hypothesis to the clinical, laboratory, and epidemiologic facts. In addition, development of a new hypothesis for re-testing may also occur.
8. Perform additional studies (if needed) by better defining the extent of the epidemic, evaluating new laboratory methods and case-finding techniques, or conducting an environmental investigation.
9. Implementation of control measures.
10. Communicate findings for community awareness.

5.5 Emergency Preparedness and Contingency Planning [46]

As per OIE guidelines, all the countries should develop emergency preparedness and contingency plans for immediate action for diseases fulfilling the provisions of Article 1.1.3.1. of the Terrestrial Code. The emergency response plans should be up-to-date tested (e.g., simulation exercise) and should be embedded in the legal framework. There must be proper chain of command and coordination with relevant support services in order to ensure the execution of rapid control efforts. A contingency plan is a set of activities, including immediate actions and longer-term measures, for responding to an animal health emergency such as disease outbreaks [46]. The contingency plan should be simple to understand and implement that involve organizing a team of relevant authorities and stakeholders with proper identification of critical resources and functions, along with the established plan for recovery. The contingency plan should be properly documented, tested, and regularly updated. The key components in a contingency plan include established chain of command, systems for rapid detection and confirmation, outbreak investigation procedures, rapid containment measures (e.g., movement control, disinfection, vaccination, culling), and communication strategy. Following the confirmation of an outbreak, the control areas/containment zones may be established. The extent of these zones depends on a number of factors, in particular, the epidemiological characteristic of the agent and disease. The control measures imposed in these areas will often include movement restrictions, intensified surveillance, emergency vaccination, targeted culling (in cases of highly infectious animal diseases), and other relevant specific measures applied to affected premises.

6 Implementation of Viral Disease Control Program at Various Levels

Level	Control measures
On individual level	• Periodic screening of animals • Segregation and/or culling (in case of highly infectious diseases) infected animals • Separation of offspring from the infected dam
On herd level	• Implementation of strict biosecurity measures • Regular screening of the herd In case of high seroprevalence of infectious diseases, slaughter of entire herd is feasible option
On regional and national levels	• Establishment of regional/national reference laboratories for diagnosis of important exotic as well as diseases of economic importance

(continued)

Level	Control measures
	• Proper implementation of surveillance program and epidemiological investigation of outbreaks (tracing of contact flocks) • Proper reporting of cases at regional and national level due to its notifiable status

7 Prevention and Control Measures for the Viral Disease Outbreaks in Swine Population

Occurrence of diseases leads to a huge economic loss in terms of livestock health and production. As the time passes the advancement that occur in the animal health are expected to play a major role in the progress of livestock industry. A lot of control on animal diseases assumes a prime importance in the crucial time when the animal agriculture is shifting from extensive to intensive and commercial system of management. Presence and accumulation of infectious agents/pathogens in the environment leads to reduction in quality and quantity of products produced by animals. Strategic control and eradication of diseases will result enhancing pig production worldwide. The various prevention and control measures for viral disease outbreak are mentioned in the following.

1. Biosecurity management: The biosecurity management must not be compromised at any cost in the farm. Good herd biosecurity is essential to maintain herd health status and also for undertaking control or eradication program of diseases. Biosecurity is a cornerstone of herd health maintenance.
2. Prevention measures against the virus introduction into farms through commercial pig feeds or feed ingredients. It has been shown previously that multiple swine viruses could survive in certain feed ingredients for a very long time [47, 48]. For example, one study has shown that the half-life of ASFV in various feed or feed ingredients ranged from 9.6 days (conventional soybean meal) to 14.2 days (complete feed) [48]. There are some strategies that might be used for reducing the infectivity of viruses contaminating the feed, such as heat treatment, e.g., pelleting the feed using higher temperature; chemical mitigation, e.g., treating the feed with a formaldehyde and propionic acid solution; and storage period management, e.g., using the knowledge on the half-life of the virus to adjust the feed storage time and conditions [7].
3. Decontamination of infected farms with effective disinfectant, i.e., 2% caustic soda should be made available (FAO animal production and health manual).

4. Vehicles used during farm visit should be properly disinfected after use (FAO animal production and health manual).
5. Exit of live pigs should be banned (FAO animal production and health manual).
6. Perception of risk management of farmers and other stakeholders could be fundamental to the disease controls. Continuing education of farmers and other stakeholders in the supply chains is required to prevent the emergence and reemergence of infectious disease outbreaks in swine. Collaboration: It takes shared knowledge and communication to prepare for and manage a disease outbreak. The disease eradication and prevention on the farm might not be successful if the diseases are still active on neighboring farms. This might also be applied to the international level. Collaboration among neighboring countries should be strengthened. Transboundary disease transmission through smuggled pigs and pork products has been suspected in many diseases across the globe [31, 32].
7. Livestock traders: They are the important group for public awareness, as the movement of animals through livestock traders is often the key epidemiological factor in the spread of diseases. Therefore, for the prevention of disease there is the need to build up a climate of trust and confidence between animal health officials and livestock traders (FAO animal production and health manual).
8. Improved control and tracing of consignments of animals and animal products by development of an integrated database, introduction of a central alert system, and risk assessment systems.
9. Isolate the infected animals and immediately slaughter those who are showing the severe symptoms of disease (FAO animal production and health manual).
10. Safe disposal of the carcasses of animals that have either slaughtered or naturally died should be done as soon as possible, so that no longer constitute a risk of further spread of pathogen to other animals (FAO animal production and health manual).
11. Public awareness campaigns through newspapers, radio, and television were also useful for prevention and control of disease (FAO animal production and health manual).

8 Conclusion

The unprecedented increase in the frequency of infectious disease outbreaks in swine industry requires meticulous planning and decision-making to curtail the outbreak progression. Biosecurity at farm level aims to prevent the introduction and spread of

pathogenic organisms, thus ensures better farm health management. Vaccination against important infectious diseases also aims to keep the disease spread at check. An outbreak is designated when disease rates are higher than normal. The outbreak of viral diseases in swine industry cause significant economic loss and the outbreak investigation is one of the critical components of infectious disease epidemiology and efficiently performed outbreak investigation helps the identification and removal of a potential cause of the outbreak and provides post-exposure prophylaxis to affected individuals. An outbreak investigation requires knowledge of descriptive and analytical epidemiological methods and it includes several steps. The most critical part in the investigation is to clearly define the problem, i.e. case definition. Outbreak investigations tries to find the answer for five Ws such as what (disease event), who (animal affected), where (place), when (time), and why/how (causes, risk factors, and modes of transmission). Furthermore, outbreak investigations frequently result in identification of new infections and diseases. Effective outbreak investigations play a vital role in widening the knowledge about infectious diseases, and also assist in the development of regulations and prevention guidelines. Since outbreak is different, there is no readymade recipe for success. By adopting the basic outbreak techniques, the investigator may efficiently contain the disease progression. In few situations, the need may arise for intricate analysis demanding the specialists such as epidemiologists, medicos, laboratory diagnosticians, and others. The successful outbreak investigation should end in communication of the outbreak results to public platform or needy people.

References

1. Statista (2020) Number of pigs worldwide in 2020, by leading country (in million head). https://www.statista.com/statistics/263964/number-of-pigs-in-selected-countries
2. Bhadauria P, Sharma A, Verma HK, Singh I, Singh R (2019) Pig farming: promising agribusiness in Punjab. ICAR-Agricultural Technology Application Research Institute, Ludhiana
3. Mpofu I, Makuza SMM (2003) In: Shonhiwa A (ed) Pig production science and technology, 1st edn. Upfront Publishing, Leicestershire
4. VanderWaal K, Deen J (2018) Global trends in infectious diseases of swine. Proc Natl Acad Sci 115(45):11495–11500
5. Razia SJ, Vidya B, Sushma K, Raghunandan T (2017) Pig mortality and its management. Theriogenol Insight 7(3):139–144
6. Iowa State University. Exotic viral diseases. https://vetmed.iastate.edu/vdpam/FSVD/swine/index-diseases/exotic-viral-diseases
7. Kedkovid R, Sirisereewan C, Thanawongnuwech R (2020) Major swine viral diseases: an Asian perspective after the African swine fever introduction. Porcine Health Manag 6(1):1–11
8. King AM, Lefkowitz EJ, Mushegian AR, Adams MJ, Dutilh BE, Gorbalenya AE, Harrach B, Harrison RL, Junglen S, Knowles NJ, Kropinski AM (2018) Changes to taxonomy and the international code of virus classification and nomenclature ratified by the international committee on taxonomy of viruses (2018). Arch Virol 163(9):2601–2631
9. Zhou B (2019) Classical swine fever in China-an update minireview. Front Vet Sci 6:187. https://doi.org/10.3389/fvets.2019.00187
10. Donaldson AI (1997) Foot-and-mouth disease in Taiwan. Vet Rec 140(15):407
11. Dunn CS, Donaldson AI (1997) Natural adaption to pigs of a Taiwanese isolate of foot-and-mouth disease virus. Vet Rec 141(7):174–175

12. DeLay J, McEwen B, Carman S et al (2005) Porcine circovirus type 2-associated disease is increasing. AHL Newslett 9(3):22
13. Cheung AK, Lager KM, Kohutyuk OI, Vincent AL, Henry SC, Baker RB, Rowland RR, Dunham AG (2007) Detection of two porcine circovirus type 2 genotypic groups in United States swine herds. Arch Virol 152(5): 1035–1044
14. Ni J, Yang S, Bounlom D, Yu X, Zhou Z, Song J, Khamphouth V, Vatthana T, Tian K (2012) Emergence and pathogenicity of highly pathogenic Porcine reproductive and respiratory syndrome virus in Vientiane, Lao People's Democratic Republic. J Vet Diagn Investig 24(2):349–354
15. Nilubol D, Tripipat T, Hoonsuwan T, Kortheerakul K (2012) Porcine reproductive and respiratory syndrome virus, Thailand, 2010–2011. Emerg Infect Dis 18(12): 2039–2043
16. Tornimbene B, Frossard JP, Chhim V, Sorn S, Guitian J, Drew TW (2015) Emergence of highly pathogenic porcine reproductive and respiratory syndrome (HP-PRRS) in medium-scale swine farms in southeastern Cambodia. Prev Vet Med 118(1):93–103
17. Puranaveja S, Poolperm P, Lertwatcharasarakul P, Kesdaengsakonwut S, Boonsoongnern A, Urairong K, Kitikoon P, Choojai P, Kedkovid R, Teankum K, Thanawongnuwech R (2009) Chinese-like strain of porcine epidemic diarrhea virus, Thailand. Emerg Infect Dis 15(7):1112–1115
18. Duy DT, Toan NT, Puranaveja S, Thanawongnuwech R (2011) Genetic characterization of porcine epidemic diarrhea virus (PEDV) isolates from southern Vietnam during 2009-2010 outbreaks. Thai J Vet Med 41(1):55–64
19. Wang D, Fang L, Xiao S (2016) Porcine epidemic diarrhea in China. Virus Res 226:7–13
20. Bora M, Bora DP, Manu M, Barman NN, Dutta LJ, Kumar PP, Poovathikkal S, Suresh KP, Nimmanapalli R (2020) Assessment of risk factors of African swine fever in India: perspectives on future outbreaks and control strategies. Pathogens 9(12):1044. https://doi.org/10.3390/pathogens9121044
21. Ciarello FP, Capucchio MT, Ippolito D, Colombino E, Gibelli LRM, Fiasconaro M, Moreno Martin AM, Di Marco Lo Presti V (2020) First report of a severe outbreak of Aujeszky's disease in cattle in Sicily (Italy). Pathogens 9(11):954
22. Janke BH, Paul PS, Landgraf JG, Halbur PG, Huinker CD (2001) Paramyxovirus infection in pigs with interstitial pneumonia and encephalitis in the United States. J Vet Diagn Investig 13(5):428–433. https://doi.org/10.1177/104063870101300513
23. Johansen CA, Van den Hurk AF, Pyke AT, Zborowski P, Phillips DA, Mackenzie JS, Ritchie SA (2001) Entomological investigations of an outbreak of Japanese encephalitis virus in the Torres Strait, Australia, in 1998. J Med Entomol 38(4):581–588
24. Liu W, Fu S, Ma X, Chen X, Wu D, Zhou L, Yin Q, Li F, He Y, Lei W, Li Y (2020) An outbreak of Japanese encephalitis caused by genotype Ib Japanese encephalitis virus in China, 2018: a laboratory and field investigation. PLoS Negl Trop Dis 14(5):e0008312. https://doi.org/10.1371/journal.pntd.0008312
25. Wu Q, Zhao X, Bai Y, Sun B, Xie Q, Ma J (2017) The first identification and complete genome of Senecavirus A affecting pig with idiopathic vesicular disease in China. Transbound Emerg Dis 64(5):1633–1640
26. Looi LM, Chua KB (2007) Lessons from the Nipah virus outbreak in Malaysia. Malays J Pathol 29(2):63–67
27. Acland HM, Littlejohns IR (1975) Encephalomyocarditis virus infection of pigs. 1. An outbreak in New South Wales. Aust Vet J 51(9): 409–415. https://doi.org/10.1111/j.1751-0813.1975.tb15789.x
28. Maurice H, Nielen M, Vyt P, Frankena K, Koenen F (2007) Factors related to the incidence of clinical encephalomyocarditis virus (EMCV) infection on Belgian pig farms. Prev Vet Med 78(1):24–34. https://doi.org/10.1016/j.prevetmed.2006.09.002
29. Vansteenkiste K, Van Limbergen T, Decaluwé R, Tignon M, Cay B, Maes D (2016) Clinical problems due to encephalomyocarditis virus infections in two pig herds. Porcine Health Manag 2(1):1–8. https://doi.org/10.1186/s40813-016-0036-z
30. Paton DJ, Simpson V, Done SH (1992) Infection of pigs and cattle with bovine viral diarrhoea virus on a farm in England. Vet Rec 131: 185–188
31. Chenais E, Depner K, Guberti V, Dietze K, Viltrop A, Ståhl K (2019a) Epidemiological considerations on African swine fever in Europe 2014–2018. Porcine Health Manag 5(1): 1–10. https://doi.org/10.1186/s40813-018-0109-2
32. Chenais E, Lewerin SS, Boqvist S, Ståhl K, Alike S, Nokorach B, Emanuelson U (2019b) Smallholders' perceptions on biosecurity and disease control in relation to African swine

fever in an endemically infected area in Northern Uganda. BMC Vet Res 15(1):1–13

33. Valeeva NI, Van Asseldonk MAPM, Backus GBC (2011) Perceived risk and strategy efficacy as motivators of risk management strategy adoption to prevent animal diseases in pig farming. Prev Vet Med 102(4):284–295
34. Food and Agriculture Organization of the United Nations/World Organisation for Animal Health/World Bank (2010) Good practices for biosecurity in the pig sector – issues and options in developing and transition countries. In: FAO animal production and health paper no. 169. FAO, Rome
35. Beltran-Alcrudo D, Falco JR, Raizman E, Dietze K (2019) Transboundary spread of pig diseases: the role of international trade and travel. BMC Vet Res 15(1):1–14. https://doi.org/10.1186/s12917-019-1800-5
36. Meng XJ (2012) Emerging and re-emerging swine viruses. Transbound Emerg Dis 59: 85–102
37. Li Y, Ji G, Wang J, Tan F, Zhuang J, Li X, Tian K (2016) Complete genome sequence of an NADC30-like porcine reproductive and respiratory syndrome virus characterized by recombination with other strains. Genome Announc 4(3):e00330–e00316. https://doi.org/10.1128/genomeA.00330-16
38. Miller RS, Sweeney SJ, Slootmaker C, Grear DA, Di Salvo PA, Kiser D, Shwiff SA (2017) Cross-species transmission potential between wild pigs, livestock, poultry, wildlife, and humans: implications for disease risk management in North America. Sci Rep 7(1):1–14. https://doi.org/10.1038/s41598-017-07336-z
39. Balkhy HH, Abolfotouh MA, Al-Hathlool RH, Al-Jumah MA (2010) Awareness, attitudes, and practices related to the swine influenza pandemic among the Saudi public. BMC Infect Dis 10(1):1–7. https://doi.org/10.1186/1471-2334-10-42
40. Guinat C, Relun A, Wall B, Morris A, Dixon L, Pfeiffer DU (2016) Exploring pig trade patterns to inform the design of risk-based disease surveillance and control strategies. Sci Rep 6(1):1–11
41. Office International des Épizooties (OIE) (2018) Terrestrial Animal Health Code (2018). http://www.oie.int/standard-setting/terrestrial-code/access-online/
42. Rodríguez-Prieto V, Vicente-Rubiano M, Sánchez-Matamoros A, Rubio-Guerri C, Melero M, Martínez-López B, Martínez-Avilés M, Hoinville L, Vergne T, Comin A, Schauer B (2015) Systematic review of surveillance systems and methods for early detection of exotic, new and re-emerging diseases in animal populations. Epidemiol Infect 143(10): 2018–2042
43. OIE South-East Asia and China Foot-and-Mouth Disease (SEACFMD) Campaign (2018) A field manual for animal disease outbreak investigation and management. OIE Sub-Regional Representation for South-East Asia, Department of Livestock Development, 69/1 Phaya Thai Road, Ratchathewi, Bangkok 10400, Thailand
44. World Health Organization (2018) Managing epidemics: key facts about major deadly diseases. World Health Organization
45. Centers for Disease Control and Prevention (2016) Investigating an outbreak. https://www.cdc.gov/csels/dsepd/ss1978/lesson6/section2.html
46. World Organisation for Animal Health (OIE) 2014 Guidelines for animal disease control – OIE. https://www.oie.int/fileadmin/Home/eng/Our_scientific_expertise/docs/pdf/A_Guidelines_for_Animal_Disease_Control_final.pdf)
47. Dee S, Neill C, Singrey A, Clement T, Cochrane R, Jones C, Patterson G, Spronk G, Christopher-Hennings J, Nelson E (2016) Modeling the transboundary risk of feed ingredients contaminated with porcine epidemic diarrhea virus. BMC Vet Res 12(1):51
48. Stoian AM, Zimmerman J, Ji J, Hefley TJ, Dee S, Diel DG, Rowland RR, Niederwerder MC (2019) Half-life of African swine fever virus in shipped feed. Emerg Infect Dis 25(12):2261

Chapter 2

Collection of Samples, Their Preservation and Transportation

Ashok Kumar, Kaushal Kishor Rajak, Ajay Kumar Yadav, Vishal Rai, Mukesh Bhatt, R. P. Singh, and R. K. Singh

Abstract

Pigs are an important livestock species raised for meat, and their products play a significant role in the livelihood of people in the country's north-eastern states. Detection of diseased porcine in the field is critical for disease treatment and control. Pigs, such as other livestock, were subjected to a slew of contagious existing, emerging, and re-emerging viral diseases, necessitating the use of a diagnostic laboratory and research organization globally. The diagnosis of viral diseases is fundamentally dependent on time and precise management. Collecting whole blood and tissues from multiple febrile or recently deceased animals is the preferred method for detecting herds early in infection. Particular tissues should be collected as aseptically as possible. Preferably, two or three humanely euthanized pigs in the early stages of disease displaying typical clinical signs and necropsied immediately will yield the most reliable diagnostic data. A detailed history of the disease outbreak, as well as a preliminary diagnosis based on clinical evaluation and necropsy findings, should be included. Animal selection, sample selection, sample handling, sample processing, necropsy technique, specimen collection media, and adequate storage all have a direct impact on the accuracy and effectiveness of laboratory results in assisting in the resolution of health problems. Existing and emerging swine pathogens, particularly those of a transboundary nature, must be closely monitored, and appropriate health interventions must be developed on a priority basis. With limited vaccine availability, the emergence of new diseases such as African swine fever (ASF) and porcine reproductive and respiratory syndrome (PRRS) are threats to pig production in India.

Key words Contagious, Emerging, Specimen, Pathogens, Necropsy, Equipment, Viral transport media (VTM), Evaluation, Backyard and Microscopy

1 Background

Pig is even-toed ungulate animal hail from genus *Sus* within family *Suidae* of the modern classification of the kingdom**.** Among the livestock species, the pig is one of the imperative species for meat production, which engages in recreation a pivotal role to provide livelihood support to the landless farmers and pitiable sections of society, especially in the North-Eastern region of the country.

Rajib Deb et al. (eds.), *Protocols for the Diagnosis of Pig Viral Diseases*,
Springer Protocols Handbooks, https://doi.org/10.1007/978-1-0716-2043-4_2,

Porcine species is closely related to humans in the mean of an anatomical, physiological, and biological system that why intensively used in the research system. According to Michael Swindle, author of "Swine in the Laboratory". If works in the pig, then it has a high possibility of working in the human. The pig industry is growing day by day, due to increased demand for pork and its products, due to the urbanization of society. The pig is considered as the only species bearing the highest litter size among meat-producing animals and the most efficient feed converting species with the shortest generation interval and the fastest growth rate. Pigs are mostly maintained in a poor sanitary condition and mostly by backyard and internal sector producers which lead to economic losses due to various viral diseases. Molecular assays for detection of nucleic acids in biologic specimens are valuable diagnostic tools supporting clinical diagnoses and therapeutic decisions.

2 Introduction

Pig is an important livestock species, which plays a crucial role in the livelihood in north-eastern states of the country. Pig is the most efficient feed converting animal species. Among meat-producing animals it is the only litter bearing animal having shortest generation interval and fastest growth rate. Pigs are mostly maintained in poor sanitary condition and mostly by backyard and internal sector producers. Pigs are susceptible to many viral diseases such as Classical Swine Fever (CSF), Foot and Mouth Disease (FMD), Swinepox, Transmissible Gastroenteritis, Calicivirus, Porcine circovirus 2, Influenza, Pseudorabies, and Adenoviruses although some emerging porcine diseases such as Porcine reproductive and respiratory syndrome virus (PRRSV), African swine fever, and Porcine respiratory coronavirus (PRCoV).

Pathogens causing significant respiratory, GIT, and skin disease in growing pigs and development of viraemia and the body distribution of susceptible macrophages lead to the shedding of pathogen [1]. The veterinary officer of various states has primary responsibility for provide tentative diagnoses of disease and final decision concerning management of swine health problems for practice [2]. The veterinary diagnostic laboratory and research institute can be an important supporting arms of the veterinary practice by different ways such as (1) *providing a consultation service*, (2) *providing technical assistance in performing laboratory testing*, and (3) *collecting and disseminating information on current research.*

Modern diagnostic laboratories have facilities and personnel highly trained in various areas of laboratory such as virology, clinical pathology, immunology, biochemistry, theriogenology, biotechnology, and microbiology. Highly sophisticated equipments for

laboratory such as real time polymerase chain reaction (PCR), scintillation counters, automated cell counters, autoanalyzers, atomic absorption spectrophotometers, Mass spectrometers, high pressure liquid and gas chromatographic equipment, next generation sequencing, elaborate equipment for culture of virus and electron microscopes are routinely used in many diagnostic laboratories today. The basic approaches for laboratory viral diagnosis are the isolation of the virus, demonstration of the virus or some viral product in clinical specimens (direct methods), and detection and measurement of viral-specific antibodies (indirect methods). Each approach has its merits, but direct demonstration of the virus and/or viral products is the most effective and useful approach for routine diagnosis [3].

Each organization is geared toward the problems that occur in the areas in which they serve. Depends upon severity of cases may referred to specialised laboratories for more details analyses if required. The technical capabilities of the organization can be used for monitoring herd health programs as well as for facilitating diagnosis in disease outbreaks. Accuracy of laboratory results and their effectiveness in helping to resolve the health problems are highly dependent on clinical evaluations of the cases by the professional practitioner and also dependent on selection, collection of specimen, processing, packaging, preservation, and transport of specimens to be submitted for the analysis.

General guidelines for the collection and submission of specimens are presented in Table 1. Most laboratories supply a specimen submission form that should be completed with the available pertinent information. In the absence of a form, the veterinarian should supply as complete a history as possible. Veterinarians should contact the diagnostic laboratory if they have any questions.

Complete history is essential for performing the appropriate tests and for proper interpretation of laboratory results. Organization should communicate with trained laboratory person in such a manner that all requests are fulfilled and steps are taken to obtain the information recorded.

3 Suitable Criteria for Collection and Transport and Preservation of Samples

The following criteria are suitable for proper selection of specimen based on viral infection like type of animals, types of specimens, type of organisms as listed in Table 1. Whenever possible, animals should be submitted directly to the diagnostic laboratory for complete necropsy examination. If a herd problem exists, more than one animal should be submitted. Bus and courier service may be used to ship small animals, provided they are packaged in leak-proof insulated containers with sufficient ice or cold packs. Do not freeze animals submitted for necropsy. For tissue specimen to minimize

Table 1
Selection site of specimen for laboratory inspection and diagnosis

S. No.	System	Selection site of cases
1	External examination	Appearance of hair coat, skin lesion location and description, conjunctiva, eyes, ears, feet, hooves, nostrils, mouths, anus, and vulva
2	Respiratory system	Nasal cavity, larynx, bronchi, lungs, and pleura
3	Circulatory system	Heart, pericardium, and blood vessels
4	Digestive system	Oral cavity, teeth, oesophagus, stomach, duodenum, jejunum, ileum, colon, liver, gall bladder, and pancreas
5	Urinary system	Kidney, ureters, and urinary bladder
6	Genital system	Ovaries, uterus, vagina, testicles, spermatic cord, and male accessory sex glands
7	Endocrine system	Thyroids, parathyroid, adrenals, and pituitary
8	Lymphoreticular and hematopoietic system	Lymph node, spleen, thymus tonsil, and bone marrow
9	Musculoskeletal system	Muscles, bones, cartilages, and tendons
10	Nerves system	Brain, spinal cord, peripheral nerves, and meninges

contamination during necropsy, it is best to collect a routine set of tissues prior to thorough examination. Recommended tissues are lung, kidney, liver, spleen, small intestine, large intestine, and mesenteric lymph nodes. Brain tissue or head should also be collected if central nervous system disease is suspected [3]. Other tissues containing abnormalities noted during the thorough examination should also be collected. A portion of these tissues should be placed in leak-proof plastic bags and placed under refrigeration. While it is recommended that each tissue be placed in a separate bag, it is absolutely essential that intestine be separated from other tissues; otherwise, biological examinations will be compromised. Tissues should be brought directly to the laboratory or shipped under refrigeration by over-night mail, bus, or courier service. Tissues collected during the latter part of the week should be frozen and shipped on Monday [4].

Since many viruses produce characteristic microscopic lesions, small pieces (1/4 inch thick) of each tissue should be placed in 10% buffered formalin for histopathologic examination. An entire longitudinal half of the brain should be submitted. These samples should not be frozen. The feces type specimen feces should be collected from acutely ill animals and placed in leak-proof containers [5]. While well-saturated swabs are adequate for many individual virologic examinations, several milliliters or grams of feces permit a more complete diagnostic work-up including

bacteriologic and parasitologic examinations. Samples should be submitted to the laboratory using cold packs as coolant. According to WHO the swabs specimens nasal and ocular swabs are useful for isolating viruses from animals with upper respiratory-tract infections [6]. Genital infections may also be diagnosed by examining swabs collected from the reproductive tract (vagina and penis mucosa). These swabs should be collected from acutely ill animals and placed directly into screw-capped tubes containing a viral transport medium. The sampling of several animals in different stages of the illness increases the likelihood of isolating the causative agent. Swabs are also useful for the sampling of vesicular lesions. Fresh vesicles should be ruptured and the swab saturated with the exuding fluid. Two swabs should be collected, one for virus isolation and one for electron microscopy. Major viral diseases of porcine for specimen collection and transport media to laboratory diagnosis are listed in Table 2.

The swab for virus isolation should be placed in viral transport medium and the swab for electron microscopy should be placed in a screw-capped tube containing one or two drops of distilled water. Scab material from the more advanced lesions should also be submitted. There are several commercially available viral transport media that help maintain the viability of viruses during shipment to the laboratory. Most of these transport media are balanced salt solutions containing high protein content and antibiotics to prevent bacterial overgrowth. Many diagnostic laboratories provide their own version of transport medium to practicing veterinarians upon request. For direct examination of specimen slide preparation needed at fresh specimens a number of infectious diseases can be diagnosed by examining slides prepared from blood and tissues. Conjunctival scrapings are particularly useful for diagnosing herpesvirus infections in pigs [7]. Imprints made from liver, spleen, and lungs are especially useful for diagnosing flavivirus and herpesvirus infections of swine [8, 9]. Slides should have sufficient cells to allow thorough examination but should not be so thick as to cause difficulty in staining. A conjunctival scraper or some other device (blunt end of scalpel blade) should be used to scrape the conjunctiva; cotton swabs are not adequate. Matted eyes should be cleaned and flushed prior to scraping the conjunctiva. Tissue imprints should be made by lightly touching the microscope slide with fresh cuts of tissue previously blotted with a paper towel to absorb some of the blood [10]. Slides should be air-dried and sent to the laboratory in slide holders to prevent breakage. Several slides permit a more thorough diagnostic work-up, including cytologic examinations. For the serological examination blood samples should be collected in sterile tubes containing no anticoagulants. These should be submitted to the laboratory in specially designed Styrofoam holders to avoid breakage [11]. Blood samples should not be frozen or allowed to overheat. If samples cannot be delivered

Table 2
Major viral diseases of porcine for specimen collection and transport media to laboratory diagnosis

Viral disease	Primary System involved to disease	Specimens for Isolation and of Screening of specimen	Transport conditions	Transport media
Influenza	Respiratory, digestive	Nasal swab, lung tissue (PM) Nasal swab, lung and tracheal swab (PM), serum, lymph node	4 °C/ Ambient temp/[a]	Transport medium 199, PBS-glycerol transport medium. Culturette Leibovitz CVTM Veal or tryptose broth
Transmissible gastroenteritis (TGE)	Digestive, respiratory	Jejunum, blood, serum and intestinal contents (small and large), feces, lungs, lymph node, serum	Ambient temp/[a]	PBS-glycerol transport medium, Leibovitz CVTM, RPMI, and EMEM
Rotavirus (Reoviruses)	Digestive	Intestinal contents, feces, serum	Ambient temp/[a]	PBS-glycerol and EMEM
Coronavirus	Digestive	Intestinal contents, feces, tonsil, lungs, stomach, small intestine, brain, spinal cord, serum	Ambient temp/[a]	PBS-glycerol transport medium, RPMI, and EMEM
Calicivirus	Digestive	Intestinal contents, feces, serum	Ambient temp/[a]	PBS-glycerol and EMEM
Adenovirus	Digestive, respiratory	Intestinal contents, feces, lungs, lymph node, serum	Ambient temp/[a]	PBS-glycerol and EMEM
Astroviruses	Digestive	Intestinal contents, feces, serum	Ambient temp/[a]	PBS-glycerol and EMEM
Pseudorabies	Respiratory system and nerves system	Lungs, lymph node, tonsils, serum, brain	Ambient temp/[a]	PBS-glycerol and EMEM
Vesicular stomatits	Digestive and musculoskeletal	Vesicular fluid, saliva, and affected mucous membranes collected early in the disease	Ambient temp/[a]	PBS-glycerol and EMEM
Parvovirus	Digestive, reproductive, and musculoskeletal	Mummified or aborted fetuses, placenta, fluids, and skin lesions	Ambient temp/[a]	PBS-glycerol transport medium, RPMI, and EMEM

(continued)

Table 2
(continued)

Viral disease	Primary System involved to disease	Specimens for Isolation and of Screening of specimen	Transport conditions	Transport media
Picornavirus (SMEDI, FMD, enteroviruses)	Respiratory, digestive, reproductive, and musculoskeletal	Vesicular fluid, affected skin and mucous membranes, blood with anticoagulant, and serum	Ambient temp/[a]	PBS-glycerol transport medium, RPMI, and EMEM
Porcine reproductive and respiratory syndrome	Reproductive and respiratory	Bronchoalveolar lavage (BAL), serum, lung, lymph nodes, tonsil, and spleen Aborted and mummified fetus	Ambient temp/[a]	PBS-glycerol transport medium, RPMI, and EMEM
Swinepox	Musculoskeletal and skin	Vesicular fluid, scabs, and scrapings from lesions	Ambient temp/[a]	PBS-glycerol and EMEM
Hog cholera, japans encephalitis (Flavivirus)	Digestive, reproductive, nervous, and urogenital	Kidney, spleen, tonsil, lymph nodes, brain, whole blood, and serum	Ambient temp/[a]	PBS-glycerol transport medium, RPMI, and EMEM
African swine fever	Respiratory and lymphoreticular	Blood, spleen, tonsil, and lymph nodes	Ambient temp/[a]	PBS-glycerol transport medium, RPMI, and EMEM

[a]As per specimen required temperature

to the laboratory within a reasonable time, serum should be removed and refrigerated or frozen.

4 Collection and Transport and Preservation of Samples

Sample selected for laboratory analyses depend, of course, on the nature of the problem. A live untreated pig with representative clinical signs is usually the sample of choice for laboratory analysis. Specimen must be taken as early as possible in the acute phase of sickness [12]. To check the bacterial contamination all the specimen including blood should be collected aseptically. Swab and other specimen should be placed in virus transport medium

Table 3
Equipment and supplies for use in performing necropsies and collecting laboratory samples

Equipment	Supplies
Knife	Swabs
Steel	Blood vials
Stone	Plastic bags
Foreceps	Sterile syringe
Scissors	Sterile needles
Saw	Wide mouth container with 10% formalin
Cleaver	pH paper
Gloves	Shipping container
Coveralls	Refrigerant
Boots	Marking pen or pencil notepad
Pail brush	Disinfectant

(VTM). VTM should be prepared sterile and distributed in 2 ml quantities in suitable sterile glass/plastic container [13]. Therefore it may be more expedient for the practitioner to collect samples during a field necropsy or from live animals and send them to the laboratory via an acceptable mode of transportation. Certain equipment and supplies are designed for the use in performing necropsies and collecting laboratory samples (Table 3) [14].

Transmission from infected to susceptible animals via aerosols has been demonstrated under experimental conditions for porcine reproductive and respiratory syndrome virus (PRRSV) influenza virus porcine respiratory coronavirus (PRCoV) [4, 9]. Viral transport media for use in collecting nasal swab and throat swab are as follows (1) Add 10 g veal infusion broth and 2 g bovine albumin fraction to sterile distilled water (to 400 ml) (2) Add 0.8 ml Gentamycin sulfate solution (50/ml) and 3.2 ml amphotericin B (250 μg/ml) sterilized by 0.22 μm filtration. Another viral transport media PBS-Glycerol transport medium (1) Phosphate-buffered saline (PBS):—NaCl 8 g—KCl 0.2 g—Na2HPO4 1.44 g—KH_2PO_4 0.24 g—Distilled water to make 1 l (2) Autoclave PBS and mix 1:1 with sterile glycerol to make 1 l (3) To 1 l PBS/glycerol add:—benzylpenicillin (2 × 106 IU/l)—streptomycin (200 mg/liter)—polymyxin B (2 × 106 IU/l)—gentamicin (250 mg/liter)—nystatin (0.5 × 106 IU/l)—ofloxacin hydrochloride (60 mg/l), and—sulfamethoxazole (0.2 g/l) Dispense 1.0–2.0 ml of transport medium into sterile plastic screw-cap vials (Cryovials). It is best to store these vials at −20 °C until used. However, they can be stored at +4 °C for 48–96 h (optimally less than 48 h) or at room temperature for short periods of 1–2 days.

After collecting specimens in VTM, it is to be packed properly before sending to a suitable laboratory. Packaging has three main aims to maintain the specimen viability, to prevent it leaking outside the package and to prevent cross contamination [11]. Biological materials survive better at low temp. So it is adviced to maintain the low temperature during transport. Low temperature may be achieved by using solid CO_2, wet ice or frozen pads. Specimens in VTM can be sent to a nearby laboratory for diagnosis by keeping it on ice through messenger, mail, freight, and courier.

5 Conclusion

The disease can be diagnosed in laboratory by histopathology, electron microscopy (EM), detection of viral antigens by immunofluorescence and polymerase chain reaction (PCR) with suitable and sufficient specimens. Sampling carefully to include acutely affected pigs will be the greatest help to the producer, the pathologist, and the veterinarian seeking treatment or prevention strategies. As a result of several intense research efforts the disease etiology, its pathogenesis, pathology, immunology were well studied. With the use of this knowledge a wider development in extension activities, anti-viral therapeutics and vaccine strategy for both humans and susceptible animals is needed to eradicate viral disease from our globe.

References

1. Murphy F, Fauquet C, Bishop D, Gharial S, Jarvis A, Martelli G, Mayo M, Summers M (1995) Virus taxonomy: classification and nomenclature of viruses. Sixth report of the international committee on taxonomy of viruses. Springer-Verlag, New York, pp 79–91
2. Grandien M (1988) Paramyxoviridae: the parainfluenza viruses. In: Lennette EH, Halonen P, Murphy FA (eds) Laboratory diagnosis of infectious diseases, principles and practice, vol 2. Springer-Verlag, New York, pp 490–491
3. Johnson FB (1990) Transport of viral specimens. Clin Microbiol Rev 3(2):120–131
4. Cavanagh D (1997) Nidovirales: a new order comprising Coronaviridae and Arteriviridae. Arch Virol 142:629–633
5. World Organisation for Animal Health (2019) Terrestrial animal health code. OIE, Paris
6. Kwon HM (1996) Analysis of the spike glycoprotein gene and nonstructural protein gene of transmissible gastroenteritis virus using PCR and RFLP analysis. Kor J Vet Res 36 (3):627–633
7. The Pig Site (2008) Handling and restraining pigs. The pig site, Sheffield, England. http://www.thepigsite.com/articles/2392/handling-and-restraining-pigs
8. Gloster J, Donaldson AI, Hough MN (1984) Analysis of a series of outbreaks of Aujeszky's disease in Yorkshire in 1981–1982: the possibility of airborne disease spread. Vet Rec 114: 234–239
9. Juozaitis A, Willeke K, Grinshpun SA, Donnelly J (1994) Impactiononto a glass slide or agar versus impingement into a liquid forthe collection and recovery of airborne microorganisms. Appl Environ Microbiol 60:861–870
10. Lin X, Willeke K, Ulevicius V, Grinshpun SA (1997) Effect of sampling time on the collection efficiency of all-glass impingers. Am Ind Hyg Assoc J 58:480–488
11. Pirbright Institute (2015) Requirements for packaging and dispatch of biological materials to the Pirbright Institute, Reference Laboratories, Pirbright, U.K. http://www.pirbright.ac.uk/ref_Labs/Default.aspx

12. Meinkoth JH, Cowell RL (2002) Sample collection and preparation in cytology: increasing diagnostic yield. Vet Clin North Am Small Anim Pract 32(6):1187–1207
13. Hermann JR, Hoff SJ, Yoon KJ, Burkhardt AC, Evans RB, Zimmerman JJ (2006) Optimization of a sampling system for recovery and detection of airborne porcine reproductive and respiratory syndrome virus and swine influenza virus. Appl Environ Microbiol 72:4811–4818
14. Madeley CF, Lennette DA, Halonen P (1988) Specime collection and transport. In: Lennette EH, Halonen P, Murphy FA (eds) Laboratory diagnosis of infectious diseases, principles and practice, vol 2. Springer- Verlag, New York, pp 7–11

Chapter 3

Methods for Quantification of Viruses

Mukesh Bhatt, Chris Einstein, Kiran, Arfa Fayaz, Vishal Rai, Monu Karki, Ashok Kumar, Ajay Kumar Yadav, and Kaushal Kishor Rajak

Abstract

Virus quantification is widely practised in both commercial and academic laboratories involved in research or production of viral vaccines, recombinant proteins, viral antigens, or antiviral agents. For this, the cell culture-based endpoint dilution assays are the most widely used methods. However, these infectivity assays are laborious, time consuming, and susceptible to failures due to the contamination of cells. With the advancement in science, a number of other methods based on chemical or physical principles have been developed for determining the viral load in a given sample. These methods include electron microscopy, hemagglutination assay, qPCR, flow cytometry, and serological assays such as ELISA. However, all of these methods have their own limitations and advantages associated with them and therefore one must be careful while selecting an appropriate method to determine the virus titer and interpretation of results. Here, we describe the theory and practical aspects of the most commonly used methods for virus quantification and their practical utility in the field of virology.

Key words Virus titer, Infectivity assays, Quantal assays, Endpoint dilution

1 Introduction

Since the recognition of viruses as important pathogen for human and animals, the search for ideal diagnostic tests has continued. Most of the viral diagnostic assays developed till date focus primarily on the detection of the causative agent rather than actually quantifying the number of virions. Virus quantification involves counting the number of viruses in a specific volume to determine the virus concentration. Although the procedure for virus quantification is complex and is not useful in clinical practice, it is utilized in both research and development (R&D) in commercial and academic laboratories as well as production situations where the quantity of virus at various steps is an important variable [1].

Methods quantifying the viruses fall into two discrete categories; infectivity assays and the tests that measures specific virion components such as specific viral protein or the viral genome

Rajib Deb et al. (eds.), *Protocols for the Diagnosis of Pig Viral Diseases*,
Springer Protocols Handbooks, https://doi.org/10.1007/978-1-0716-2043-4_3,

[2]. Infectivity assays are the tests that measure virions that can successfully infect a cell to produce infectious progeny. The inactivated or non-infectious virion are not counted in such assays. The second type of virus quantification assays is based on the principle of chemical/physical measurement of virus particles and includes serologic assays, polymerase chain reaction (PCR), and hemagglutination assays (HA). Such methods are not able to differentiate the infective and non-infective viral particles and thus give the result even if the sample does not contain a single infective virus [3].

2 Infectivity Assays

In infectivity assays, viruses are inoculated onto a monolayer of susceptible host cells, embryonated eggs, or animals. An infectivity assay measures the virus particles capable of replicating in a particular cell type or animal. These assays can further be divided into two types: (1) quantitative assays (e. g.: plaque assay) and (2) quantal assays or end-point dilution assays.

2.1 Quantitative Assay (Plaque Assay)

Plaque assay is the standard method that has long been used to determine the virus titer (i.e., infectious dose). It determines the number of plaque-forming units (pfu) in a sample and the titer of a virus stock is represented by plaque-forming units per milliliter (pfu/mL). Typically, tenfold serial dilutions of the virus stock are inoculated into each well of six-well plate or animals in replicates and after incubation periods extending from few days to weeks, infectious particles produce visible zones of infected cells called plaques (Fig. 1). The inoculated cell cultures are laid with a semi-solid media (overlay medium) to localize the spread of infection to the immediate vicinity of originally infected cell. The commonly used overlay media include agar, methyl cellulose, tragacanth, and starch gel [4]. Agar is the most commonly used overlay media; however, it is inhibitory to some viruses. Methyl cellulose which is overlaid at 37 °C or lower temperature is preferred for the thermolabile viruses [4]. The pfu/mL result represents the number of infectious particles in the sample, assuming that each plaque is caused by a single infectious virus particle. Plaques are easily seen following staining of monolayers either by incorporating neutral red in overlay media or direct staining with methylene blue or crystal violet [4, 5]. The important points to be taken into account while performing plaque assay include host cellular compatibility with the virus in question, appropriate viral growth conditions, sufficient dilution ranges in order to clearly differentiate plaques, and correct overlay selection followed by staining for the cells and virus in question [6].

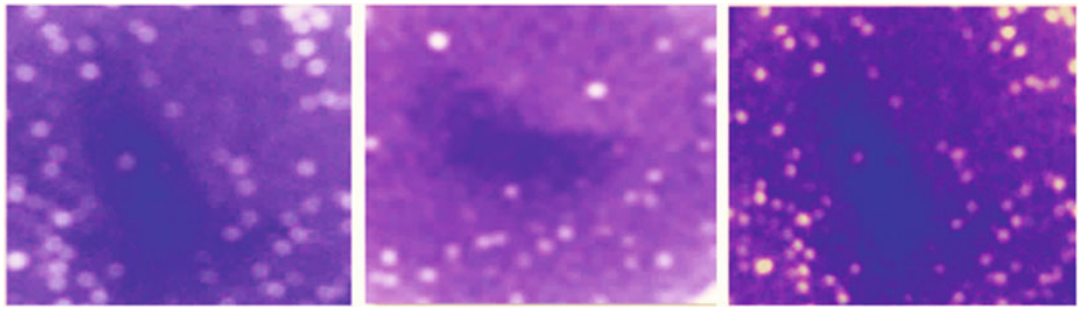


Fig. 1 Plaque assay using foot and mouth disease virus in BHK-21 cells

Viruses that are non-cytocidal and do not produce plaques can be titrated by counting other types of infective centers. Haemadsorbing viruses can be titrated by counting the number of focal areas to which RBCs are adsorbed. Groups of infected cells are called foci and the assay is termed as focus-forming assays (FFA) [2]. Although the FFA is a variation of the plaque assay, it employs immunostaining techniques using fluorescently labelled antibodies specific for a viral antigen to detect infected host cells and infectious virus particles before an actual plaque is formed. The FFA delivers the results in lesser time as compared to other methods of virus titration and the virus titer is expressed as FFU/mL [7].

Plaque assay protocol [5] *for FMD virus:*

1. Culture the BHK-21 cells in growth medium (GMEM+10% FBS) in a six-well plate to give 90–100% confluence by the following days.
2. Dilute the virus suspension tenfold serially from 1:10^{-1} to 1:10^{-6}.
3. Discard the growth medium and wash the cells with serum-free GMEM.
4. Infect the confluent monolayers of BHK-21 cells with 100 μL volume of diluted virus (each dilution per well) and 400 μL volume of GMEM (total 500 μL volume/well) keeping a well seeded with cells in 500 μL of serum-free GMEM as control.
5. Incubate the plates at 37 °C under 5% CO_2 tension for 1 h for virus adsorption with a gentle shaking at 30 min.
6. Discard the unadsorbed virus after 1 h and overlay the monolayer with 3 mL of autoclaved agar overlay (at about 40 °C). After the gel solidifies, incubate the plates at 37 °C for up to 48 h.
7. After 48 h, remove the agar overlay from wells and for fixation of cells, add 10% formalin to each well of plate followed by incubation at 37 °C for 1 h. After one hour, stain the plates with 0.1% crystal violet in 10% formalin for 15 min (1 mL/well) followed by washing with distilled water and air dry at 37 °C.

8. Count the plaques and record the morphology.
9. Calculate the virus titer using the following formulas:

$$\text{Pfu/mL} = \text{Average no.of plaques per dilution}/(D \times V).$$

where; D: Dilution factor (for dilution 10^{-2}; D is 0.01 and for dilution 10^{-4}; D is 0.0001).

V: Volume of virus added per well (in mL; 0.1 mL in the above protocol).

2.2 Quantal Assay or End-Point Dilution Assay

Many animal viruses do not form plaques on the cell monolayer but induce a visible cytopathic effect (CPE). These morphological changes that are observable under microscope can be exploited for virus quantification in "end-point dilution assays." These assays are called "quantal assay" as the end-point titer is based on the outcome of infection and does not represent the absolute number of virus particles in a sample. Consequently, the unit of infectivity measured by this method may require more than one infectious particle [8]. There are different ways of expressing the titer such as $TCID_{50}$, LD_{50}, and EID_{50}, depending on the host system that is used. When end-point titration assay is performed in cultured cells, the titer of the virus is recorded as $TCID_{50}$ or median tissue culture infectious dose. EID_{50} or median egg infectious dose is the titer when embryonated eggs are inoculated with the virus for the determination of titer. The titer obtained is referred to as LD_{50} or median lethal dose if the virus can cause the death in 50% of the animals. ID50 or median infectious dose is the titer of virus that would infect 50% of the test animals [8, 9].

Due to distinct differences in assay methods and principles, $TCID_{50}$ and pfu/mL are not equivalent. According to the Poisson distribution, which describes the number of random events (virus particles) occurring at a known average rate (virus titer) in a fixed space (the amount of virus medium in a well); the theoretical relationship between $TCID_{50}$ and PFU is approximately 0.70 PFU = 1 $TCID_{50}$ (thneedle; http://www.protocol-online.org/biology-forums/posts/1664.html). However, a study using two different strains of *Enterovirus* demonstrated that it is difficult to establish the relationship between PFU and $TCID_{50}$ and it was found to be different for two different strains [10].

There are three methods to determine the end-point titer including a more recent method given by Ramakrishnan [11] that has been proposed to be used along with the existing methods:

(a) Reed–Muench method.
(b) Improved Karber Method.
(c) Ramakrishnan method.

Protocol for determining end-point titer:

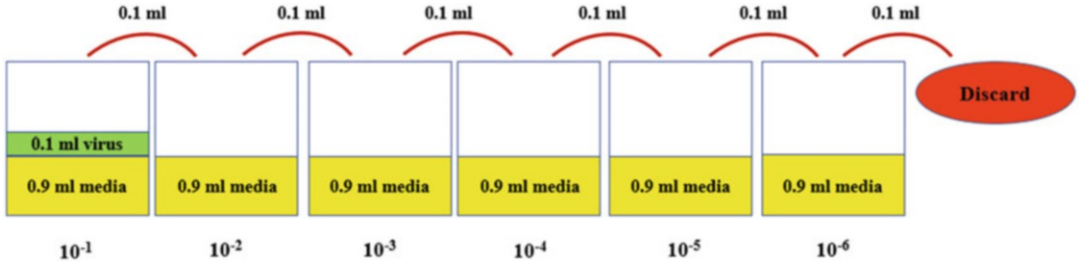


Fig. 2 Preparing ten-fold serial dilution of virus sample

	Virus 1						Virus 2					
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}	CC	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}	CC
B	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}	CC	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}	CC
C	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	CC	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	CC
D	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	CC	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	CC
E	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	CC	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	CC
F	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	CC	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	CC
G	10^{-7}	10^{-7}	10^{-7}	10^{-7}	10^{-7}	CC	10^{-7}	10^{-7}	10^{-7}	10^{-7}	10^{-7}	CC
H	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC

Fig. 3 Microtitration plate format for determining end-point titer (Note: 10^{-1} to 10^{-6}; serial virus dilutions and CC; uninfected cell control)

1. Dilute the virus sample (tenfold dilution; Fig. 2) in growth medium for titration on a single 96-well cell culture plate as mentioned in the following. Change tips between the dilutions and use a fresh tip for each transfer.
2. Dispense 0.1 mL virus suspension to each well (Fig. 3) keeping five replicates per dilution (10^{-1} to 10^{-6}).
3. Using a multichannel micropipette add 0.1 mL of cell suspension first into all the control wells (without virus) and then to virus wells.
4. Incubate the plates in 5% CO_2 atmosphere at 37 °C.
5. Carefully change the medium every second day. For this, the microplate is held upside down and the contents are discarded by gentle thrust downwards once or twice. Then, put two drops of maintenance media in each of the wells using a 10 mL serological pipette.
6. Observe for cytopathic effect (CPE) and record the results.

	1	2	3	4	5	6	7	8	9	10	11	12
A	+	+	+	+	+							
B	+	+	+	+	+							
C	+	-	+	+	+							
D	-	+	+	-	+							
E	-	+	+	-	-							
F	-	-	-	+	-							
G	-	-	-	-	-							
H	-	-	-	-	-							

Fig. 4 Microtitration plate reading indicating presence or absence of CPE in respective wells

3 Calculation of End-Point Titer

3.1 Reed–Muench Method

Let us take an example for the determination of the end-point titer. The positive (+) sign in Fig. 4 indicates the presence of CPE and the minus (−) sign indicates no CPE.

The above results (Fig. 4) can be summarized in the tabular form as given in Table 1.

Accumulated values for the total number of wells showing CPE are obtained by adding in the direction of lowest to the highest values. The accumulated infected ratios and the percentage infected for each dilution are calculated.

In the example depicted in the Table 1 it can be seen that infectivity in the dilution 10^{-4}, is higher than 50% (67) and in the next higher dilution, 10^{-5} it is only 33%. Therefore, to find the 50% endpoint dilution, which obviously lies between these two dilutions (10^{-4} to 10^{-5}) first, proportionate distance (PD) is required to be calculated using a simple formula.

$$\mathrm{PD} = \frac{\%\text{Infection above } 50\% - 50\%}{\%\text{Infection above } 50\% - \%\text{Infection below } 50\%}$$

$$\mathrm{PD} = \frac{67 - 50}{67 - 33} = \frac{17}{34} = 0.50$$

The proportionate distance obtained, thus, has to be corrected to find out exactly 50% infectivity by the dilution factor, also called exponential of dilution (ED). The exponential of dilution of exactly 50% infectivity = PD × (ED next below 50% − ED next above 50%) + ED next above 50%.

$$\begin{aligned}\text{Log10 TCID}_{50} &= \mathrm{PD} \times (\text{ED next below } 50\% - \text{ED next above } 50\%) + \text{ED next above } 50\% \\ &= 0.50 \times [(-5) - (-4)] + (-4) \\ &= -4.50\end{aligned}$$

Table 1
Tissue culture infectivity data for determination of 50% end-point by Reed–Muench method

Virus dilution	Test results		Accumulative value		Ratio infected	% infected
	No. of culture infected	No. of culture not infected	Infected	Not infected		
10^{-1}	5	0	20	0	20/20	100
10^{-2}	5	0	15	0	15/15	100
10^{-3}	4	1	10	1	10/11	91
10^{-4}	3	2	6	3	6/9	67
10^{-5}	2	3	3	6	3/9	33
10^{-6}	1	4	1	10	1/11	9
10^{-7}	0	5	0	15	0/15	0

Table 2
Tissue culture infectivity data for determination of 50% end-point by Spearman–Karber method

Virus dilution	Ratio infected	Proportion infected
10^{-1}	5/5	1.0
10^{-2}	5/5	1.0
10^{-3}	4/5	0.80
10^{-4}	3/5	0.60
10^{-5}	2/5	0.40
10^{-6}	1/5	0.20
10^{-7}	0/5	0

Hence, the titer of the virus is: $10^{4.50}$ $TCID_{50}$/0.1 mL or $10^{5.50}$ $TCID_{50}$/mL.

3.2 Improved Karber Method

For determining end-point using the Karber method, the infectivity assay results given in Fig. 4 can be summarized as seen in Table 2.

TCID50 can be calculated by Karber method using the following formula:

$$\text{Log10 TCID50} = L - d(s - 0.5)$$

where L = log 10 of the most concentrated virus dilution tested, d = log dilution factor, s = sum of the proportion (1.0 + 1.0 + 0.80 + 0.60 + 0.40 + 0.20 + 0 = 4.0).

Table 3
Tissue culture infectivity data for determination of 50 % end-point by Ramakrishnan method (based on Fig. 4)

Virus dilution	Died/infected	Inoculated/replicates per dilution	Death score
10^{-1}	5	5	5/5 = 1.0
10^{-2}	5	5	5/5 = 1.0
10^{-3}	4	5	4/5 = 0.80
10^{-4}	3	5	3/5 = 0.60
10^{-5}	2	5	2/5 = 0.40
10^{-6}	1	5	1/5 = 0.20
10^{-7}	0	5	0/5 = 0

Therefore, Log10 TCID50 $= (-1) - (1)[4 - 0.5] = -4.50$

Hence, the titer of the virus would be $= 10^{4.50}$ $TCID_{50}$/0.1 mL, or $10^{5.50}$ $TCID_{50}$/mL.

3.3 Ramakrishnan Method

Ramakrishnan [11] has recently proposed two formulas for determining the virus titer using the end-point dilution method which can be used to quantify the virus in a given sample in addition to the existing methods, but not exclusively. The virus titer can be calculated as follows (Table 3).

Formula 1:

$$\mathrm{Log}_{10}50\%\text{end-point dilution} = -\left[\left(\frac{\text{total no.of animals died}}{\text{number of animals inoculated per dilution}}\right) + 0.5\right] \times \text{log dilution factor.}$$

$$\mathrm{Log}_{10}50\%\text{end-point dilution} = -[4 + 0.5] \times 1 = -4.50$$

Therefore, titer of the virus would be $= 10^{4.50}$ $TCID_{50}$/0.1 mL, or $10^{5.50}$ $TCID_{50}$/mL.

Formula 2 (if any accidental death occurred):

$$\mathrm{Log}_{10}50\%\text{end-point dilution} = -(\text{total death score} + 0.5) \times \text{log dilution factor} = -(4 + 0.5) \times 1 = -4.50$$

Hence, the titer of the virus would be $= 10^{4.50}$ $TCID_{50}$/0.1 mL, or $10^{5.50}$ $TCID_{50}$/mL.

The determination of virus titer as calculated using the three formulae gave the same results for virus quantification assay.

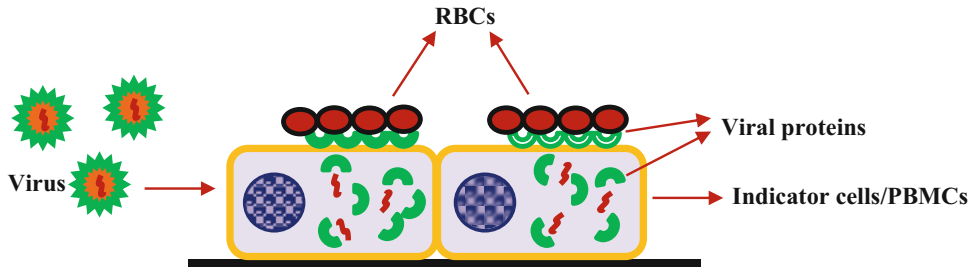


Fig. 5 Schematic representation of haemadsorption phenomenon

4 Haemadsorption Assay-Based Infectivity Assay for African Swine Fever Virus (ASFV)

The haemadsorption (HAD) test [12] is based on the characteristic of ASFV infected monocytes to form a rosette of erythrocytes around the infected cell. A positive result in the HAD test is definitive for ASF diagnosis and is represented as rosette formation around the infected leukocytes (Fig. 5).

The end-point titer is determined by any of the methods as described earlier and is represented as HAD units yielding a 50% of cumulative infection ($HADU_{50}$) per milliliter. The test is semi-quantitative in nature and the only limitation of this assay is the maintenance of primary cells which can be more difficult to manage than established cell lines [12].

Briefly, the HAD test is performed in Microtest I plates (Falcon) with 60 wells per plate, which is inoculated with five serial tenfold dilutions of a virus sample (12 wells per dilution). The indicator cell must be an ASFV-sensitive cell capable of adhering erythrocytes on its surface membrane when infected with the virus, as peripheral blood monocytes or alveolar macrophages, prepared from swine are used for ASFV in this assay. The detailed protocol for haemadsorption assay for ASFV has been described by Carrascosa and colleagues [13].

5 Chemical/Physical Methods of Virus Quantitation

Chemical/physical methods of virus quantitation measure the amount (or relative amount) of a viral protein, genome, or enzyme in a sample. Although these assays provide no information about the amount of infectious virus in a sample, they are often convenient, quick, and quite reproducible and can often be correlated back to infectivity assays as a quick way to estimate the infectivity of a sample. Few examples of such methods are as follows:

1. Direct visualization of virions by electron microscopy (EM).
2. Hemagglutination (HA) assay.
3. Genome quantification by PCR.
4. Serological assays.
5. Flow cytometry or flow virometry.

5.1 Direct Visualization of Virions by EM

With the advancements in electron microscopy, it has become possible to determine the virus titer [14]. However, it has some limitations like the cost of the procedure, the expertise required and the limited sensitivity (at least 10^6 particles/mL must be present) [15]. It is reported that the titer obtained by quantitative transmission electron microscopy (TEM) are often higher than the results from other assays as all particles, regardless of infectivity are quantified. Because of high instrument cost and the amount of space and support facilities needed, TEM equipment is available in a limited number of facilities and hence this method has limited utility in virus quantification.

5.2 Hemagglutination (HA) Assay

The viruses that have the ability to agglutinate the RBCs (e.g. influenza viruses) can be quantified by hemagglutination (HA) assay and the results are expressed in terms of hemagglutination units (HAU). It relies on the fact that hemagglutinin, a surface protein of influenza viruses, agglutinates red blood cells and causes red blood cells to clump together. The assay takes shorter time of around 1–2 hrs to complete and is based on the technical expertise of the operator. A haemagglutination assay has been developed for the detection of porcine circovirus 2 (PCV2) and it was found that the assay could detect $10^{4.09}$ $TCID_{50}$/mL of PCV2 [16]. The detection limit was found to be even lower than immunocapture ELISA for PCV2 which could detect as low as 400 $TCID_{50}$/mL of PCV2 [17]. Similarly, HA properties have also been reported for other porcine viruses such as porcine *Deltacoronavirus* [18], PRRSV [19], and porcine haemagglutinating encephalomyelitis virus [20].

5.3 Genome Quantification by PCR

Quantitative real-time PCR (qPCR) is a reliable, rapid, highly sensitive, and specific assay for nucleic acid quantification. Due to its ability to detect and measure minute amount of nucleic acid in a wide range of samples, it has become a yardstick in the field of molecular biology with its wide array of applications including microbial quantification, gene expression analysis, microarray verification, identification of transgenes, etc. Conventional quantitative PCR has already proven its utility in the monitoring of viral load as a useful marker of disease progression and as a component to study the effect of antiviral compounds [21–23]. The severity of a number of diseases has been linked to the viral load and thus real-time

PCR quantification can be used as an important tool to estimate viral load and to study its role in disease progression, latency, and reactivation [24–30].

Real-time polymerase chain reaction (PCR) or quantitative PCR (qPCR) is used in both ways; qualitative test for diagnosis of viral infections and as a quantitative test for quantifying the virus load. Quantitative detection is based on the fluorescence detection activity either by using fluorescent dyes such as SYBER Green or using sequence-specific probes tagged with a fluorophore attached to one end. The results of qPCR for virus quantification are expressed as genome copies/mL. The viral load can be quantified by two methods: relative quantification and absolute quantification. Relative quantification is based on the comparison between the expression of a target gene versus a reference/internal/endogenous control gene. Generally, relative quantification is sufficient on most occasions and is simpler to develop [28]. However, absolute quantification is useful when the results are required to be provided in terms of units or a sufficient number of reference/controls are not available or the quantification is required to access the progression or recovery from virus infection. Absolute quantification employs a standard curve to interpolate the copy number in a given sample. The standard curve is obtained by plotting Ct values (PCR cycles that show statistically significant increases in the product) obtained by testing a sample of known concentration against log-transformed concentrations of serial tenfold dilutions of the target nucleic acid.

In comparison to other methods of virus quantification, real-time PCR is far more convenient, reliable, and better suited to quick decision making in a clinical situation [30, 31]. In addition, qPCR can also provide information related to strain/genotype of virus along with the early diagnosis and quantification of virus which may help in adopting a timely treatment regimen for a specific viral infection. Other advantages of using qPCR include higher reproducibility and less inter and intra-assay variability as compared to other methods [28]. However, it is noteworthy that the quantitative estimates of qPCR are generally higher than other methods of virus quantification. This is due to the reason that qPCR amplifies all the target nucleic acids including infectious and non-infectious particles, defective interfering particles, and free nucleic acids in sample, while in other methods, either the virus particles (electron microscopy) or infective units are counted (end-point dilution assays).

Table 4
Common enzymes and substrate combinations used in ELISA

Enzyme	Complementary substrate	Wavelength for detection (nm)
Alkaline phosphatase (AP)	pNPP: p-nitrophenyl-phosphate	405
ß-galactosidase (ß-gal)	ONPG: Ortho-Nitrophenyl-β-galactoside	405
Horseradish peroxidase (HRP)	OPD: o-phenylenediamine dihydrochloride TMB: 3,3′,5,5′-tetramethylbenzidine	492 450

5.4 Serological Assays or Enzyme Linked Immunosorbent Assay (ELISA)

Quantitative serological assays such as ELISA are based on the antigen antibody interaction which is measured by enzyme's ability to convert a reagent to a detectable signal that can be used to calculate the concentration of the antigen in the sample [32]. The common enzymes and substrate combinations used in different ELISA formats are given in Table 4. ELISA can be used to detect and quantify both antigen and antibody in different formats and is comparatively less time consuming and high throughput method for simultaneous detection and quantification of protein antigens and antibody. Antigen detection ELISA can be performed in three formats which include direct ELISA, sandwich ELISA, and competitive ELISA. For quantification of viral antigen in ELISA test, the absorbance (OD values) of the test samples is compared with the standard curve obtained by parallel testing of sample containing known concentration of target protein. However, as these assays do not provide the results in absolute units, they cannot be used for absolute virus quantification.

Cell-ELISA can be used in conjunction with end-point dilution methods to determine virus titer in terms of $TCID_{50}$/mL. This strategy can be utilized in non-cytopathic viruses that do not cause visible CPE. In such cases, the cell monolayer is first infected with different dilutions of viruses as described in end-point dilution method and then after the incubation period is over, the monolayer is fixed with fixative agents such as paraformaldehyde followed by staining with antigen specific antibody conjugated to enzymes. The color development is then measured in ELISA plate reader after addition of substrate specific for the particular enzyme. The positive or negative results are recorded based on the OD values obtained for the non-infected control wells.

5.5 Flow Cytometry or Flow Virometry

Flow cytometry and its derivative fluorescence-activated cell sorting (FACS) have been methods of choice since the 1970s to analyze and purify individual cells. In early days, the quantification of viruses through flow cytometry was challenging due to the very

small size of virus particle in contrast to the resolution limit of standard flow cytometers, which was supposed to be about 300–500 nm [33]. Although flow cytometry has been used since long for virus characterization and enumeration [34] and the term "flow virometry" was given by Grivel et al. in 2013, it has been used for a wide array of viruses only recently [35].

In flow cytometry the number of intact virus particles is quantified when the fluorescent tagged virus particles are passed through a laser beam. The analysis of samples requires careful preparation of the samples, fixing, labeling, and heating to promote the penetrance of the dye [36, 37]. Dyes such as SYBR Green-I or SYBR Gold at 80 °C are used to stain the viral nucleic acids. For RNA viruses, potential RNA nucleic acid stains such as Styryl-TO TOTO-1, SYTO 12, and 14, SYTO RNASelect, and LDS 751 are employed [35]. The buffers in which the viruses are suspended previous to flow cytometric analysis are also important. Out of different buffers used for virus suspension which include tris-EDTA buffer, 5% sucrose-NTE buffer, PBS or 0.1–1% PFA, EDTA containing buffers have been reported to prevent the aggregate formation and allow the distribution of viruses during flow cytometric analysis [37–40]. To achieve the optimum results, final viral suspension should be filtered several times to remove the particulate matter and contaminants.

The results of flow cytometric analysis are represented in virus particles per mililitre (vp/mL). However, the quantification results are higher than that of end-point dilution methods and are comparable to electron microscopic assays (TEM). This is due to the reason that flow cytometry counts the infectious and non-infectious particles while end-point dilution methods only take into account the infectious virus particles only [41]. Bonar and colleagues showed that flow virometry is a much more sensitive technique when compared to ELISA and has similar detection of viruses to qPCR assays [42]. Overall although flow virometry requires sophisticated equipment and sample preparation, it is a very sensitive method for virus quantification with a linear working range of 10^5–10^9 vp/mL and an analysis time of ~10 min with a short sample preparation time.

6 Applications of Virus Quantification

6.1 Vaccine Production

Determination of virus load in vaccines is an important step to avoid any harmful effects related to vaccines. Generally, the amount of viral antigen in inactivated or live attenuated vaccines is represented in terms of $TCID_{50}$/mL. The virus quantification is also vital in the process of vaccine development and production as determination of ideal multiplicity of infection (moi) is very crucial to grow the virus in high titer in production facilities.

6.2 Antiviral Development

Before recommending the antivirals for treatment of a particular viral infection, a number of trials are conducted in vitro and in animal models to determine the safety and therapeutic limit of the concerned drug [43]). The specific antiviral activity is measured using a quantitative assay to measure virus replication in the presence of increasing concentrations of the product compared to replication in the absence of product. The effective concentration (EC_{50}) is the concentration of product at which virus replication is inhibited by 50%. The EC_{50} value is then used to calculate the therapeutic index (i.e., CC50 value/EC50 value) of an antiviral and it is desirable to have a high therapeutic index giving maximum antiviral activity with minimal cell toxicity.

6.3 Viral Therapeutics

Virus quantification is also applied as a tool to determine the response of the animals or individual to the treatment protocol. Besides this, quantification of virus titer, viral antigen or viral RNA in the patient can also be employed for management of viral diseases and determination of infectivity (transmissibility) of an individual to susceptible population [1].

6.4 Routine Virological Assays

Virus titer determination is a routine procedure in laboratories conducting the research in the field of virology. Virus quantification is performed and used as a guiding stone in number of assays such as virus neutralization test (VNT), growth kinetics studies, optimization of different assays such as ELISA and PCR. Besides this, virus titer is also taken into account during experimental animal studies for the purpose of raising hyper-immune sera and to determine the expression of certain proteins in host system of in vitro in response to different concentration of virus antigens.

7 Conclusion

Virus quantification is of prime importance for commercial and academic laboratories involved in the research or production of viral vaccines, recombinant proteins, viral antigens, or antiviral agents. The most common and accurate methods for determining the virus titer include plaque assay and 50% tissue culture infectious dose ($TCID_{50}$). These tests can be used to determine the infectious virus titer in a sample and there is no replacement for these tests. However, these tests are time consuming and need utmost caution as they are susceptible to failures due to contamination of cell culture system. Although with the advancement in science a number of other methods have been used for virus quantification including transmission electron microscopy (TEM), qPCR, ELISA, and flow virometry, there remain significant drawbacks associated with all of these assays. Although the modern methods

are rapid and provide results in a single day or span of hours, they require costly equipment and expertise for performing these assays. In addition, these assays are at backfoot when the infectious virus titer with high precision is required for sensitive virological procedures such as vaccine production and antiviral optimization. In general, there is still a need for new analytical methods that can rapidly quantify viral concentration to reduce costs and alleviate bottlenecks associated with current assays.

References

1. Berger A, Preiser W (2002) Viral genome quantification as a tool for improving patient management: the example of HIV, HBV, HCV and CMV. J Antimicrob Chemother 49 (5):713–721. https://doi.org/10.1093/jac/dkf050
2. Payne S (2017) Methods to study viruses. In: Viruses. Academic Press, London, pp 37–52
3. Fox JC, Kidd IM, Griffiths PD, Sweny P, Emery VC (1995) Longitudinal analysis of cytomegalovirus load in renal transplant recipients using a quantitative polymerase chain reaction: correlation with disease. J Gen Virol 76 (Pt 2):309–319. https://doi.org/10.1099/0022-1317-76-2-309
4. Mohanty SB, Dutta SK (1981) Cultivation and assay of viruses. In: Veterinary virology. Lea & Febiger, Philadelphia, PA
5. Bhatt M, Mohapatra JK, Pandey LK, Mohanty NN, Das B, Prusty BR, Pattnaik B (2018) Mutational analysis of foot and mouth disease virus nonstructural polyprotein 3AB-coding region to design a negative marker virus. Virus Res 243:36–43. https://doi.org/10.1016/j.virusres.2017.10.010
6. Baer A, Kehn-Hall K (2014) Viral concentration determination through plaque assays: using traditional and novel overlay systems. J Vis Exp (93):e52065. https://doi.org/10.3791/52065
7. Flint SJ, Enquist W, Racaniello VR, Skalka AM (2009) Virological methods. In: Principles of virology. ASM Press, Hoboken, NJ
8. Condit CR (2013) Principles of virology. In: fields virology, 6th edn. Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia
9. MacLachlan NJ, Dubovi EJ (2017) Virus replication. In: Fenner's veterinary virology, 5th edn. Academic Press, London
10. Pourianfar HR, Javadi A, Grollo L (2012) A colorimetric-based accurate method for the determination of enterovirus 71 titer. Indian journal of virology 23(3):303–310. https://doi.org/10.1007/s13337-012-0105-0
11. Ramakrishnan MA (2016) Determination of 50% endpoint titer using a simple formula. World J Virol 5(2):85–86. https://doi.org/10.5501/wjv.v5.i2.85
12. de León P, Bustos MJ, Carrascosa AL (2013) Laboratory methods to study African swine fever virus. Virus Res 173(1):168–179. https://doi.org/10.1016/j.virusres.2012.09.013
13. Carrascosa AL, Bustos MJ, de Leon P (2011) Methods for growing and titrating African swine fever virus: field and laboratory samples. Curr Protoc Cell Biol Chapter 26:Unit 26.14
14. Malenovska H (2013) Virus quantitation by transmission electron microscopy, TCID50, and the role of timing virus harvesting: a case study of three animal viruses. J Virol Methods 191(2):136–140
15. Fox JC, Emery VC (1992) Quantification of viruses in clinical samples. Rev Med Virol 2: 195–203
16. Cheng H, Yang L, Cai Z, Qiao X, Du L, Hou J, Chen J, Zheng Q (2020) Development of haemagglutination assay for titration of porcine circovirus type 2. Anal Biochem 598:113706
17. Huang L, Wei Y, Xia D, Liu D, Zhu H, Wu H, Li F, Liu C (2019) A broad spectrum monoclonal antibody against porcine circovirus type 2 for antigen and antibody detection. Appl Microbiol Biotechnol 103:3453–3464. https://doi.org/10.1007/s00253-019-09715-0
18. Zhang Y, Han L, Xia L, Yuan Y, Hu H (2020) Assessment of hemagglutination activity of porcine *deltacoronavirus*. J Vet Sci 21(1):e12. https://doi.org/10.4142/jvs.2020.21.e12
19. Jusa ER, Inaba Y, Kohno M, Mashimo H, Hirose O (1996) Hemagglutination with porcine reproductive and respiratory syndrome virus. J Vet Med Sci 58(6):521–527. https://doi.org/10.1292/jvms.58.521

20. Mora-Díaz JC, Piñeyro PE, Houston E, Zimmerman J, Giménez-Lirola LG (2019) Porcine Hemagglutinating encephalomyelitis virus: a review. Front Vet Sci 6:53. https://doi.org/10.3389/fvets.2019.00053
21. Clementi M (2000) Quantitative molecular analysis of virus expression and replication. J Clin Microbiol 38:2030–2036
22. Holodniy M, Katzenstein D, Sengupta S, Wang AM, Casipit C, Schwartz DH, Konrad M, Groves E, Merigan TC (1991) Detection and quantification of human immunodeficiency virus RNA in patient serum by use of the polymerase chain reaction. J Infect Dis 163:862–866
23. Menzo S, Bagnarelli P, Giacca M, Manzin A, Varaldo PE, Clementi M (1992) Absolute quantitation of viremia in human immunodeficiency virus infection by competitive reverse transcription and polymerase chain reaction. J Clin Microbiol 30(7):1752–1757. https://doi.org/10.1128/jcm.30.7.1752-1757.1992
24. Kearns AM, Turner AJL, Taylor CE, George PW, Freeman R, Gennery AR (2001) LightCycler-based quantitative PCR for rapid detection of human herpesvirus 6 DNA in clinical material. J Clin Microbiol 39:3020–3021
25. Kimura H, Morita M, Yabuta Y, Kuzushima K, Kato K, Kojima S, Matsuyama T, Morishima T (1999) Quantitative analysis of Epstein–Barr virus load by using a real-time PCR assay. J Clin Microbiol 37(1):132–136. https://doi.org/10.1128/JCM.37.1.132-136.1999
26. Lallemand F, Desire N, Rozenbaum W, Nicolas JC, Marechal V (2000) Quantitative analysis of human herpesvirus 8 viral load using a real-time PCR assay. J Clin Microbiol 38 (4):1404–1408. https://doi.org/10.1128/JCM.38.4.1404-1408.2000
27. Laue T, Emmerich P, Schmitz H (1999) Detection of dengue virus RNA inpatients after primary or secondary dengue infection by using the TaqMan automated amplification system. J Clin Microbiol 37(8):2543–2547. https://doi.org/10.1128/JCM.37.8.2543-2547.1999
28. Mackay IM, Arden KE, Nitsche A (2002) Real-time PCR in virology. Nucleic Acids Res 30 (6):1292–1305. https://doi.org/10.1093/nar/30.6.1292
29. Ohyashiki JK, Suzuki A, Aritaki K, Nagate A, Shoji N, Ohyashiki K, Ojima T, Abe K, Yamamoto K (2000) Use of real-time PCR to monitor human herpesvirus 6 reactivation after allogeneic bone marrow transplantation. Int J Mol Med 6(4):427–432. https://doi.org/10.3892/ijmm.6.4.427
30. Tanaka N, Kimura H, Iida K, Saito Y, Tsuge I, Yoshimi A, Matsuyama T, Morishima T (2000) Quantitative analysis of cytomegalovirus load using a real-time PCR assay. J Med Virol 60 (4):455–462. https://doi.org/10.1002/(sici)1096-9071(200004)60:4<455::aid-jmv14>3.0.co;2-q
31. Locatelli G, Santoro F, Veglia F, Gobbi A, Lusso P, Malnati MS (2000) Real-time quantitative PCR for human herpesvirus 6 DNA. J Clin Microbiol 38(11):4042–4048
32. Kemeny DM, Challacombe SJ (1988) ELISA and other solid phase immunoassays: theoretical and practical aspects. Wiley, Chichester
33. Chandler WL (2016) Measurement of microvesicle levels in human blood using flow cytometry. Cytometry B Clin Cytom 90:326–336. https://doi.org/10.1002/cyto.b.21343
34. Marie D, Brussaard CPD, Thyrhaug R, Bratbak G, Vaulot D (1999) Enumeration of marine viruses in culture and natural samples by flow cytometry. Appl Environ Microbiol 65 (1):45–52. https://doi.org/10.1128/AEM.65.1.45-52.1999
35. Lippé R (2018) Flow Virometry: a powerful tool to functionally characterize viruses. J Virol 92(3):e01765–e01717. https://doi.org/10.1128/JVI.01765-17
36. Khalil JY, Langlois T, Andreani J, Sorraing JM, Raoult D, Camoin L, La Scola B (2016) Flow cytometry sorting to separate viable giant viruses from amoeba co-culture supernatants. Front Cell Infect Microbiol 6:202. https://doi.org/10.3389/fcimb.2016.00202
37. Brussaard CP, Marie D, Bratbak G (2000) Flow cytometric detection of viruses. J Virol Methods 85(1-2):175–182. https://doi.org/10.1016/s0166-0934(99)00167-6
38. Chen F, Lu JR, Binder BJ, Liu YC, Hodson RE (2001) Application of digital image analysis and flow cytometry to enumerate marine viruses stained with SYBR gold. Appl Environ Microbiol 67(2):539–545. https://doi.org/10.1128/AEM.67.2.539-545.2001
39. Ferris MM, McCabe MO, Doan LG, Rowlen KL (2002) Rapid enumeration of respiratory viruses. Anal Chem 74(8):1849–1856. https://doi.org/10.1021/ac011183q
40. Landowski M, Dabundo J, Liu Q, Nicola AV, Aguilar HC (2014) Nipah virion entry kinetics, composition, and conformational changes determined by enzymatic virus-like particles and new flow virometry tools. J Virol 88 (24):14197–14206. https://doi.org/10.1128/JVI.01632-14
41. Shen CF, Meghrous J, Kamen A (2002) Quantitation of baculovirus particles by flow

cytometry. J Virol Methods 105(2):321–330. https://doi.org/10.1016/s0166-0934(02)00128-3

42. Bonar MM, Tilton JC (2017) High sensitivity detection and sorting of infectious human immunodeficiency virus (HIV-1) particles by flow virometry. Virology 505:80–90. https://doi.org/10.1016/j.virol.2017.02.016
43. CDER (2006) Guidance for industry antiviral product development — conducting and submitting virology studies to the agency. Center for Drug Evaluation and Research (CDER) at the Food and Drug Administration, Rockville, MD
44. Brussaard CP (2004) Optimization of procedures for counting viruses by flow cytometry. Appl Environ Microbiol 70:1506–1513. https://doi.org/10.1128/AEM.70.3.1506-1513.2004

Chapter 4

Protocols for Isolation of Genetic Materials from RNA Viruses

Nihar Nalini Mohanty, Vikas Gupta, Laxmi Narayan Sarangi, Rohini Bhat, and Sathish B. Shivachandra

Abstract

The isolation of viral RNA with purity and integrity is a critical element for the overall success of viral diagnosis. The era of classical virology has transcended way beyond the labor-intensive manual method of RNA extraction to the modern-age efficient and simpler protocols. With an aim to obtain a RNA material free from carry over contaminants such as protein, unwarranted cellular genome, and chemicals, etc. there are three major techniques followed worldwide such as organic extraction viz phenol-guanidine isothiocyanate (GITC)-based solutions, silica-membrane-based spin column technology, and paramagnetic particle technology. The method of extraction and the flow of processes within a particular method would vary with the type of material being handled. The major considerations while extracting RNA from tissue sample would be eliminating endogenous RNase that would compromise RNA integrity. The final step of RNA extraction is the storage of the isolated genome, which solely depend upon the purpose with which the extraction was carried out. If the sample is not intended for immediate application, then several commercially available formulations such as FORMAzol and RNA stable have been found suitable for long-term storage.

Key words RNA, Phenol-Guanidine, Silica-membrane, Paramagnetic particle

1 Introduction

The isolation of viral RNA with purity and integrity is a critical element for the overall success of viral diagnosis and RNA-based assays [1]. A low quality RNA may compromise the results of downstream applications which are often labor-intensive, time-consuming, and very expensive [2]. To ensure acceptable viral RNA quality, the RNA extraction procedure must fulfill a number of requirements with the final preparation must be free from protein, genomic DNA, nucleases, and carryover phenol or alcohol [3, 4], Buckingham,2007]. Structurally viruses could be divided into two major types (a) enveloped viruses and (b) non-enveloped viruses. In enveloped viruses, the nucleocapsid core is housed within a

Rajib Deb et al. (eds.), *Protocols for the Diagnosis of Pig Viral Diseases*,
Springer Protocols Handbooks, https://doi.org/10.1007/978-1-0716-2043-4_4,

glycolipid shell or envelope, whereas in case of the non-enveloped or naked viruses the nucleocapsid lacks the glycolipid cover. The presence and absence of shell though not remarkable but alters the accessibility to the viral RNA. The viral RNA encased within the capsid are broadly classified in two types single-stranded RNA and double-stranded RNA.

The extraction techniques vary significantly with the type of genetic material under study. There are three major techniques extensively used for RNA extraction: organic extraction, such as phenol-guanidine isothiocyanate (GITC)-based solutions, silica-membrane-based spin column technology, and paramagnetic particle technology. The method used for RNA isolation is very similar to those described for DNA; however, special precautions need to be taken during RNA isolation. RNA molecules are relatively short and single stranded, therefore easily damaged by mechanical shearing during the process of sample preparation. Furthermore, RNA is very vulnerable to digestion by ribonucleases (RNase) which are present endogenously in various concentrations in certain cell types as well as exogenously on hands and surfaces [5]. The extraction tactics must be formulated in such a way, so as to include sufficient management of endogenous RNase activity. Failure to eliminate potential sources of RNase contamination is likely to yield a degraded RNA sample for utility. Even traces of carry over contamination could compromise the RNA integrity. Since, the RNA have a short half-life, experiments involving RNA are most judiciously planned in close proximity of RNA isolation [6]. It is always important to use RNAse-free solution during extraction procedure as well as RNAse-free pipette tips and glassware/plastic wares.

Chemically and biologically, the RNA is more labile than DNA, particularly at elevated temperatures and in the presence of alkali. The activity of a variety of resilient ribonucleases (RNase), further adds to the handling difficulties. Therefore, proper collection, shipment, and processing of the sample specimens are crucial for diagnosis of diseases caused by RNA viruses. Proper maintenance of cold chain, use of virus transport medium, and addition of glycerol to the transport media are suggested to preserve the integrity of the infecting virus during sample transport. However, if the virion rupture, the RNA comes out to the solution and the endogenous nucleases degrades the RNA thereby resulting in false negative test results. In order to overcome such scenarios, many researchers are using solutions such as RNAlater which is a storage reagent that stabilizes the RNA thereby preserving the RNA integrity. It can be used both for preserving the RNA in the tissue and clinical samples but as far as the question of its utility, in storing extracted RNA is concered, it is debatable. Furthermore, the importance of ultra-low temperature shipment of the specimen has always been exigent.

Efficient methodologies have been designed for isolation of RNA, which in due course should be refined and optimized as per use. The protocol for isolation of viral RNA depends upon the base material which could be anything starting from cell pellet to, blood, tissue, cell culture supernatant, etc. It is important to consider the source and type of specimen during selection of the extraction procedure to be adopted; for example, the method/kit used for extraction of RNA directly from clinical specimen may not be suitable for extraction of RNA from virus infected cell culture supernatant. Irrespective of the source, the primary aim involves the release of the viral RNA from its protein and glycolipid housing and the cellular structures encompassing them. Protocols for the isolation of RNA begin with lysis mediated by buffers that typically consists of harsh chaotropic agents such as one of the guanidinium salts (guanidinium thiocyanate, guanidinium hydrochloride) sodium dodecyl sulfate (SDS), lithium dodecyl sulfate (LiDS), N-laurylsarcosine (sarcosyl), urea, phenol, or chloroform. All these agents act by disrupting the cellular and subcellular structures and help in releasing the viral RNA while maintaining an RNase-free environment. Appropriate steps must be taken to break through the cell wall in order to access the cellular contents. It is worth noting that an endless list of permutations on fundamental RNA extraction techniques exists; for example, some techniques support isolation of poly(A) material directly from a cellular lysate without prior purification of total RNA. As far as which RNA extraction procedure is to be used always depends on the investigator. It always helps in devising the proper extraction protocol and further downstream applications [6].

2 Important Considerations for Purification of the RNA from Clinical Specimen

Any worthwhile strategy for RNA purification must achieve specific objectives, if data derived from the final RNA preparation are to be meaningful and applicable for downstream processes. There are many procedures for isolating viral RNA from various biological sources, and each procedure in some way accomplishes these targets. One should follow few core concepts that accompanies most of the RNA extraction procedures, such as:

1. Selection of an appropriate method of membrane solubilization.
2. Ensuring total inhibition of nuclease activity.
3. Selection of a method for deproteinization of the sample.
4. Selection of a method for nucleic acid concentration.
5. Selection of proper storage conditions for purified RNA.

The first consideration towards viral RNA isolation strategy is the method of cellular disruption to release the viral RNA from its protein housing. The method of lysis will determine the extent of subcellular disruption of the sample and the subsequent access to the viral RNA. For example, a lysis buffer that is used successfully with tissue culture cells may be entirely inappropriate for whole tissue samples. The method by which membrane solubilization is accomplished will dictate the requirement of any additional steps that may be required in viral RNA extraction.

The second one is to inhibit nuclease activity. Some lysis reagents do act as strong nuclease inhibitor while others require additional nuclease inhibitors to safeguard the RNA during the isolation procedure.

The third factor is complete deproteinization of the sample. The complete removal of protein from a cellular lysate is of paramount importance in the isolation of both DNA and RNA. Proteinase K/Protease can be used to digest the proteins whereas RNAse-free DNAse can be added to degrade the contaminating DNA. Organic extraction using phenol and chloroform or dissolving the sample in guanidium salt buffers are also used for removal of the proteins.

Concentration of RNA is very important in the purification schemes. The most versatile method for concentrating nucleic acids is precipitation using various combinations of salt and buffer. For example, one common method is to add 0.1 vol of 3 M sodium acetate (pH 5.2) to a nucleic acid sample, followed by the addition of 2.5 vol of 95–100% ethanol. Addition of carrier RNA (usually poly(A)-homopolymers or other molecules that mimic nucleic acid) is another concept used by many researchers for concentration of RNA, especially for purification of RNA at low concentrations. To form a precipitate, a large number of molecules need to aggregate. In cell free sample specimens, viz. cerebrospinal fluid, synovial fluids, nasal secretions, etc. the concentration of RNA/DNA is low and addition of carrier RNA increases the yield of the RNA. Furthermore, it also protects the target RNA by reducing the damage caused by RNAses. It may not be required when sample material contains large quantity of DNA/RNAs such as blood, stool, Semen, etc., [6].

Because of the naturally labile character of RNA, improper storage of excellent RNA samples will often result in degradation in a relatively short time. There are many opinions as to the proper temperature, buffer, and storage for the purified viral RNA.

3 Lysis Buffer

There are a number of formulations available for lysis buffers which depends on the degree of cellular disruption required and RNase

inhibition. There is no one right way to extract RNA from cells and tissues the only thumb rule is to avoid RNase activity. Gentle lysis buffers such as NP-40 are normally not preferred due to their inability to inhibit the RNase activity and with the rupture of cell membrane the sequestered RNase are suddenly liberated compromising the RNA integrity.

3.1 Chaotropic Lysis Buffers

The best suited way to deal with stubborn RNases is to disrupt cells in guanidinium lysis buffer [7]. Guanidinium buffers efficiently denature and solubilize proteins, including RNase. It is not necessary to add additional RNase inhibitors to such lysis buffers, and even the RNA isolation procedures can be performed with room temperature reagents. RNA lysis buffers that contain guanidinium thiocyanate or guanidinium-HCl reproducibly yield very high-quality RNA samples, which is because of the extremely chaotropic nature of these chemicals. They have been reported to be the most effective protein denaturants [8–10]. The efficiency of protein denaturation, including disruption of RNases, may be enhanced by the inclusion of β-mercaptoethanol (β-ME) which acts to break intramolecular protein disulfide bonds. However common reducing reagent, dithiothreitol (DTT) should be avoided in this particular application because of its chemical reactiveness with guanidinium.

In this approach, the salient chemical differences between RNA, protein, and DNA are exploited by creating an acidic pH environment and judiciously blending organic solvents. As a result of the ease of this approach, numerous products and methodologies have been developed for the rapid, efficient purification of both RNA and DNA (and protein) from the same biological source [11, 12]. The principal drawback of all of these procedures is that it is very difficult to discriminate between cytoplasmic and nuclear RNA. Again the isolated RNA becomes susceptible to nuclease degradation when the denaturants have been removed. Therefore, it is necessary to guard against the RNase peril consistently.

3.2 Guanidinium Acid-Phenol Extraction

This method of extraction is a modification of the procedure of Chomczynski and Sacchi [13]. The highest quality RNA indisputably results from the extraction of RNA mediated by chaotropic lysis buffers, and guanidinium-containing buffers are among the most effective. Chomczynski and Sacchi [13] eased the purification of undegraded RNA by treatment of cells with guanidinium thiocyanate-containing lysis buffers, without subsequent CsCl ultracentrifugation. In subsequent modifications, the RNA is isolated in a very short time by extraction of a guanidinium cell or tissue lysate with an acidic phenol solution, followed by the addition of chloroform to facilitate partitioning of the aqueous and organic material. Now a days several formulations of guanidinium thiocyanate and phenol are readily available commercially [e.g.,

TRI Reagent (Molecular Research Center), TRIzol (Thermo Fisher Scientific)]. Upon phase separation, RNA is retained in the aqueous phase, while DNA and proteins partition into the organic phase. RNA is then recovered by precipitation with isopropanol and collected by centrifugation. These commercial formulations have become an inseparable partner to systems such as spin column or in magnetic bead format for ease in processing and to maximize purity [e.g., Direct-zol (Zymo Research) and QIAzol 1 RNeasy (Qiagen)]. In general, RNA can be efficiently isolated from less than 1 mg of tissue or 10^5 cells, usually in less than 1 h, using these procedures. A protocol by Farrell [6] is hereby exampled below for extraction of viral RNA and as such protocols are subject to differ from different labs. However, the overall principle of extraction remains fundamentally consistent.

1. Wear gloves and appropriate protective wear.
2. Harvest cells by centrifugation and re-suspend cell pellets in 100 μL of the following formulation (solution D) per 10^6 cells:
   Solution D:
   (a) 4 M guanidinium thiocyanate
   (b) 25 mM sodium citrate, pH 7.0
   (c) 0.5% sarcosyl
   (d) 100 mM β-ME.

   Pipette up and down or gently vortex in order to break up the cell pellet.
3. Transfer the lysate to a centrifuge tube. For each 1 mL of solution D lysis buffer used in **step 2**, add:
   (a) 0.1 mL 2 M sodium acetate, pH 5.2,
   (b) 1 mL water-saturated phenol (molecular biology grade),
   (c) 0.2 mL chloroform:isoamyl alcohol (49:1).

   Cap the tube and mix carefully and thoroughly by inversion following the addition of each reagent and invert vigorously for an additional 30 s after all reagents have been added.
4. Cool sample on ice for a minimum of 15 min; centrifuge at 4 °C at high speed to separate the phases.
5. Transfer aqueous (upper) phase containing the RNA to a fresh tube and mix with 0.75 vol of ice-cold isopropanol. Store at −20 °C for at least 1 h to precipitate RNA.
6. Collect precipitate by centrifugation at 10,000 × *g* for 20 min at 4 °C. Carefully decant and discard supernatant.
   Caution: Do not exceed the recommended maximum g-force for any of the tubes used in this protocol.

7. Completely dissolve RNA pellet in 300 μL of solution D (*see* **step 2**) and then transfer to a RNase-free 1.7 mL microfuge tube.
8. Reprecipitate the RNA by the addition of 0.75 vol of ice-cold isopropanol and store at −20 °C for 1 h.
9. Collect precipitate at 12,000 × *g* in a microcentrifuge for 10 min at 4 °C. Carefully decant and discard supernatant.
10. Wash pellet three times with 500 μL 70% ethanol per wash, followed by a final wash with 500 μL 95% ethanol. If the RNA does not dislodge during these washes, there is no need to re-centrifuge. Allow tubes to air-dry to remove residual ethanol. Store the RNA as an ethanol precipitate until it is to be used.

    Note: The final wash with 95% ethanol will accelerate the drying process.
11. Re-dissolve RNA in the smallest possible volume of TE (Tris EDTA)buffer or nuclease-free water. Incubation at 65 °C for 10 min may facilitate solubilization, though this is unnecessary if the RNA did not dry out completely following the ethanol washes. Following the determination of concentration, store the RNA in suitable aliquots at −80 °C. Avoid repeated freezing and thawing.

4 Density Gradient Centrifugation

The several procedures for the isolation of viral RNA from biological sources enriched in RNase employs cell and tissue disruption with guanidinium buffer. The resulting intermixing of subcellular components mandates the separation of these biochemical macromolecules from each other, with particular regard to the removal of DNA from RNA preparations. This is attributable to the measurable differences in the densities of DNA, RNA, and protein which allow partitioning by banding or pelleting them in a density gradient. Isopycnic centrifugation, a type of density gradient centrifugation, is a technique in which macromolecules move through a density gradient until they find a density equal to their own. Macromolecules therefore accumulate at that position in the gradient, floating there until the end of the centrifugation run. In some cases, RNA is of greater density than any position in the gradient and accumulates as a pellet at the bottom of the tube. The classical gradients used for this type of separation are cesium chloride (CsCl), cesium trifluro acetate (CsTFA), and cesium sulfate (Cs_2SO_4). Other materials such as glycerol, ficoll, metrizamide, and sucrose also have specific applications; they are readily available at low cost and high purity. Nucleic acid resolution based on

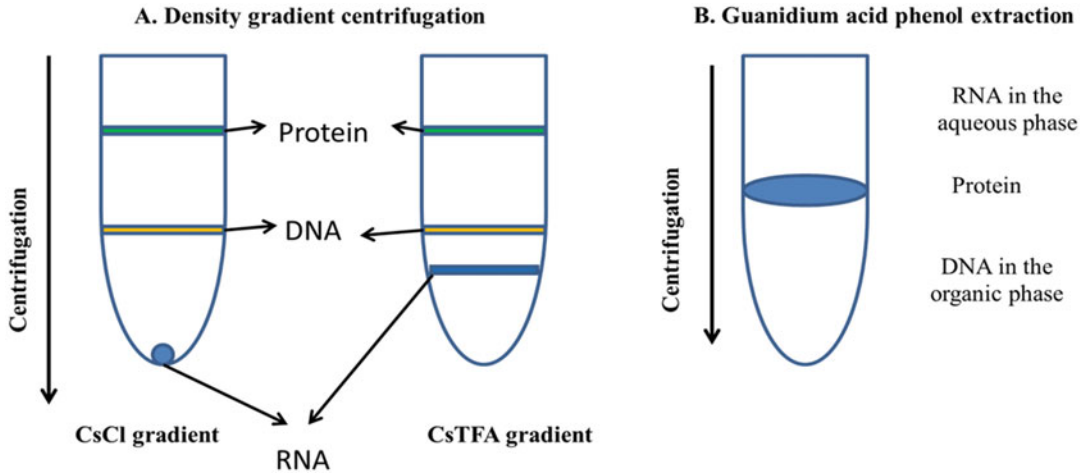


Fig. 1 Nucleic acid resolution based on differences in density

differences in density is best performed using one of the cesium salts (*see* Fig. 1).

CsTFA (GE Healthcare Life Sciences) is an excellent CsCl alternative for density gradient separation of RNA and DNA by isopycnic centrifugation. At ultracentrifugation RCFs, CsTFA will likewise self-form a gradient. The CsTFA has the advantage of easy dissociation of proteins from nucleic acids, banding of RNA, simultaneous recovery of DNA, RNA, and protein from the same gradient, greater yield of RNA and efficient inhibition of RNase activity. However, the requirement for a very expensive ultracentrifuge, the relatively long duration of centrifugation, the labor-intensive clean-up of gradient-purified RNA, and fewer samples at a time has replaced isopycnic centrifugation with newer reagents and kits [6].

A protocol, as described by [14], has been highlighted below for extraction by isopycnic extraction of RNA.

- Extraction buffer.
  - 100 mM Tris–HCl, pH 7.5.
  - 50 mM EDTA, pH 8.0.
  - 500 mM NaCl.
  - 5% β-Mercaptoethanol.
- CsCl Cushion.
  - 96% CsCl.
  - 100 mM EDTA, pH 8.0.
- 14% Sarcosyl.
- 1 M NaCl.

4.1 Procedure

The following method is based on 0.5 g of starting material but it can be applied to larger tissue samples by increasing buffer volumes and the size of the ultracentrifuge tube.

1. Homogenize 0.5 g of tissue in liquid nitrogen and transfer the resulting powder to a 15 mL centrifuge tube.
2. Immediately add 3.33 mL of extraction buffer and 1 mL of 14% Sarcocyl, vortex briefly.
3. Add 0.65 g of CsCl powder, mix well, and incubate at 65 °C for 15 min.
4. Centrifuge at 4 °C for 20 min at 9500 rpm/9383 × *g* (C1015 in GS-15/Allegra21/Allegra X-22 Series rotor).
5. Filter the supernatant through Miracloth (Calbiochem) and store on ice while preparing the CsCl cushion.
6. Add 1.6 mL of CsCl to a 5 mL ultracentrifuge tube.
7. Apply 3.4 mL of the filtered supernatant carefully to the top of the cushion.
8. Centrifuge at 20 °C for 20 h at 40,000 rpm/2,84,061 × *g* (SW40Ti rotor).
9. The centrifugation results in two phases. Genomic DNA is located at the interface, whereas RNA is located at the bottom of the tube below the cushion phase. Remove 1/3 of the upper phase and carefully add a similar volume of DEPC(Diethylpyrocarbonate) water to the tube (referred to subsequently as a wash step).
10. Repeat this washing step 2 times removing more of the upper phase each time without disturbing the cushion. Make sure to remove the genomic DNA at the interface is completely.
11. Remove ½ of the cushion and wash with DEPC water.
12. Remove the second half of the cushion, re-suspend the RNA in 133 μL DEPC water, and transfer to a 1.5 mL microcentrifuge tube.
13. Add 2.5 vol absolute ethanol (EtOH), mix, and incubate at −20 °C for a minimum of 2 h.
14. Centrifuge at 4 °C for 30 min at 1000 rpm/10,732 × *g* (S0410 rotor) in a microcentrifuge.
15. Wash the RNA pellet in 0.5 mL 70% ethanol. Repeat the centrifugation and re-suspend the pellet in 133 μL DEPC water.
16. Precipitate overnight by adding 1/10 vol of 1 M NaCl and 2.5 vol of absolute EtOH.

17. Centrifuge at 4 °C for 30 min at 10,000 rpm, wash with 0.5 mL 70% EtOH, air-dry at room temperature, and re-suspend the pellet in 30–50 μL of DEPC water.
18. Store the RNA at −80 °C.

The concentration and quality of the RNA is determined by measuring the absorbance at both 260 nm and 280 nm. The purity of RNA is estimated by the ratio of absorbance at 260–280 nm with RNA absorbing at 260 nm and proteins absorbing at 280 nm. A ratio of 1.8–2.0 indicates high purity.

Molecule	Density (g/mL)
Protein	1.2–1.5
DNA	1.5–1.7
RNA	1.8–2.0

5 Silica Technology

Currently, the use of glass fiber filters (a.k.a. silica binding technology) is the most popular method for small-scale RNA isolation and is used in conjunction with guanidinium-based cell lysis. The silica filter columns that are small enough for use with a standard microcentrifuge. These filters consist of glass microfibers that are positioned in the bottom of small plastic column that fits inside a standard 1.5 mL microcentrifuge tube. The nucleic acid purification and clean-up procedure is efficient and is performed in a remarkably short time. In general, RNA (or DNA) binds to silica in a high-salt, chaotropic environment. Such affinity-based nucleic acid isolation is referred to as solid-state extraction. Following a series of washes, the purified material is eluted from the silica matrix under very low-salt conditions and in a very small elute (*see* Fig. 2). Care should be taken to avoid any of the ethanol-containing wash buffer in the eluted RNA, which will make the sample completely useless for applications such as electrophoresis (the sample will float out of the well, even after the addition loading buffer) and for any enzymatic manipulation (due to enzyme inhibition).

For example, a protocol from a commercial kit (Thermo Scientific GeneJET Viral DNA and RNA Purification Kit #K0821) for extraction of viral RNA is detailed. This is a sample protocol for RNA purification from 200 μL of EDTA- or citrate-treated plasma, serum, blood or milk samples. Similar such protocols are available from other manufacturers which could be referred for the use in one laboratory.

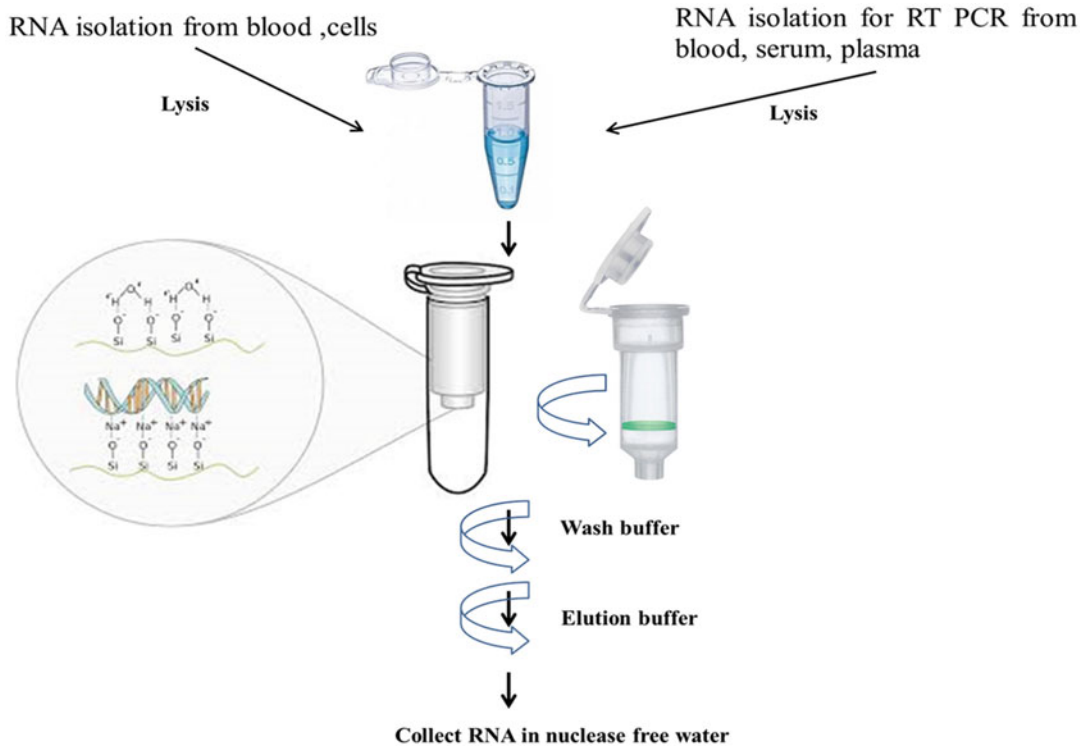


Fig. 2 Silica-based purification

5.1 Step Procedure

1. Add 50 μL of column preparation liquid to the center of spin column membrane, so that the membrane is entirely moistened.

   Notes:

   (a) Before starting the procedure, each new spin column must be prepared by treating it with Column Preparation Liquid. Column treatment maximizes binding of the nucleic acids to the membrane, resulting in more consistent yields.

   (b) Do not centrifuge the prepared column. The prepared column should be stored at room temperature until it is used for sample processing.

2. Load 200 μL of sample to an empty 1.5 mL microcentrifuge tube.

   Add 200 μL of Lysis Solution (supplemented with Carrier RNA) and 50 μL of Proteinase K, mix thoroughly by vortexing or pipetting.

3. Incubate the sample for 15 min at 56 °C in a thermomixer/water bath. Leave thermomixer turned on for eluent preheating during later steps of the procedure.

4. Centrifuge for 3–5 s at full speed to collect any sample solution from the inside of the lid.

   Note: Supplement Lysis Solution with Carrier RNA prior to use.

5. Add 300 µL of ethanol (96–100%) and mix by pipetting or vortexing. Incubate the sample at room temperature for 3 min.
6. Centrifuge for 3–5 s at full speed to collect drops from the inside of the lid.
7. Transfer the lysate to the prepared spin column preassembled within the wash tube.
8. Centrifuge the column for 1 min at 6000 × *g*.
9. Discard the wash tube containing flow-through.
10. Place the spin column into a new 2 mL wash tube.

    Notes:

    (a) Ensure that a new spin column has been prepared as described in **step 1**.

    (b) Close the bag with spin columns tightly after each use.

11. Add 700 µL of wash buffer 1 supplemented with ethanol to the spin column.
12. Centrifuge the column for 1 min at 6000 × *g*.
13. Discard the wash tube containing flow-through.
14. Place the spin column into a new 2 mL wash tube.
15. Add 500 µL of wash buffer 2 supplemented with ethanol to the spin column.
16. Centrifuge the column for 1 min at 6000 × *g*.
17. Discard the wash tube containing flow-through.
18. Place the spin column into a new 2 mL wash tube.

    Note: Supplement concentrated wash buffer 2 with ethanol prior to the first use.

19. Add 500 µL of wash buffer 2 supplemented with ethanol to the spin column.
20. Centrifuge the column for 1 min at 6000 × *g*.
21. Discard the wash tube containing flow-through.
22. Place the spin column into a new 2 mL wash tube.
23. Centrifuge the column for 3 min at 16,000 × *g*.
24. Discard the wash tube containing remaining flow-through.
25. Place the spin column into a new 1.5 mL elution tube.
26. Add 50 µL of eluent preheated to 56 °C to the center of spin column membrane.
27. Incubate for 2 min at room temperature.

28. Centrifuge the column for 1 min at 13,000 × *g*.
29. Discard the spin column.

    Note: Lower volume of eluent (30–40 μL) can be used in order to concentrate eluted nucleic acids. Larger elution volumes (up to 100 μL) can also be used but may result in the dilution of the viral nucleic acid sample. Keep the elution tube containing pure viral nucleic acids. Use the purified nucleic acids immediately or store at −20 °C or −70 °C.

6 Affinity Matrices

In addition to the approaches described above for the isolation of RNA using silica technology, there are products available which capture polyadenylated transcripts/RNA. For example, mRNA isolation kits with tracts of oligo(dT) covalently linked to a solid support. In such cases the polyadenylated transcripts are captured through canonical base-pairing between the poly(A) tail and the oligo(dT) in a high-salt environment. The linkage material could be cellulose, polystyrene, and/or latex beads. An older type of affinity selection in which poly(A)1 mRNA was affinity-captured using a column packed with poly(U) linked to sepharose beads [15] is no longer favored because of the relatively poor binding capacity of poly(U) matrices and the fact that quantitative recovery of RNA from a poly(U) matrix usually required formamide-based elution buffers. The nucleic acid species on the column is often referred to as the bait, while the target that can bind to it are known as the prey.

7 Purification Using Magnetic Beads

Magnetic beads are made up of tiny (20–30 nm) particles of iron oxides, such as magnetite (Fe_3O_4), which gives them super paramagnetic properties. Super paramagnetic beads are different to ferromagnets in a manner that they exhibit magnetic behavior only in the presence of an external magnetic field, which enables the beads to be separated in suspension, along with the target they are bound to. Since they do not attract each other outside of a magnetic field, they can be used without any concern about unwanted clumping. The types of magnetic beads are based on the different surface coatings and chemistries which give each type of bead its own binding properties.

This extraction approach relies on using magnetic beads with a coating that can bind nucleic acids reversibly by just adjusting buffer conditions. After binding target nucleic acid, an external magnetic field attracts the beads to the outer edge of the tube, thus immobilizing them. While the beads are immobilized, the

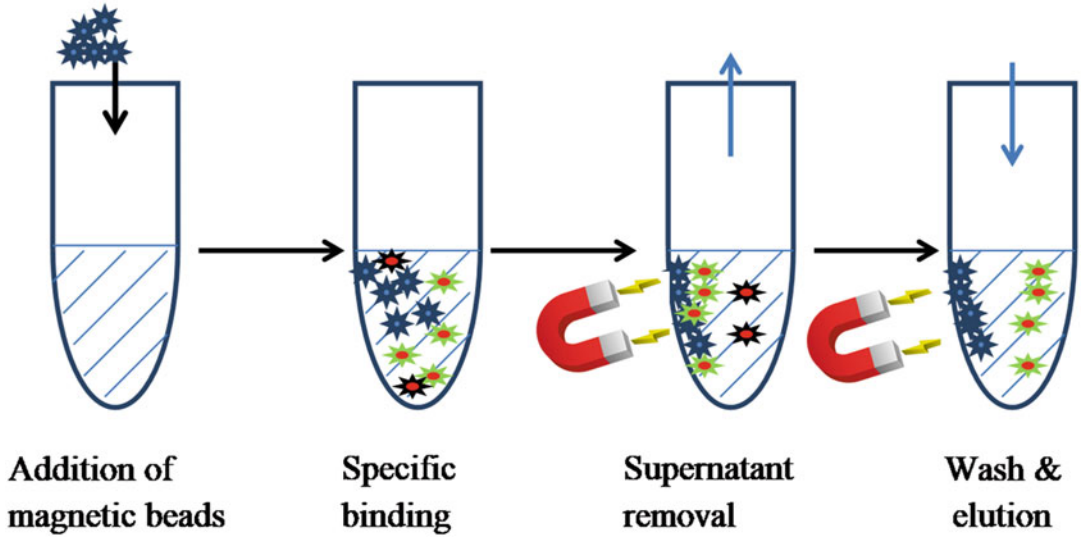


Fig. 3 Magnetic bead-based purification

bead-bound nucleic acid is retained during the washing steps. Adding elution buffer, and removing the magnetic field then releases the nucleic acid as a purified sample, ready for quantification and analysis.

This approach removes the need for vacuum or centrifugation, which minimizes stress or shearing forces on the target molecules, requires fewer steps and reagents than other extraction protocols. An illustration is provided in Fig. 3.

8 Separation of Double-Stranded RNA from Single-Stranded RNA

There are viruses which houses double-stranded RNA such as viruses belonging to family *Reoviridae*. Double-stranded RNA can be isolated from cells and tissues by several different methods. The use of conventional techniques for extraction and purification of dsRNA from the infected cell culture is tedious and time-consuming. Hence, double-stranded RNA is first isolated and purified with phenol-chloroform extraction followed by differential lithium chloride (LiCl) precipitation to remove the single-stranded RNA (ssRNA). This involves several sequential steps and takes around 30–48 h to purify the dsRNA bluetongue virus (BTV) [16].

The procedure is based on differential solubility of different types of nucleic acid in LiCl. LiCl offers major advantages over other RNA precipitation methods in that it does not precipitate DNA, protein or carbohydrate [17]. LiCl precipitation also removes inhibitor of translation or cDNA synthesis from RNA preparations [18].

A much quicker method, which requires only 24 h for purification of dsRNA, has been described by Attoui et al. [19]. This method is currently in vogue for purification of dsRNA of BTV and other dsRNA viruses [20–23]. In this method the total RNA pellet is dissolved in 100 μL of RNase-free water, mixed with an equal volume of 4 M LiCl solution and incubated overnight at 4 °C. This is followed by centrifugation at 18,000 × *g* for 5 min to precipitate ssRNA. The dsRNA in the supernatant is precipitated at −20 °C for 2 h by addition of 200 μL of isopropanol and 50 μL of 7.5 M ammonium acetate, and pelleted by centrifugation at 18,000 × *g*. the pellet is finally washed with 75% ethanol.

The dsRNA can also be purified by CF11 cellulose chromatography [24]. This is a rapid method but requires column conditions to be strictly followed and has not been in wide use.

Several authors have used nuclease such as RNaseT1for degradation of ssRNA from a mixed population of ssRNA and DsRNA. Several nucleases are available commercially which are used which have been used as per suitability for differentiation, characterization, and purification of RNA.

A protocol for extraction of double-stranded RNA [25] is briefed as follows:

1. Infected cells were pelleted by centrifugation at 500 × *g* for 10 min at 4 °C.
2. Pellet was re-suspended in 1 mL of lysis buffer (1% SDS, 0.1 M sodium acetate, pH 5.0) and incubated at 37 °C for 30 min.
3. 100 μg/mL proteinase K (Final concentration) was added to it and incubated at 56 °C for 1 h.
4. The mixture was then centrifuged twice (12,000 × *g* for 15 min at 4 °C) with an equal volume of 3:2 phenol-chloroform suspensions.

   Note: phenol guanidinium method or commercially available combinations such as Trizol could also be used.
5. The aqueous phase was removed and mixed with two volumes of ethanol and 0.3 M sodium acetate, pH 5.0 (final concentration).
6. Precipitated at −70 °C for 2 h.
7. Total RNA was recovered by centrifugation (12,000 × *g* for 15 min at 4 °C) and dissolved in 0.5 mL Tris-EDTA (TE, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA).
8. Single-stranded RNA (ssRNA) was removed by precipitation at 4 °C for 8 h with 2 M LiCl (Final concentration).
9. The supernatant, which contained dsRNA was precipitated with 4 M LiCl (Final concentration) at 4 °C for 8 h. The dsRNA was recovered by centrifugation (12,000 × *g* for 15 min at 4 °C).

10. The pellet was washed with 75% ethanol and re-suspended in 20 μL TE by heating at 60 °C for a few minutes.

9 Filter Paper Matrix Cards

Detection and characterization of viral pathogens from field samples owing to the instability of RNA molecules has always remained challenging. Further the need of appropriate transport temperature and media in resource poor settings mostly compromise the test results. In recent developments filter paper-based system in specimen collection and transport is gaining momentum as it maintains the integrity of the nucleic acid during sample transport and storage [26]. In this process the test samples (blood, plasma, serum, cerebrospinal fluid, tongue epithelium, etc.) is spotted onto the specialized filter paper and allowed to dry. These specialized filter papers are impregnated with chemical mixture that lyse the cells and organelles and also inactivate the pathogenic virus upon contact. The nucleic acid is entangled in the matrix and remains tightly bound. The proteins including the ribonucleses are denatured and thus allowing the long storage of the nucleic acid for longer period. Literatures have cited several researches that have evaluated these cards by storing the card at temperature ranging from −20 to 37 °C (median 25 °C) for period varying from 1 to 8 months (median 30 days). At the laboratory, the viral RNA is retrieved from the matrix depending upon the type of filter paper used. The purified RNA is then used for molecular detection assays. These specialized cards have been successfully used for molecular detection of various RNA pathogens such as porcine reproductive and respiratory syndrome virus (+ssRNA), avian influenza virus (-ssRNA), measles (-ssRNA), foot and mouth disease virus (+ssRNA), infectious bronchitis virus (+ssRNA), infectious bursal disease virus (ds RNA), rabies virus (-ssRNA), west nile virus (+ssRNA), etc. (Reviewed by in [27]). Several such filer papers could be sourced from FTA® [(Flinders Technology Associates) cards, GE Healthcare Life Sciences] which are most commonly used [27]. The other filter paper matrix that has been used for detection of infectious disease diagnosis include ADVANTEC chromatography paper No. 526, ADVANTEC filter paper No. 2, Whatman filter paper 903.

10 Storage of Purified RNA

The correct storage conditions for the purified viral RNA samples have remained pivot for any study. Improper storage, over a period of merely a few hours or as long as several months, is likely to have a profound negative impact on the probable utility of purified RNA.

The key considerations that have to be taken into account is the source of RNA and the timeline within which the RNA sample is to be used and the solution/buffer in which the RNA has been dissolved.

Purified RNA is most stable when stored as an ethanol precipitate at −80 °C. Under these conditions RNA can be stored confidently for several months or even longer.

RNA species also show enhanced stability when stored for weeks in hydrated form at −20 °C. If the sample is to be used within the week of purification, the RNA may be stored stably at −20 °C, again as an ethanol precipitate.

The "clock begins ticking" when a purified sample of RNA is dissolved in aqueous buffer, either sterile water or modified TE buffer (10 mM Tris; 0.1 mM EDTA; overall pH 7.5). TE buffer has the advantage of being able to chelate the magnesium ions that are often carried over from certain isolation procedures, and the low EDTA concentration is unlikely to cause problems in downstream PCR procedures. The low concentration of EDTA can prevent magnesium-induced strand breakage in later steps of an isolation procedure, particularly at elevated temperatures. The most suited action is to determine the RNA concentration and then store the remaining RNA sample in suitable aliquots at −80 °C.

Conventionally, RNA has also been stored in varying concentrations of SDS, VDR, and ultrapure formamide. However, the incomplete removal of these "protectant" compounds will reduce the utility of the RNA in most downstream applications. Other options for long-term storage of RNA include highly purified 100% formamide [28], a commercially available stabilized form of formamide known as FORMAzol (Molecular Research Center, Cincinnati, OH). Some authors have found the use of FORMAzol very promising for preserving RNA and have successfully demonstrated the stability up to 2 years at 20 °C. RNAstable (Biomatrica, San Diego, CA) is suitable for long-term storage or shipping of purified samples of RNA. The manufacturers claim that RNA stable can preserve the total RNA/ mRNA/ miRNA for 12 years at ambient temperature and thereby they can eliminate sample loss due to freeze/thaw cycles [29].

References

1. Wink M (2006) An introduction to molecular biotechnology: molecular fundamentals, methods and application in modern biotechnology. Wiley-VCH, Weinheim
2. Tan SC, Yiap BC (2009) DNA, RNA, and protein extraction: the past and the present. J Biomed Biotechnol
3. Cseke LJ, Kaufman PB, Podila GK, Tsai C-J (2004) Handbook of molecular and cellular methods in biology and medicine, 2nd edn. CRC Press, Boca Raton, FL
4. Buckingham L, Flaws ML (2007) Molecular diagnostics: fundamentals, methods, & clinical applications. F.A. Davis, Philadelphia, PA
5. Amani P, Hofmann A (2018) Basic principles. In: Hofmann A, Clokie S (eds) Wilson and Walker's principles and techniques of biochemistry and molecular biology.

Cambridge University Press, Cambridge, pp 8–39. https://doi.org/10.1017/9781316677056.004
6. Farrell RE (2017) RNA isolation strategies. In: RNA methodologies, 5th edn. Academic Press, pp 75–115. https://doi.org/10.1016/B978-0-12-804678-4.00003-8
7. Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294–5299
8. Cox RA (1968) The use of guanidinium chloride in the isolation of nucleic acids. In: Grossman L, Moldave K (eds) Methods in enzymology, vol 12. Academic Press, Orlando, FL, pp 120–129, part B
9. Gordon JA (1972) Denaturation of globular proteins. Interaction of guanidinium salts with three proteins. Biochemistry 11: 1862–1970
10. Nozaki Y, Tanford C (1970) The solubility of amino acids, diglycine, and triglycine in aqueous guanidinium hydrochloride solutions. J Biol Chem 245:1648–1652
11. Chomczynski P (1993) A reagent for the single-step simultaneous isolation of RNA, DNA, and proteins from cell and tissue samples. BioTechniques 15:532–536
12. Majumdar D, Avissar YJ, Wyche JH (1991) Simultaneous and rapid isolation of bacterial and eukaryotic DNA and RNA: a new approach for isolating DNA. BioTechniques 11(1): 94–101
13. Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159
14. Ott T, Krusell L, Udvardi M (2005) RNA isolation using CsCl gradients. In: Lotus japonicus handbook. Springer, Dordrecht, pp 125–128
15. Lindberg U, Persson T (1974) Messenger RNA isolation with poly(U) agarose. Methods Enzymol 34:496–499
16. Squire KRE, Chuang RY, Osburn BI, Knudson DL, Doi RH (1983) Rapid methods for comparing the double-stranded RNA genome profiles of bluetongue virus. Vet Microbiol 8(6): 543–553
17. Barlow JJ, Mathias AP, Williamson R, Gammack DB (1963) A simple method for the quantitative isolation of undegraded high molecular weight ribonucleic acid. Biochem Biophys Res Commun 13:61–66
18. Cathala G, Savouret J, Mendez B, West BL, Karin M, Martial JA, Baxter JD (1983) A method for isolation of intact, translationally active ribonucleic acid. DNA 2:329–335
19. Attoui H, Billoir F, Cantaloube JF, Biagini P, de Micco P, de Lamballerie X (2000) Strategies for the sequence determination of viral dsRNA genomes. J Virol Methods 89(1–2):147–158
20. Potgieter AC, Steele AD, Van Dijk AA (2002) Cloning of complete genome sets of six dsRNA viruses using an improved cloning method for large dsRNA genes. J Gen Virol 83(9): 2215–2223
21. Singh KP, Maan S, Samuel AR, Rao S, Meyer AJ, Mertens PPC (2004) Phylogenetic analysis of bluetongue virus genome segment 6 (encoding VP5) from different serotypes. Vet Ital 40(4):479–483
22. Potgieter AC, Page NA, Liebenberg J, Wright IM, Landt O, Van Dijk AA (2009) Improved strategies for sequence-independent amplification and sequencing of viral double-stranded RNA genomes. J Gen Virol 90(6):1423–1432
23. Maan S, Rao S, Maan NS, Anthony SJ, Attoui H, Samuel AR, Mertens PP (2007) Rapid cDNA synthesis and sequencing techniques for the genetic study of bluetongue and other dsRNA viruses. J Virol Methods 143 (2):132–139
24. Herring AJ et al (1982) Rapid diagnosis of rotavirus infection by direct detection of viral nucleic acid in silver-stained polyacrylamide gels. J Clin Microbiol 16(3):473–477
25. Chomczynski P (1992) Solubilization in formamide protects RNA from degradation. Nucleic Acids Res 20:3791–3792
26. Biswas SK, Chand K, Pandey AB, Mondal B (2016) Experimental protocol for quick extraction of double-stranded rna (dsRNA) of bluetongue virus (BTV) from infected BHK-21 cells with ribonuclease T1 (RNAse T1) at different concentrations. Anim Sci 10(1)
27. Smith LM, Burgoyne LA (2004) Collecting, archiving and processing DNA from wildlife samples using FTA® databasing paper. BMC Ecol. 4, 4
28. Cardona-Ospina JA, Villalba-Miranda MF, Palechor-Ocampo LA, Mancilla LI, Sepúlveda-Arias JC (2019) A systematic review of FTA cards® as a tool for viral RNA preservation in fieldwork: Are they safe and effective?. Prev Vet Med 172:104772
29. Stevenson HS, Wang Y, Muller R, Edelman DC (2015) Long-term stability of total RNA in RNAstable® as evaluated by expression microarray. Biopreserv Biobank 13(2): 114–122. https://doi.org/10.1089/bio.2014.0068

Chapter 5

Multiplex PCR for Diagnosis of Porcine Diseases

Manjisha Choudhury, Ajay Kumar Yadav, Seema Rani Pegu, Rajib Deb, and Swaraj Rajkhowa

Abstract

Molecular diagnostics have revolutionized the efficiency of diagnosis of infectious diseases. An accurate diagnosis of a disease would prevent further infestation of infections in the healthy population. Although several laboratory techniques exists such as cell-culture, serological, and molecular based methods that were developed in the past decades for highlighting these diseases, however, because of their robustness, high sensitivity, specificity, rapidness, and suitability of the range of types of samples that can be analyzed, nucleic acid amplification tests are suitable for the diagnosis of pathogenic infections. Since the development of polymerase chain reaction (PCR), this molecular technique has been widely utilized as a nucleic acid amplification tool and besides being utilized as a laboratory tool, it has proved to have an exceptional potential in clinical applications, including detection of specific or broad spectrum pathogens, evaluation of emerging novel infections, disease surveillance, early threat of bio-threat agents, and antimicrobial resistance profiling. The multiplex PCR is the technique where we can simultaneously detect more than one organism in a single reaction. The multiplexing may be done either with conventional PCR or with real time PCR. This technique provides detection of mixed infection including both viruses and bacteria, time saving and cost effective.

Key words Molecular diagnostics, Polymerase chain reaction (PCR), Multiplex PCR, Multiplex qPCR, Mixed infections, Rapidness

1 Introduction

Molecular diagnostics have revolutionized the efficiency of diagnosis of infectious diseases. An accurate diagnosis of a disease would prevent further infestation of infections in the healthy population. Although several laboratory techniques exists such as cell-culture, serological, and molecular based methods that were developed in the past decades for highlighting these diseases, however, because of their robustness, high sensitivity, specificity, rapidness, and suitability of the range of types of samples that can be analyzed, nucleic acid amplification tests (NAATs) are suitable for the diagnosis of pathogenic infections. Since the development of polymerase chain

Rajib Deb et al. (eds.), *Protocols for the Diagnosis of Pig Viral Diseases*,
Springer Protocols Handbooks, https://doi.org/10.1007/978-1-0716-2043-4_5,

reaction (PCR) in 1985, this molecular technique has been widely utilized as a nucleic acid amplification tool and besides being utilized as a laboratory tool, it has proved to have an exceptional potential in clinical applications, including detection of specific or broad spectrum pathogens, evaluation of emerging novel infections, disease surveillance, early threat of bio-threat agents and antimicrobial resistance profiling. The initial application of PCR for diagnosis of porcine diseases dates back to 1989, reporting the wide applicability of PCR in detection of Shiga-like toxin producing *Escherichia coli* (*E. coli*) [1], verocytotoxin-producing *E. coli* in diseased pigs [2], pseudorabies virus from acutely diseased as well as latently infected pigs [3, 4], Porcine *Haemophilus pleuropneumonia* infection [5], Porcine respiratory Coronavirus infection [6], foot and mouth disease (FMD) virus in porcine tissues [7], Porcine Parvovirus (PPV) infections [8], porcine rotavirus G types [9], porcine group A rotavirus [10], porcine reproductive and respiratory syndrome virus (PRRSV) infections [11].

Although it has been three decades since its development, PCR is regarded as a gold standard and continues to be the technique of choice for nucleic acid amplification both as analytical and as a diagnostic tool. However, a number of modifications of the conventional PCR technique have been developed; one of the variant of this classic technique is the multiplex PCR. The first report of application of multiplex PCR in diagnosis of inherited genetic disease was described in 1988 for detection of majority of deletions in the Duchenne muscular dystrophy (DMD) gene in humans [12].

This chapter will highlight the parameters critical in developing a multiplex PCR, troubleshooting of commonly encountered problems during optimization, strategic actions to overcome such problems and highlight application of multiplex PCR in diagnosis of various porcine diseases.

2 Principle of Multiplex PCR

Multiplex PCR (M-PCR) is the technique of simultaneous amplification of more than two target sequences in a single reaction using more than two primer pairs.

A multiplex PCR is a modification of the conventional singleplex PCR. In a conventional singleplex PCR, one single target sequence is amplified in a single reaction tube, whereas a multiplex PCR facilitates the simultaneous amplification of multiple target sequences in a single reaction tube (Figs. 1 and 2 illustrate the technique of conventional PCR and multiplex PCR).

There are two variants of multiplex PCR. The first variant of multiplex PCR includes a single template PCR reaction. This technique utilizes a single template which could be a genomic DNA or a complementary DNA (cDNA) in a single reaction tube alongwith

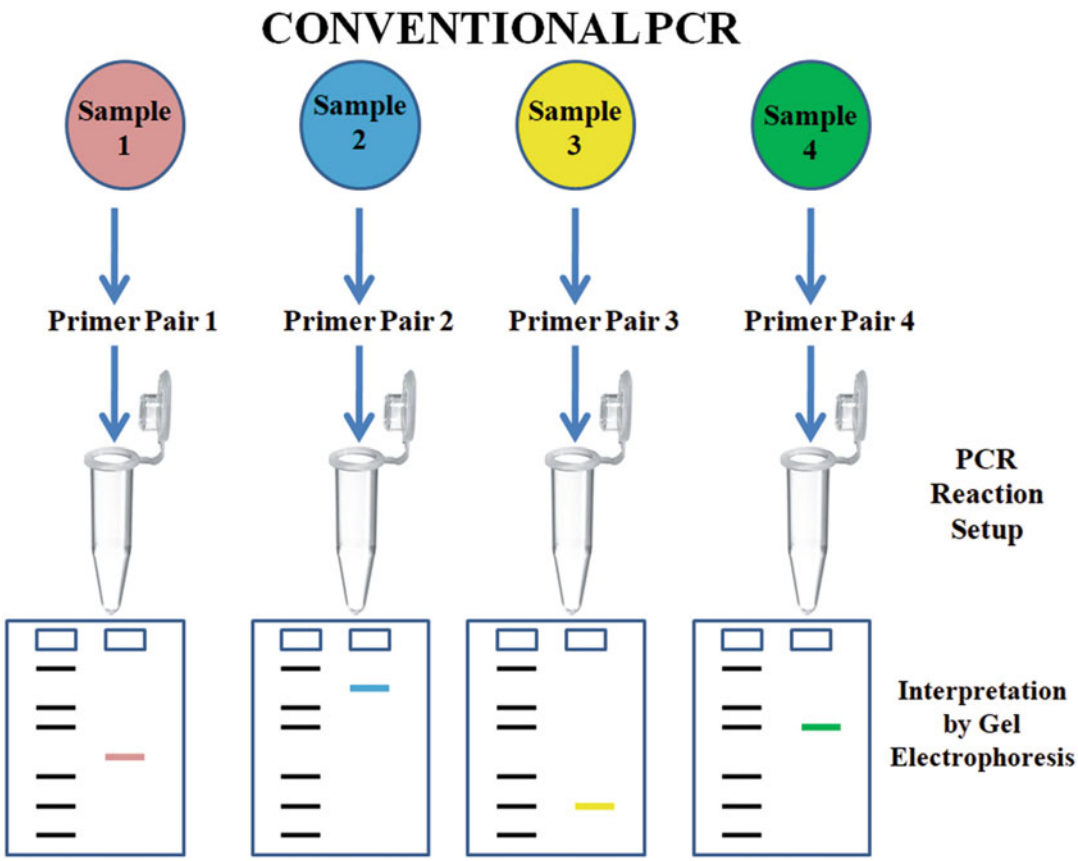


Fig. 1 Conventional PCR

multiple primer pairs to amplify multiple targets within the template (Fig. 2a). An example of a report described the rapid detection of enteric pathogens by multiplex PCR in porcine feces from a single DNA template extracted from porcine feces [13]. Fig. 3 illustrates a multiplex PCR developed by [13], designed to amplify a 655 base pair (bp) portion of the *Lawsonia intracellularis* 16S rRNA gene, a 354 bp portion of the *Brachyspira hyodysenteriae* NADH oxidase gene, and a 823 bp portion of the *Brachyspira pilosicoli* 16S rRNA gene.

The second variant of multiplex PCR includes multiple template PCR reaction. This technique utilizes multiple templates in a single reaction tube alongwith multiple primer pairs to amplify specific targets in the template (Fig. 2b). An example of a report that described the application of multiplex PCR in clinical application for detection of major swine viruses in pigs with multiple infections, considered a range of samples, viz. diarrheic stool samples, aborted fetus organs, and principal organs collected from post-weaning multisystemic wasting (PMWS)—suspected pigs [14]. Fig. 4 illustrates a multiplex PCR developed by [14] detecting

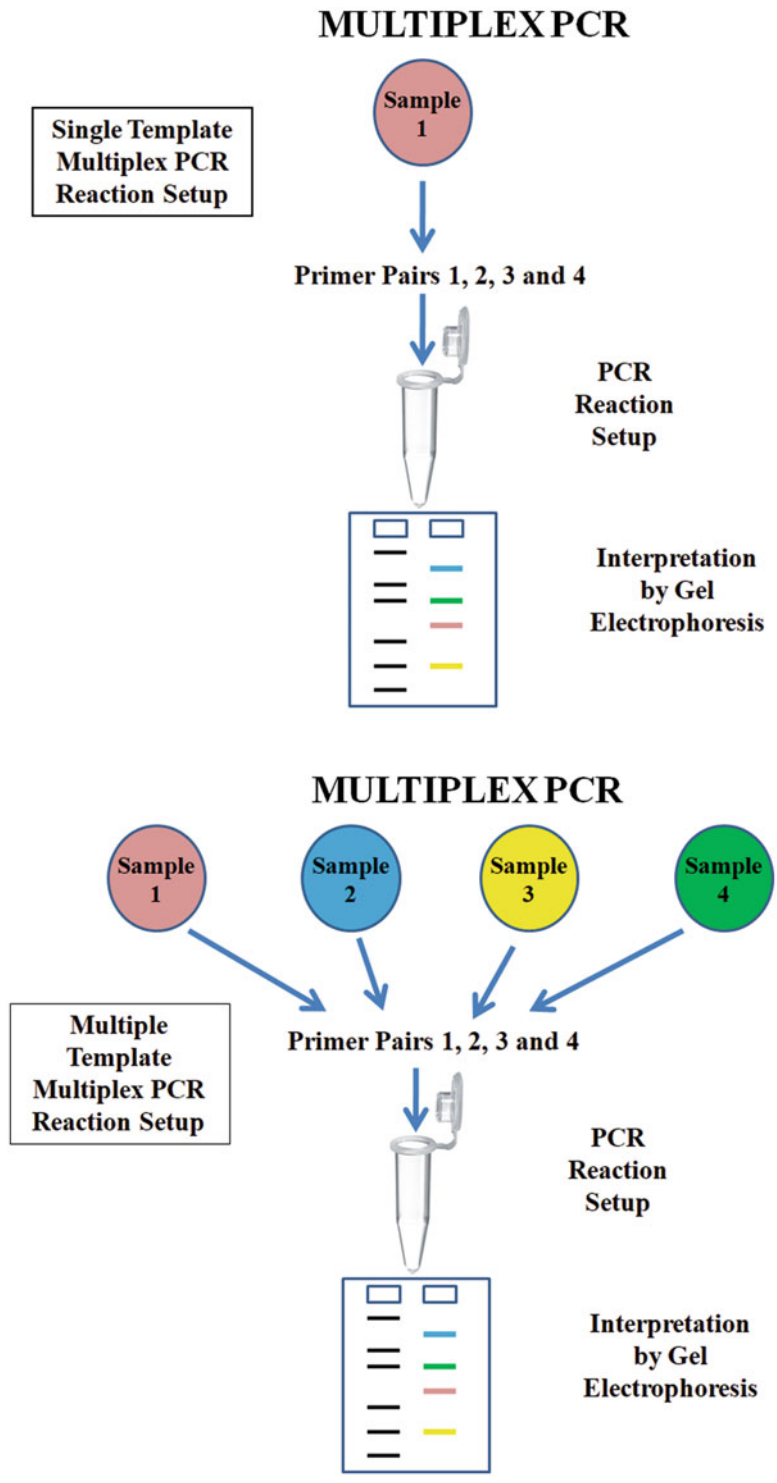


Fig. 2 Multiplex PCR

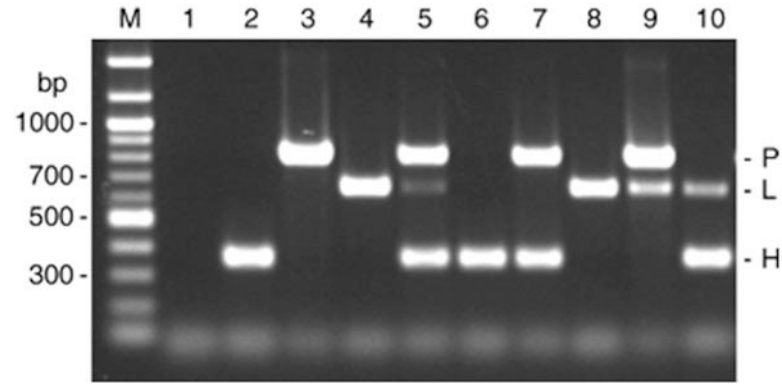


Fig. 3 Multiplex PCR for rapid detection of enteric pathogens in porcine feces in agarose gel showing the relative sizes of PCR products from three bacterial pathogens: P—*Brachyspira pilosicoli*, L—*Lowsonia intracellularis*, H—*Brachyspira hyodysenteriae*, M—molecular mass markers, Lane 1—negative control, Lane 2—*B.hyodysenteriae* strain B78, Lane 3—*B. pilosicoli* strain P43/6/78, Lane 4—*L. intracellularis* strain 2189/94, Lanes 5–10—field samples

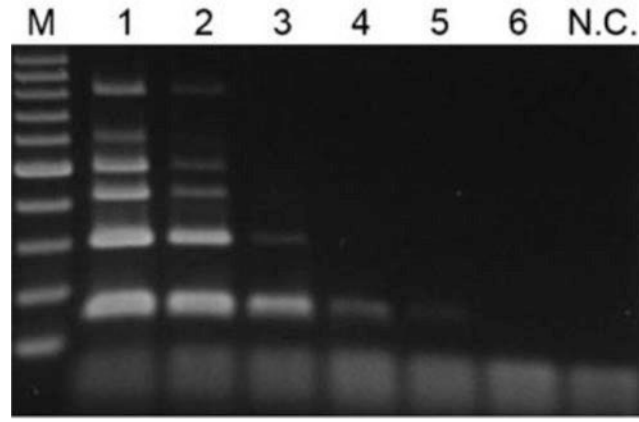


Fig. 4 Multiplex PCR for detection of six swine viruses in agarose gel showing the relative sizes of PCR products in lane 1

six viruses PRRSV, Japanese encephalitis virus (JEV), Getah virus (GETV), transmissible gastroenteritis virus (TGEV), porcine epidemic diarrhea virus (PEDV), and Porcine Rotavirus A (PoRV-A).

The most important criteria for amplification of more than two targets in a single reaction is dependent on the compatibility of the primer pairs used in the reaction. An essential requirement for all the primers in a multiplex PCR is to have similar melting temperatures (T_m) for efficient annealing and subsequent dissociation of the primers from their complementary target sequences at approximately the same temperature, permitting amplification of each target to occur at that particular temperature and time. For example, a multiplex will not at all work in case one pair of primer anneals

to the target sequence at that instant when another pair of primer is dissociating from its respective target sequence. It is, therefore, due to this reason that designing of all the primer pairs for a multiplex PCR follows the criteria of having difference of their T_ms between few degrees to each other.

Another important aspect of designing a multiplex PCR is such that relative sizes of the target amplicons are visually identifiable and distinguishable from each other on a gel electrophoresis system. For example, two amplicons of a multiplex PCR having a minimum difference of approximately 43–50 bp would be easily distinguishable on an agarose gel. [14] developed a multiplex PCR for major porcine viruses, reporting an amplicon size of 160 bp for GETV detection and 203 bp for PPV detection on agarose gel.

3 Optimization of a Multiplex PCR

Most of the parameters and strategies used for developing a conventional singleplex PCR [15] would be helpful for developing a multiplex PCR assay. However, some additional parameters are to be considered that play a critical role in optimizing a multiplex PCR for efficient detection of multiple targets.

The primary challenge while optimizing a multiplex PCR is the elimination of the formation of primer dimers. Primer dimers are basically short undesirable products that are generally formed at the room temperature prior to the first round of PCR amplification. The formation of these primer dimers are due to annealing between primers that possess 3′ end complementary overlappings of two or more nucleotides and the abundant availability of the polymerase enzyme prior to the first round of PCR amplification aids in the extension of these annealed primers to amplify such short untargeted amplicons. Therefore, the main requirement to eliminate such primer dimers is due to the fact that these short untargeted products are efficiently amplified and they effectively and equally compete with the amplification of the desired targets thereby impacting the overall efficiency of a multiplex PCR. A point to be noted here is that with the increase in the number of targets of a multiplex PCR assay, the chances of accumulation of primer dimers also increases as the chances of a number of primers to possess complementary overlappings of two or more nucleotides at their 3′ ends also significantly increases.

Another challenge while optimizing a multiplex PCR is the reproducibility of the assay in maintaining the target specific amplifications and obtaining equivalent yields of each of the targets.

A detailed analysis of each of the parameters to be considered has been discussed in the following sections that will aid in optimizing a multiplex PCR assay.

3.1 Designing of Primers

Ideally it is a pre-requirement that all the primers designed for a multiplex PCR assay should have nearly similar T_ms or a maximum Tm difference of ±5 to 6.5 degrees among the primers so that a single annealing temperature would be optimal for all the primer pairs to amplify their specific targets within a single reaction. For example, Table 1 lists five pairs of primers used in a bacterial multiplex PCR for detection of virulence associated genes of *Pasteurella multocida* from pigs developed in our laboratory [16]. The Tm of the primers designed has a range of 49.9–56 degrees with a maximum difference of 6.4 degrees between them and a minimum difference of 0 degrees between them. Most of the primers designed have Tm in and around 55–56 degrees facilitating the optimization of the annealing temperature of the assay efficiently at 56 degrees.

The primer pairs of a multiplex PCR should have nearly similar amplification efficiencies for their respective targets. This could be achieved when the designed primers have an optimal length of 18–30 bp, a GC content of 35–60% and do not possess any significant homology either internally or among one another. For example, the primers listed in Table 1 show an uniformity in their length of 20 bases, maintaining a GC content of 35–50% and 50% in majority of the primers and do not show presence of any secondary structure among them.

In order to prevent preferential amplification of shorter products over larger ones, the amplicon size range between the smallest and the largest amplicons should not exceed 500–600 bp.

Another example showing the properties of primer pairs of two independent multiplex PCRs developed in our laboratory for one-step multiplex PCR for rapid and simultaneous detection of three porcine viruses, viz. Porcine Circovirus type 2, Porcine Parvovirus, and Classical Swine Fever virus and multiplex PCR for simultaneous detection of N, M, and GP5 genes for diagnosis of PRRSV have been listed in Tables 2 and 3, respectively.

While designing the primers, the probable candidate sequences can be evaluated using an online Oligo analyzer tool (e.g. OligodT analyzer), to screen presence or absence of any secondary structures such as hairpin formations within the candidate sequences and probability of formation of any dimers. In addition, a thorough homology search (e.g. BLAST) of the candidate sequences would ensure prevention of any non-specific primer annealing to pseudogenes or partially homologous nucleotide sequences.

3.2 Reaction Component Optimization

Every component of a PCR reaction mix contributes to successful amplification of the targets. Therefore, optimization of these individual components may be needed for improvement in sensitivity or specificity of the assay.

(a) *Concentration of Magnesium chloride ($MgCl_2$)*

Table 1
Properties of primers used in a bacterial multiplex PCR assay for rapid detection of virulence associated genes of *Pasteurella multocida* from pigs [16]

Target genes	Primer Sequence	Length (bp)	GC content %	Melting temperature (Tm)°C	Amplicon size (bp)
ompH	F: CTGGTTTAGCGCTTGGTGTT	20	50	56	242
	R: TCTACCCCAAGCTGCTTCAA	20	50	56.3	
ompA	F: AGCGCGTAGATTACAGACCA	20	50	55.9	350
	R: GTGACCTGTTGCGCTGATAG	20	55	56	
plpB	F: CCAAAATTGCGAAGGAAAAA	20	35	49.9	443
	R: CGCGAAATCGACATCATCTA	20	45	52.4	
hgbA	F: AAGTCGCTAAAATCGCGAAA	20	40	52.8	548
	R: ATCCCAAAATGGCGTAACAG	20	45	53.3	
pfhA	F: TTTAGCGGGGAGTTCAGCTA	20	50	55.7	753
	R: GTGACATCGCCGGTAACTTT	20	50	55.4	

Table 2
Properties of primers used in one-step multiplex PCR for rapid and simultaneous detection of three porcine viruses

Sl. no.	Target viruses	Length (bp)	GC content %	Tm°C	Amplicon size (bp)
1.	PCV-2 forward	20	45	54.1	553
	PCV-2 reverse	20	45	53	
2.	PPV forward	20	55	57.4	326
	PPV reverse	21	52.4	56.9	
3.	CSFV forward	20	50	55.1	171
	CSFV reverse	20	55	55.4	

The concentration of magnesium chloride in the reaction mix and the annealing temperature of the primers play a vital role in specificity and yield of the products. 1.5 mM $MgCl_2$ is the recommended concentration for a standard PCR

Table 3
Properties of primers used in a multiplex PCR for simultaneous detection of three genes for diagnosis of PRRSV

Sl. no.	Target genes	Length (bp)	GC content %	Tm °C	Amplicon size (bp)
1.	N—Forward N—Reverse	24 22	48 49.1	58.9 59.6	372
2.	M—Forward M—Reverse	21 26	52 53.9	59.2 52.7	525
3.	ORF5—Forward ORF5—Reverse	21 22	57 59	57.9 57.9	603

Table 4
Detailed concentration of each reaction components optimized for most of the multiplex PCR

Reaction Component	(Amount/concentration) per 25 μl reaction
10× PCR buffer	2.5 μl
$MgCl_2$	2 mmol/l
Each dNTP	0.2 mmol/l
Taq DNA polymerase	1 U
Forward primers	20 pmol
Reverse primers	20 pmol
Template DNA	3 μl

[17]. However, it has been reported that the efficiency of certain multiplex amplifications was found to be enhanced from increased concentration of $MgCl_2$ in the range of 3–10 mM [18] and 1.8–10.8 mM [17]. Therefore, an optimal concentration of $MgCl_2$ for a reaction needs to be standardized since an addition of suboptimal concentrations of $MgCl_2$ may result in high levels of non-specific amplification and reduced product yield. Table 4 may be referred for detailed concentration of each reaction components optimized for most of the multiplex PCR working efficiently in our laboratory.

(b) *Concentration of Deoxynucleoside Triphosphates (dNTPs)*

The concentration of dNTPs in a reaction is of significance since dNTPs bind to divalent cations (magnesium ions) quantitatively, therefore variation in the concentration of dNTPs would affect the optimal concentration of $MgCl_2$ as well. DNA Polymerases also require free magnesium ions besides Mg^{2+} ions bound to dNTPs. Therefore, a suboptimal increase

Table 5
Three examples of polymerases used in multiplex PCRs

DNA polymerase	Concentration of buffer	pH of buffer	Concentration of KCl salt
AmpliTaq gold	15 mM Tris–HCl	8.0	50 mM KCL
Stoffel CM	10 mM Tris–HCl	8.0	10 mM KCL
Taq polymerase (Fermentas)	20 mM Tris–HCl	8.4	50 mM KCL

in the concentration of dNTPs in a reaction would inhibit any amplification of the targets. For example, for a standard PCR reaction, the recommended concentration of $MgCl_2$ is 1.5 mM at dNTP concentrations of around 200 μM each.

(c) *Buffer and Salt*

The optimal concentration of buffer and its pH and the concentration of KCl is dependent on the DNA Polymerase being used in the reaction. Table 5 lists three examples of polymerases used in multiplex PCRs, showing optimal activity in their respective buffer and salt concentrations.

It has been reported that generally buffers with lower salt concentrations work better for primer pairs amplifying longer targets and buffers with higher salt concentrations work better for primer pairs amplifying shorter targets [17].

(d) *Concentration of Primer*

The concentration of each primers used in multiplex PCR reactions generally ranges from 100 to 400 nM but these concentrations may vary between targets due to their difference in priming efficiencies (Fig. 5). While developing multiplex PCRs in our laboratory, initially an equimolar concentration of each primer is used in the range 100–200 nM under multiplex conditions to determine equivalent yields of all the specific targets. In certain instances, we have also encountered uneven amplification with respect to one or two targets of a multiplex assay and such targets showed faint amplifications in comparison to other targets of the same multiplex assay under the same optimized cycling conditions. In such cases, altering the proportions of various primers of a reaction, by increasing the concentrations of the weakly amplifying primers and decreasing the concentration of the strongly amplifying primers was observed to overcome such problems. Another strategy to overcome such problem is by doubling the concentration of the weakly amplifying primers of a reaction alone.

(e) *DNA Polymerase*

The enzymes used more often for multiplex PCR are recombinant thermostable DNA polymerase from *Thermus*

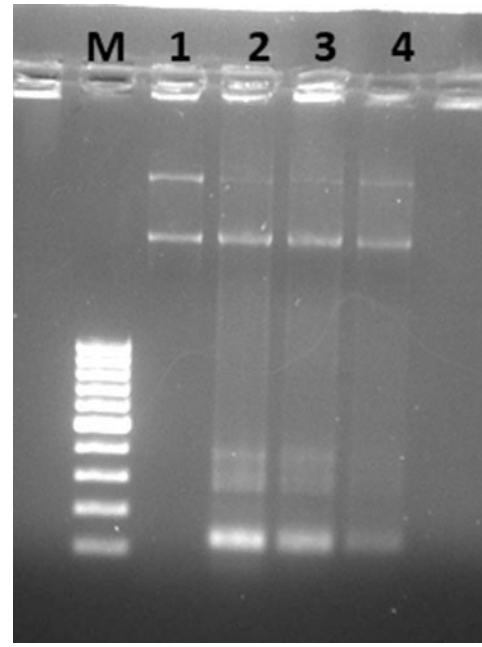


Fig. 5 Effect of concentration of primer variation on a multiplex PCR assay. Lane 1 depicts no amplification due to non-optimal primer concentration, Lane 2–4 depicts effect of varying concentrations of primer pairs

aquaticus (e.g. AmpliTaq DNA Polymerase), Stoffel fragment of Taq DNA polymerase (e.g. Stoffel CM), chemically modified version of AmpliTaq DNA polymerase, (e.g. AmpliTaq Gold), DreamTaq DNA Polymerase (Fermentas), Hot start polymerase (e.g. SapphireAmp Fast PCR master mix), Phusion high-fidelty master mix, Q5 Hot Start High Fidelty DNA polymerase, Multiplex PCR enzymes (Takara Bio.).

The concentration of the enzyme used for optimization of multiplex PCRs is 5–10 units per 100 μl reaction. The most effective concentrations of the enzyme has been reported to be 0.4 μl or 2 U/25 μl reaction [17], 0.25 μl/50 μl reaction [14], 1 U/25 μl reaction [16].

(f) *Amount of template*

A template concentration of 100 ng of bacterial or viral DNA or cDNA is more than sufficient for initial optimization of multiplex PCR assays. A common error that is committed in optimizing is addition of excess amount of template to a reaction which can inhibit a PCR, resulting in a smear-like pattern when visualized on an agarose gel or show non-specific amplification (Fig. 6). An assay can be optimized to such an extent to be efficient enough to amplify targeted products from a minimum amount of 450 pg of viral genomic DNA or RNA as a starting template [19].

Fig. 6 Gel photo of excess addition of templates. Lane 3 depicts smear-like patter due to excess amount of template added in PCR reaction

(g) *Reaction volume*

The total volume of a multiplex PCR reaction could range from a minimum of 10 μl to a maximum volume of 100 μl. The choice of the reaction volume depends on number of pathogens or genes targeted for a multiplex PCR assay and the optimization based on the cost of the reagents and the DNA polymerase consumed.

(h) *Use of adjuvants*

Certain additives although optional to use in a PCR reaction mix has been recommended in various reports [17, 20, 21] in order to improve the efficiency of amplification, sensitivity, and specificity of a multiplex PCR assay. Table 6 lists the examples of such adjuvants and the concentrations used for in multiplex PCR assay.

For example, Fig. 7 reveals a comparative difference in the detection of three genes of PRRSV on using 5% DMSO while developing a multiplex PCR in our laboratory.

There are also ample of commercially available PCR master mixes that are widely used in the development of multiplex PCRs thus, saving time and reducing the cumbersome energy and effort for optimization of each and every reaction components of a PCR mix.

Table 6
Adjuvants and the concentrations used for in multiplex PCR assay

Adjuvants	Concentration
Dimethylsulfoxide (DMSO)	5%
Glycerol	5%
Bovine serum albumin (BSA)	0.8 μg/μl
Betaine	1 M

Fig. 7 Effect of 5% DMSO in detection of three genes of PRRSV. Lane 1 depicts multiplex PCR products without addition of DMSO, Lane 2 depicts the effect of adding 5% DMSO resulting in three distinct products of multiplex PCR

4 Multiplex PCR Troubleshooting

There are many problems or difficulties that could be encountered while optimizing and developing a multiplex PCR.

1. There could be preferential amplification of certain specific targets in comparison to the other targets of the multiplex assay.
2. The assay could be poorly sensitive or specific.
3. There could be spurious amplified products due to the formation of primer dimers.
4. The primer pairs of the multiplex assay may not have similar amplification efficiencies for their respective targets.

5. There could be inhibitors of *Taq* polymerase enzyme present in the clinical samples that would prevent the detection of specific targets.

Optimizing the multiplex PCR with the primer pairs may yield multiple non-specific products which may mask the amplification of the desired products, impacting the overall sensitivity and specificity of the assay. Such non-specific PCR products can be strategically eliminated partially or completely by increasing the annealing temperature of the primer pairs which would increase the efficiency of annealing of primer to its target and prevent the formation of primer dimers. Following that, it may be necessary to increase or decrease the concentration of magnesium chloride ($MgCl_2$) in the reaction mix, or to lower the concentration of primer pairs in the reaction mix, or to add certain additives to the reaction mix, such as DMSO, BSA, betaine, or glycerol, if the annealing temperature is increased. The temperature at which the primer pairs are annealed does not remove non-specific products.

Certain substances may be present in the clinical samples which act as inhibitors of *Taq* polymerase enzyme, thereby inhibiting the detection of pathogens by PCR. These substances are generally proteinaceous in nature and stall the activity of the enzyme by modifying the quaternary structure of the enzyme or by blocking the active site of the enzyme [21]. For example, salts such as oxalates and urea, heparin, urine, fecal, semen, hair, and iron-containing compounds such as hemoglobin in blood, serum, or plasma can inhibit the activity of the enzyme. Therefore, these samples require prior treatment of deproteination with organic solvents such as phenol or chloroform.

Preferential strategies such as "hot-start" or use of uracil N-glycosylase alone or combination of both is known to enhance the specificity of the reaction. The hot-start protocol is basically increasing the reaction temperature to a temperature (above 80 degrees or at 95 degrees) higher than the Tm of the primers and then subsequently adding the *Taq* polymerase enzyme or primers to the reaction tube prior to the initiation of the multiplex PCR program, to enhance annealing of primers to its specific target sequences in the template(s) and this would eliminate non-specific amplification and primer dimers. The use of enzymatic method such as uracil N-glycosylase has been reported to minimize PCR product carryover and enhance the sensitivity of the PCR [22].

During troubleshooting, an initial strategy would be to ensure that the corresponding singleplex PCRs of the targets have been successfully working fine but it is the multiplex reaction that is not successfully giving the desired specificity, results and/or yield. Based on our laboratory experiences while developing multiplex

PCR and literature reports [18, 21], Table 7, therefore, lists the commonly encountered problems after visualization on gel electrophoresis while developing a multiplex PCR assay, the possible causes of these problems and the recommended strategies and solutions to overcome these problems.

5 Key Pointers for Multiplex PCR Development

1. The developed multiplex PCR should be robust, sensitive, and rational for detection of specific pathogens or targets in the assay.
2. The primer pairs designed should efficiently and accurately detect the targeted pathogens without raising any ambiguity of presence or absence of the pathogens and the amplicons should be easily interpretable in the detection system.
3. The sensitivity and specificity aspect of the multiplex assay needs to be evaluated prior to any clinical application as a diagnostic tool and should be supported with a well-documented comparison of their optimization with their corresponding singleplex PCRs by serial dilutions of the target template(s) as well as clinical sample(s).
4. Strategies and precautions should to be considered to prevent false negative results due to reaction failure.
5. Validation of the assay should be confirmed using external or internal control known targets in addition to the unknown samples being screened to indicate reaction failure.

6 Evaluation as a Potential Diagnostic Tool

Any developed assay to be proposed as a potential diagnostic tool involves comparison of its performance to a gold standard technique in terms of its percentage sensitivity and specificity. Analytical specificity is the ability of the assay to accurately distinguish between targeted and non-targeted pathogens or genes. Analytical sensitivity refers to the minimum concentration of a pathogens or targets that can efficiently amplified by the assay. The sensitivity of an assay is expressed as pg or fg of template or as copy numbers of template by performing serial ten-fold dilutions of the template(s). Multiplex PCRs having sensitivity up to 0.1 fg template or 1–5 copies of template detection have been reported [23, 24].

Table 7
Troubleshooting of Multiplex PCR

Sl. no.	Common problem	Possible causes	Recommended solutions
1.	Problems due to carryover contamination observed	Residual or carryover templates or reaction components may be accumulated on pipettes	The routine use of autoclaved filter tips while setting up PCR reactions is mandatory Pipettes needs to be UV sterilized prior to use and after use Pre- and post-sterilization of work benches and glasswares needs to be followed
		Specimen handling, PCR setup and amplicon detection performed in the same work bench and in the same room	The usage of four separate designated laboratory/rooms/areas needs to be followed 1. Sample or template handling and preparation 2. Setting up of PCR mix 3. Addition of template(s) 4. Amplicon detection
2.	PCR assay initially was working successfully with the designed primer pairs but suddenly shows non-specific with a different set of the same primer pairs or with a new batch of the same primer pairs		The routine use of both positive and negative controls needs to be followed to ensure the assay is valid
3.	No PCR products observed at all	Enzyme activation is not complete	The cycling parameters and pre-PCR heat activation of chemically modified enzymes needs to be checked
		One or more component(s) have been left out of the reaction	A new reaction mix needs to be prepared
		Inhibitors may be present in the template(s)	Alternate extraction procedure needs to be implemented or extraction of template (s) needs to be repeated on a sterile work bench
		Product may be degraded due to UNG activity	The PCR product needs to be kept on ice immediately after completion of PCR

4.	Low yield of all products observed	Incomplete amplification may have occurred	Pre-PCR enzyme activation time needs to be increased Cycle number of PCR needs to be increased Concentration of enzyme needs to be increased Concentration of $MgCl_2$ needs to be increased
		pH of Tris–HCl buffer may be too high (sub-optimal activation of the chemically modified enzymes)	pH of Tris–HCl buffer needs to be decreased to 8.0
		Concentration of the primers may be too low	Concentration of the primers needs to be increased
		The annealing temperature may be too high	The annealing temperature needs to be decreased
		The template(s) may be insufficient for the reaction or have been degraded	The concentration and the quality of the template to be used in the reaction needs to be checked
5.	Low yield of one of the products is observed	The concentration of the primer pair specific to the low yield PCR product may be too low	The concentration of the primer pair specific to the low yield PCR product needs to be increased
		The primer pair specific to the low yield PCR product may be poorly designed	The primer pair needs to be redesigned in case of necessity
		Secondary structure may be present in the template(s)	Alternate enzymes may be utilized (e.g. DreamTaq, sapphire, Phusion)
		dUTP may be incorporated inefficiently	Some TTP may be added to the reaction The concentration of the enzyme needs to be increased
		The annealing temperature may be too high	The annealing temperature needs to be decreased
6.	Low yield or loss of one or more large fragments may be observed	There could be preferential amplification of smaller products	The primer concentration of the large PCR products needs to be increased
		The concentration of the enzyme may be too low	The concentration of the enzyme may be increased
		The extension time may be too short	The extension time may be increased
		The template may be degraded	The quality of the template needs to be checked
		The concentration of the template may be too low	The concentration of the template needs to be checked

(continued)

Table 7
(continued)

Sl. no.	Common problem	Possible causes	Recommended solutions
7.	High background and non-specific PCR products observed	The concentration of the enzyme may be too high	The concentration of the enzyme needs to be decreased
		Presence of too much enzyme at early cycles	Hot-start protocols needs to be preferred (hot start manually or use of simplified hot-start enzymes)
		The pre-PCR heat cycle may be too long	The pre-PCR heat activation of chemically modified enzymes needs to be decreased
		The concentration of $MgCl_2$ may be too high	The concentration of $MgCl_2$ needs to be decreased
		The primers may be poorly designed	The primers needs to be redesigned
		There could be sub-optimal amount of template used in the reaction	The concentration of the template to be used in the reaction needs to be checked
		The annealing temperature could be too low	The annealing temperature needs to be increased
			Certain additives such as DMSO, glycerol, BSA, betaine is needed to be added to avoid non-specific amplification
			Preparation of reaction mix needs to be performed in ice to prevent annealing of primers to non-complementary sequences at room temperature

7 Advantages of Multiplex PCR

1. *Analysis of multiple targets in a single assay*
   Rather than performing multiple number of PCR amplifications for detecting multiple regions of a gene or a pathogen or detecting multiple viruses or bacteria in an infected sample, for example, respiratory tract secretions, it would be rational to amplify all targets of interest simultaneously within a single assay.
2. *Possess high degree of sensitivity*
   The assay is highly sensitive in detecting both non-cultivable virus and neutralized virus present in antigen-antibody complexes.
3. *Internal controls*
   False negatives in comparison to singleplex PCRs can always be revealed within an assay since every amplicon may serve as an internal control for all the other amplified amplicons.

8 Application of Multiplex PCR in Porcine Diseases

(a) Detection of bacterial and viral pathogens in clinical and epidemiological studies.

(b) Routine screening of individual pathogens, symptom-associated pathogens and evaluating various pathogens associated with diseases.

(c) Typing and sub-typing of strains of various pathogens in epidemiological studies.

(d) Detection of co-infections of pathogens during outbreaks.
   Typing of PRRSV by a multiplex PCR assay, developed by [25] was one of the early reports of application of multiplex PCR in detection of porcine diseases. Another exceptional utilization of multiplex PCR is reported by Ogawa et al. in 2009 for detection of major swine DNA and RNA viruses in pigs with multiple infections. This report includes detection of nine viruses, viz. PCV-2, PPV, PRRSV, JEV, PoRV-A, PEDV, TGEV, Getahvirus, suid herpesvirus 1 within a single assay. There has been an extensive use of multiplex PCRs in detection of porcine diseases irrespective of the type of template (s) that has been utilized. For example.

1. *Multiplex PCR for detection of porcine DNA virus(s)*
   There are reports of multiplex PCR for detection of the DNA contained emergent disease agents such as African swine fever virus (ASFV), Aujesky disease and PCV [26]. Yang et al.

in 2019 [27] developed a multiplex assay to detect and discriminate porcine circoviruses such PCV-1, PCV-2, and PCV-3 in clinical specimens.

2. *Multiplex PCR for detection of porcine RNA virus(s)*
   There are reports of multiplex RT-PCR for simultaneous detection of North American Genotype of PRRSV, Swine Influenza virus (SIV), and Japanese encephalitis virus (JEV) [28]. Fujii et al. in 2019 [29] developed a semi-nested multiplex PCR for genotyping of Rotavirus. We have developed a multiplex PCR assay for detection of three genes of PRRSV in our laboratory (Table 3).
3. *Multiplex PCR for detection of porcine DNA and RNA viruses*
   There are reports of multiplex PCR for simultaneous detection of six swine DNA and RNA viruses, viz. Classical Swine Fever Virus (CSFV), PRRSV, JEV, PCV-2, PPV, and Porcine Pseudorabies virus (PRV) [19]. Hu et al. in 2015 [30] had developed a multiplex PCR for simultaneous detection of CSFV, ASFV, PRRSV, and porcine pseudorabies virus. We have developed a multiplex PCR assay for simultaneous detection of PCV-2, PPV, and CSFV in our laboratory (Table 2).
4. *Multiplex PCR for detection of bacterial DNA pathogens in swine*
   Phillips et al. in 2009 [31] had developed a multiplex PCR to detect *Lawsonia intracellularis*, *Brachyspira hyodysenteriae*, and *Brachyspira pilosicoli* in feral pigs. Elder et al. in 1997 [32] had developed a multiplex PCR for simultaneous detection of *Lawsonia intracellularis, Serpulina hyodysenteriae,* and *Salmonellae* in porcine intestinal specimens. A rapid multiplex PCR for detection of virulence associated genes of *Pasteurella multocida* from pigs [16] and a multiplex PCR assay for simultaneous detection of three important pathotypes of Escherichia coli from diarrheic piglets Rajkhowa et al. [33] has been developed in our laboratory.

8.1 Porcine Respiratory Diseases

Porcine respiratory disease complex, commonly known as PRDC, is one of the major concerns for pig rearers and involve multiple viral and bacterial pathogens. Lung et al. in 2017 [34] had reported a multiplex PCR assay for detection of bacteria and viruses associated in swine respiratory diseases such as PRRSV, Influenza A virus, PCV-2, porcine Coronavirus, *Mycoplasma hyopneumoniae*, *Pasteurella multocida*, Salmonella, and *Streptococcus suis*.

8.2 Porcine Reproductive Diseases

Major economic losses are faced in the piggery sector due to reproductive problems such as stillbirths, mummified fetus, embryonic death, and infertility. Such problems are due to infection by opportunistic bacteria, viruses, and sometimes fungi and protozoa

that are often endemic in herds. PRRSV, Pseudorabies virus (Aujeszsky's disease), PCV-2, PPV, CSFV, Swine Influenza virus (SIV), Porcine enterovirus, *Brucella suis*, *Leptospira pomona*, *Leptospira bratislava*, *Erysipelotrix rhusiopathiae* are some of the pathogens associated with reproductive diseases of pigs. There are numerous reports on utilization of multiplex PCRs for diagnosis of such pathogens associated with reproductive diseases. In 2003, Kim and Chae [35] developed a multiplex nested PCR for differentiation of PCVs and PPV from pigs with Post-weaning multisystemic wasting syndrome (PMWS). Pan et al. in 2005 [36] reported multiplex PCR for rapid detection of pseudorabies virus, PPV, and PCV-2.

8.3 Clostridial Enteritis

The disease is caused by *Clostridium perfringens*, which is an inhabitant of the large intestine. It usually occurs when the piglet has had insufficient intake of colostrums and is one of the causes of mortality in neonates. Meer and Songer in 1997 [37] reported a multiplex PCR assay for genotyping of the major toxins of *Clostridium perfringens*. Kanakaraj et al. in 1998 [38] had reported a multiplex assay for detection of *Clostridium perfringens* in feces and intestinal contents of pigs and in swine feed.

8.4 Porcine Diarrhea

Diarrhea or scours in piglets are common at both neonatal and post-weaning stage and is a common cause of mortality in piglets. *E. coli*, TGEV, clostridial diseases, coccidiosis, and PoRV-A are some of the pathogens associated with pre-weaning diarrhea. The potential pathogens causing diarrhea in post-weaning piglets include *E. coli*, PoRV-A, TGEV, Salmonellosis, and Campylobacter. Seungtae et al. in 2014 [39] had reported a diagnostic test for enteric diarrhea in pigs utilizing an efficient multiplex PCR. In 2019, Liu et al. [40] had reported detection and differentiation of five diarrhea related pig viruses such as PEDV, TGEV, PoRV-A, PoRV-C, and PCV-2 utilizing a multiplex PCR assay. A multiplex PCR assay for simultaneous detection of three important pathotypes of Escherichia coli from diarrheic piglets was reported by Rajkhowa et al. [33]. Ding et al. in 2019 [41] had reported a multiplex RT-PCR for detection of major enteric RNA viruses in pigs such as PEDV, TGEV, PoRV-A, porcine kobuvirus (PKV), porcine sapovirus (PSaV), and porcine deltacoronavirus (PDCoV).

8.5 Porcine Parasitic Diseases

The diagnosis of parasites in pig management is also emphasized as the parasites can infect them and produce a wide range of clinical manifestations. Beck et al. in 2009 [42] reported the utilization of a multiplex PCR in detecting *Trichinella pseudospiralis* in muscle tissue of domestic pig in Croatia. Lin et al. in 2008 [43] had reported the utilization of multiplex PCR for differentiation of two porcine nodule worms, viz. *Oesophagostomum dentatum* and *Oesophagostomum quadrispinulatum*. Sato et al. in 2006 [44] had

reported utilization of a multiplex PCR in identifying Afro-American genotype of *Taenia solium* in cysts samples obtained from pigs. The report revealed occurrence of porcine cysticercosis in Brazilian pigs.

9 Conclusion

Multiplex PCRs have proved to be robust, rapid, precise, and a comprehensive diagnostic tool in effective management of pigs and the diseases associated with them. The potential of this technique to detect multiple pathogens infecting the pig population is itself significant in routine screening of individual pathogens, symptom-associated pathogens, evaluating various pathogens associated with diseases, detection of co-infections, assessment of status of a disease, and identification of carriers of infection(s) in order to reduce morbidity and mortality. Although this technique can sometimes be cumbersome in terms of its optimization but once developed, the efficiency of multiplex PCRs in minimizing the need of additional confirmatory diagnostic tests, possessing characteristic feature of high sensitivity, and specificity in identification of infectious agents, reducing the detection time and cost involved over other traditional cultivation methods in identification of pathogens from clinical samples and its rapidity in implementation of effective therapies, promotes this technique to have an excellent utility in clinical diagnosis of porcine diseases.

References

1. Karch H, Meyer T (1989) Single primer pair for amplifying segments of distinct Shiga-like toxin genes by polymerase chain reaction. J Clin Microbiol 27(12):2751–2757
2. Meyer T, Bitzan M, Sandkamp O, Karch H (1989) Synthetic oligodeoxyribonucleotide probes to detect verocytptoxin – producing *Escherichia coli* in diseased pigs. FEMS Microbiol Lett 57(2):247–252
3. Belak S, Ballagi-Pordany A, Flensburg J, Virtanen A (1989) Detection of pseudorabies virus DNA sequences by polymerase chain reaction. Arch Virol 108(3–4):279–286
4. Jestin A, Foulon T, Pertuiset B, Blanchard P, Labourdet M (1990) Rapid detection of pseudorabies virus genome sequences in biological samples from infected pigs using polymerase chain reaction DNA amplification. Vet Microbiol 1(4):317–328
5. Xufu Y, Faquan P (1990) Confirmation and diagnosis of Porcine Haemophilus pleuropneumonia infection in China. Chinese J Anim Vet Sci
6. Rasschaert D, Duarte M, Laurde H (1990) Porcine respiratory coronavirus differs from transmissible gastroenteritis virus by a few genomic deletions. J Gen Virol 71 (11):2599–2607
7. Meyer RF, Brown CC, House C, House JA, Molitor TW (1991) Rapid and sensitive detection of foot-and-mouth disease virus in tissues by enzymatic RNA amplification of the polymerase gene. J Virol Methods 34(2):161–172
8. Molitor TW, Oraveerakul K, Zhang QQ, Choi CS, Ludemann LR (1991) Polymerase chain amplification (PCR) amplification for detection of porcine parvovirus. J Virol Methods 32 (2–3):201–211
9. Gouvea V, Santos N, Timenetsky MDC (1994a) Identification of bovine and porcine rotavirus G types by PCR. J Clin Microbiol 32(5):1338–1340

10. Gouvea V, Santos N, Timenetsky MDC (1994b) VP4 typing of bovine and porcine group A rotaviruses by PCR. J Clin Microbiol 32(5):1333–1337
11. Suarez P, Zardoya R, Prieto C, Solana A, Tabares E, Bautista JM, Castro JM (1994) Direct detection of the porcine reproductive and respiratory syndrome (PRRS) virus reverse polymerase chain reaction (RT-PCR). Arch Virol 135:89–99
12. Chamberlain JS, Gibbs RA, Ranier JE, Nguyen PN, Caskey CT (1988) Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. Nucleic Acids Res 16:11141–11156
13. La T, Collins AM, Phillips ND, Oksa A, Hampson DJ (2006) Development of multiplex PCR for rapid detection of enteric pathogens Lawsonia intracellularis, Brachyspira hyodysenteriae, and Brachyspira pilosicoli in porcine faeces. Lett Appl Microbiol 42(3):284–288
14. Ogawa H, Taira O, Hirai T, Takeuchi H, Nagao A, Ishikawa Y, Tuchiya K, Nunoya T, Ueda S (2009) Multiplex PCR and multiplex RT-PCR for inclusive detection of major swine DNA and RNA viruses in pigs with multiple infections. J Virol Methods 160:210–214
15. Saiki RK (1989) The design and optimization of the PCR. In: PCR technology, principles and applications for DNA amplification. Stockton Press, New York, pp 7–16
16. Rajkhowa S (2015) Development of a novel multiplex PCR assay for rapid detection of virulence associated genes of *Pasteurella multocida* from pigs. Lett Appl Microbiol 61 (3):293–298
17. Henegariu O, Heerema NA, Dlouhy SR, Vance GH, Vogt PH (1997) Multiplex PCR: critical parameters and step-by-step protocol. BioTechniques 23:504–511
18. Zangenberg G, Saiki R, Reynolds R (1999) Multiplex PCR: Optimization Guidelines. In: Chapter 6, PCR applications protocols for functional genomics. Academic Press, pp 73–94
19. Xu XG, Chen GD, Huang Y, Ding L, Li ZC, Chang CD, Wang CY, Tong DW, Liu HJ (2012) Development of multiplex PCR for simultaneous detection of six swine DNA and RNA viruses. J Virol Methods 183(1):69–74
20. Apte A, Daniel S (2003) PCR primer design. In: Chapter 7, PCR Primer A laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbour, NY, pp 61–74
21. Mahony JB, Chernesky MA (1995) Multiplex polymerase chain reaction. In: Chapter 10, Molecular methods for virus detection. Academic Press, San Diego, pp 219–236
22. Loewy ZG, Mecca J, Diaco R (1994) Enhancement of *Borrelia burgdorferi* PCR by uracil N-glycosylase. J Clin Microbiol 32(1):135–138
23. Erlich H, Gelfand D, Sninsky J (1991) Recent advances in the polymerase chain reaction. Science 252:1643–1651
24. Mahony J, Luinstra K, Sellors J, Chernesky M (1993) Comparison of plasmid and chromosome based polymerase chain reaction assays for detecting *Chlamydia trachomatis* nucleic acids. J Clin Microbiol 9:2241–2245
25. Gilbert SA, Larochelle R, Magar R, Cho HJ, Deregt D (1997) Typing of porcine reproductive and respiratory syndrome viruses by a multiplex PCR assay. J Clin Microbiol 35 (1):264–267
26. Gerilovych A, Stegniy B, Golovko V, Solodiankin O, Gerilovych I, Smolyaninova Y, Rudova N (2015) Development of the multiplex PCR for detection of the DNA contained emergent diseases agents in pigs (African swine fever, Aujeszky disease, Circoviral disease). Online J Public Health Inform 7(1):e131
27. Yang K, Jiao Z, Zhou D, Guo R, Duan Z, Tian Y (2019) Development of a multiplex PCR to detect and discriminate porcine circoviruses in clinical specimens. BMC Infect Dis 19:778
28. Chen HY, Wei ZY, Zhang HY, Lu XL, Zheng LI, Cui CA, Liu J, Zhu QL, Wang ZX (2010) Use of multiplex RT-PCR assay for simultaneous detection of North American genotype PRRSV, swine influenza virus and Japanese encephalitis virus. Agric Sci China 9 (7):1050–1057
29. Fujii Y, Doan YH, Wahyuni RM, Lusida MI, Utsumi T, Shoji I, Kazuhiko K (2019) Improvement of rotavirus genotyping method by using the semi-nested multiplex PCR with new primer set. Front Microbiol 10:647
30. Hu L, Lin XY, Yang ZX, Yao XP, Li GL, Peng SZ, Wang Y (2015) A multiplex PCR for simultaneous detection of classical swine fever virus, African swine fever virus, highly pathogenic porcine reproductive and respiratory syndrome virus, porcine reproductive and respiratory syndrome viruses and pseudorabies in swine. Pol J Vet Sci 18(4):715–723
31. Phillips ND, La T, Adams PJ, Harland BL, Fenwick SG, Hampson DJ (2009) Detection of *Brachyspira hyodysenteriae*, *Lowsonia intracellularis*, and *Brachyspira pilosicoli* in feral pigs. Vet Microbiol 134(3–4):294–299
32. Elder RO, Duhamel GE, Mathiesen MR, Erickson ED, Gebhart CJ, Oberst RD (1997) Multiplex polymerase chain reaction for

simultaneous detection of *Lowsonia intracellularis, Serpulina hyodysenteriae* and *salmonellae* in porcine intestinal specimens. J Vet Diagn Investig 9(3):281–286

33. Rajkhowa S, Sarma D, Shakuntala I, Sahoo N (2015) Multiplex PCR assay for simultaneous detection of three important pathotypes of *Escherichia coli* from diarrhoeic piglets. Indian J Anim Sci 85(11):1202–1204
34. Lung O, Adjei SO, Buchanan C, Joseph T, King R, Erickson A, Detmer S, Ambagala A (2017) Multiplex PCR and microarray for detection of swine respiratory pathogens. Transbound Emerg Dis 64(3):834–848
35. Kim J, Chae C (2003) Multiplex nested PCR compared with insitu hybridization for the differentiation of porcine circoviruses and porcine parvovirus from pigs with postweaning multisystemic wasting syndrome. Can J Vet Res 67 (2):133–137
36. Pan QX, Chen D, He KW, Huang KH (2005) Multiplex PCR for rapid detection of pseudorabies virus, PPV and PCV-2. Virol Sin 20 (6):603–606
37. Meer RR, Songer JG (1997) Multiplex polymerase chain reaction assay for genotyping *Clostridium perferingens.* Am J Vet Res 58 (7):702–705
38. Kanakaraj R, Harris DL, Songer JG, Bosworth B (1998) Multiplex PCR assay for detection of *Clostridium perferingens* in feces and intestinal contents of pigs and in swine feed. Vet Microbiol 63(1):29–38
39. Seungtae K, Sharma N, Seog KH, Young MG, Kyoo OS, Yi PS, Yeun LJ, Jeong DK (2014) Establishment of diagnostic test for enteric diarrhea in pigs using efficient multiplex polymerase chain reaction. Indian J Anim Sci 84 (11):1157–1162
40. Liu G, Jiang Y, Opriessnig T, Gu K, Zhang H, Yang Z (2019) Detection and differentiation of five diarrhea related pig viruses utilizing a multiplex PCR assay. J Virol Methods 263:32–37
41. Ding G, Fu Y, Li B, Chen J, Wang J, Yin B, Sha W, Liu G (2019) Development of a multiplex RT-PCR for the detection of major diarrhoeal viruses in pig herds in China. Transbound Emerg Dis 67(2):678–685
42. Beck R, Beck A, Lucinger S, Florijancic T, Boskovic I, Marinculic A (2009) Vet Parasitol 159(3–4):304–307
43. Lin RQ, Ai L, Zou FC, Verweij JJ, Jiang Q, Li MW, Song HQ, Zhu XQ (2008) A multiplex PCR tool for the specific identification of *Oesophagostomum* spp. from pigs. Parasitol Res 103 (4):993–997
44. Sato MO, Cavalcante TV, Sako Y, Nakao M, Yamasaki H, Yatsuda AP, Nakaya K, Ito A (2006) Evidence and potential for transmission of human and swine *Taenia solium* Cysticercosis in the Piracuruca region Piaui, Brazil. Am J Trop Med Hyg 75(5):933–935

Chapter 6

Protocols for Isolation of Plasmid DNA

Vinod Kumar Singh, Vikas Gupta, and Chayanika Das

Abstract

Plasmid DNA isolation is indivisible step in the development of diagnostic assays based on recombinant proteins and other molecular biology experiments. There are hundreds of protocols published for isolation and purification of plasmid DNA and still the search is on for the cost and time saving methods. Each protocol published has its own advantages and limitation and the plasmid DNA obtained by different protocols vary in purity and yield. Many of these are the modification of the classical alkaline lysis method while the available rapid isolation methods employ different solid phase minicolumns. To discuss all the methods of plasmid isolation will require volumes of book space hence in this chapter the most common and trusted protocols used invariably in diffferent laboratories around the world are described.

Key words Plasmid, Isolation, Methods, Molecular assays

1 Introduction

Among the various signature molecules in a pathogen cell, nucleic acid or genetic material is one of the important targets for diagnostics assays. The nucleic acids content of a cell comprise of genomic or chromosomal DNA, plasmid DNA, and the different types of RNAs. The chromosomes are simply arrays of genetic material which translates into a single DNA molecule per chromosome at molecular level but structurally, it is a complicated structure involving proteins as well [1]. In most of the bacteria, single copy of closed circular chromosome is present that encodes all the essential functions. However, in higher organism the genetic information is distributed among number of chromosomes [2]. Furthermore, the plasmids are double-stranded circular DNA molecules independent of chromosomal DNA and are found in most of the bacteria [3]. These self-replicating genetic elements have also been reported in some of the lower eukaryotic organisms such as yeasts [4]. *Escherichia coli* (*E. coli*) fertility factor F was the first plasmid discovered for its ability to mediate transfer of chromosome markers from one strain to another [5]. However, until the discovery of plasmids

Rajib Deb et al. (eds.), *Protocols for the Diagnosis of Pig Viral Diseases*,
Springer Protocols Handbooks, https://doi.org/10.1007/978-1-0716-2043-4_6,

encoded resistance to antibiotics in epidemic strains of *Shigella* in late 1950s in Japan, plasmids research did not receive much importance [6]. Thereafter, in the next two decades, much research was centered on plasmids imparting drug resistance, particularly on the mechanism of resistance. It also became apparent during this period that the plasmids were not just responsible for fertility or drug resistance but they confer much greater variety of host properties. Apart from the defined benefits plasmids specify to the bacterium, the naturally occurring plasmids have also been tailored to produce new plasmids, which are being used as cloning vectors in recombinant DNA research [7]. While the presence of a plasmid can be detected genetically as a change in the phenotype of the bacterial cell, often the isolation of plasmid is required to carry out studies in different scientific contexts, including restriction enzyme mapping, as factors in the spread of antibiotic resistance, determination of size and number of plasmids, nucleotide sequencing, or for constructing new hybrid plasmids. The degree of purity of isolated plasmid will depends upon the methods employed for its extraction. In biochemical aspects, to purify plasmid from bacteria is to isolate plasmid DNA from the mixture of proteins, chromosomal DNA, plasmid DNA, and different RNAs. Of these biopolymers, chemical properties of protein are very different from nucleic acids (DNA and RNA) and it is rather easy to separate nucleic acids from proteins. However, DNA and RNA are very similar molecules exhibiting same basic biochemical properties. Uracil nucleoside and ribose sugar in RNA at the place of thymine and deoxyribose sugar in DNA, respectively, are the only noticeable structure difference but may have quite different conformational forms [8]. Furthermore, plasmids and chromosomal DNA are both deoxyribose nucleic acid and share similar biochemical properties. Also, in most of the bacteria, the chromosomal DNA is closed circular form and so is the form of plasmids, and the distinguishable difference between chromosomal DNA and plasmid, is the reasonably smaller size of plasmids compared to large chromosomal DNA. The tridimensional structural differences along with the molecular size difference form the basis for the isolation of plasmid from other nucleic acids. However, any nucleic acid extraction method can be broadly divided into four basic steps, viz. lysis or disruption of cell, removal of membrane lipids, proteins, and other nucleic acids, purification and concentration of nucleic acid [9]. Following this basic principle, plasmids can be isolated by a variety of methods, many of which rely on the differential denaturation and reannealing of plasmid DNA compared to chromosomal DNA. Moreover, with the advancement in science and technology, various modifications were introduced to make the process more efficient and many rapid extraction kits using minispin column were also developed for an early laboratory diagnosis. However, the need of the hour is the diagnosis at the site of patient and to achieve this in molecular

diagnosis we need to take the nucleic acid extraction protocols from the laboratory settings to the field/onsite in an easy and rapid format for providing point-of-care diagnosis. However, this chapter is mostly focused on the isolation of bacterial plasmid only and describes the classical as well as new rapid methods of plasmid purification.

2 Materials

2.1 Media (# Note 1)

1. *Luria Bertani Broth*: Tryptone 10 g/L, NaCl 5 g/L, Yeast Extract 5 g/L.
2. *Brain-Heart Infusion Broth*: Calf brain, infusion from 200 g/L, Beef heart, infusion from 250 g/L, Proteose peptone 10 g/L, Dextrose 2 g/L, Sodium chloride 5 g/L, Disodium phosphate 2.5 g/L.
3. *Luria Bertani Agar*: Tryptone 10 g/L, NaCl 5 g/L, Yeast Extract 5 g/L, Agar 15 g/L.
4. *Brain-Heart Infusion Agar*: Calf brain, infusion from 200 g/L, Beef heart, infusion from 250 g/L, Proteose peptone 10 g/L, Dextrose 2 g/L, Sodium chloride 5 g/L, Disodium phosphate 2.5 g/L, Agar 15 g/L.

   Adjust the pH to 7.4 ± 0.2 using 10 N NaOH and sterilized by autoclaving.

2.2 Chemicals and Other

1. Antibiotics
2. Agarose
3. Lysozyme: Dry powder. Store at −20 °C.
4. Ficoll 400,000
5. Ethidium bromide (EtBr) solution
6. Cesium chloride (CsCl)
7. Ethanol (70%)
8. Potassium acetate, pH 4.8 (3 M Potassium, 5 M Acetate)
9. Phenol, chloroform, and isoamyl alcohol (25:24:1 v/v)
10. Isopropyl alcohol
11. Isoamyl alcohol
12. Nuclease free water (NFW)

2.3 Major Equipment

1. Refrigerated microcentrifuge
2. pH meter
3. Ultracentriguge
4. UV-transilluminator or Gel documentation system

5. Water bath
6. Power supply and accessories for electrophoresis

2.4 Solutions and Buffers (# Note 2)

1. *TE buffer*: 10 mM Tris, pH 8.0, 1 mM EDTA
2. *1% Sodium dodecyl sulfate (SDS) in 0.2 N NaOH*
3. *Tris-borate buffer: pH 8.2; 89 mM Tris base, 12.5 mM Disodium EDTA, and 8.9 mM Boric acid*
4. *Lysozyme Mixture*
   (a) *For Gram-negative bacteria*: Lysozyme, 7500 U/mL; Ribonuclease I, 0.3 U/mL; 0.05% Bromphenol blue in Tris-borate buffer; 20% Ficoll 400,000 (# **Note 5**)
   (b) *For Gram-positive bacteria*: Lysozyme 75,000 U/mL, 50 mM Disodium EDTA (pH 8.0); 0.1 M Sodium chloride
5. *SDS mixture.*
   (a) *For Gram-negative bacteria*: 0.2% SDS in Tris-borate buffer in 10% Ficoll 400,000
   (b) *For Gram-positive bacteria*: 2.0% SDS in Tris-borate buffer in 10% Ficoll 400,000
6. *Overlay mixture* 0.2% SDS in Tris-borate buffer in 5% Ficoll 400,000
7. *STET*: 8% (w/v) Sucrose, 5% (v/v) Triton X-I 00, 50 mM EDTA, pH 8.0, 50 mM Tris–HCl, pH 8.0
8. *Glucose buffer*: 50 mM Glucose, 25 mM Tris–HCl, pH 8.0, and 10 mM EDTA
9. *Resuspension solution*: 6.5% Sucrose, 50 mM Tris–HCl, and 1 mM EDTA; pH 8.0
10. *Lysis solution:* 3% SDS, 50 mM Tris–HCl, and 5 mM EDTA. Adjust the pH to 12.2–12.4 with 5 N Sodium hydroxide just prior to use.
11. *Neutralizing solution* (Potassium acetate: 3 M Potassium/5 M Acetate): Dissolve 29.4 g of Potassium acetate in 88.5 mL distilled water, and 11.5 mL of Glacial acetic acid. Store at room temperature.

2.4.1 For Extraction and Purification of Plasmid DNA (# Notes 3 and 4)

2.4.2 For Analysis of Plasmid DNA

1. *Electrophoresis buffer* (# **Note 6**)
   (a) *Tris-borate-EDTA (TBE) Buffer, 1×*: 89 mM Tris base (pH 7.6), 89 mM Boric acid and 2 mM Disodium EDTA
   (b) *Tris-acetate-EDTA (TAE) Buffer, 1×*: 40 mM Tris (pH 7.6), 20 mM Acetic acid and 1 mM EDTA
2. *EtBr solution (10 mg/mL)*: 10 mg/mL EtBr in double distilled water. Store in light proof vials at room temperature.

3. *Sample loading Buffer (6×)*: 0.25% (w/v) Bromophenol blue, 0.25% (w/v) Xylene cyanol FF, 30% (v/v) Glycerol in double distilled water. Make aliquot and store at −20 °C.

3 Methods of Plasmid Isolation

Plasmid isolation is a crucial step in most routine laboratory experiments in biochemistry and molecular and cell biology. Over the century the procedure has also been used as an indivisible step in recombinant DNA technology. There are several methods published for isolation of plasmid, majority of which makes use of the difference in size of the chromosomal and plasmid DNAs to give preferential release of plasmid [10–23]. The alkaline-sodium dodecyl sulfate (SDS) method is the classical and most popular method for purification of plasmid [13]. With time and necessities several modifications were introduced and new methods are still developing for purity and/or quantity and time and cost saving.

3.1 Classical Methods of Plasmid Purification

The separation of plasmid DNA from chromosomal DNA is the major problem to overcome during purification of plasmid DNA. The physical characteristics that permit the separation of plasmids are its relatively smaller size, covalently closed circular structure, and the fact that they are not bound to other cellular components in the lysate. There has been several methods developed and are being used for the purification of plasmid DNA. Among the several methods, the choice of the method for plasmid extraction depends on the purpose and the required purity of extracted plasmid DNA with each method having its own advantages and limitations. The chromosomal DNA presence in the plasmid preparation, if any, may interfere in downstream processes such as hybridization, RE analysis, transformation and also with agarose gel electrophoresis. Also, the other impurities such as nucleases could degrade the plasmid; high molecular weight RNA will inhibit restriction endonucleases while detergents and salts left over from the purification medium may interfere with the subsequent processing. Therefore, one should always go for some cleaning-up of the plasmid preparation after its isolation from the bacterium. The classical methods commonly used for demonstrating the plasmids include agarose electrophoresis method, rapid boiling method, dye-CsCl gradient method, column chromatography and the most popular is the alkaline lysis method.

3.1.1 Agarose Electrophoresis Method

Among the classical methods of plasmid isolation, agarose electrophoresis method described by Eckhardt [24] is the simplest in principle. It involves the lysis of the bacterial cells in the well of agarose gel using lysozyme and sodium dodecyl sulfate (SDS) followed by electrophoresis of the gel. Upon electrophoresis, the

chromosomal/genomic DNA hardly drifts into the gel matrix due to its larger size while the much smaller plasmid DNA can penetrate and are displayed in the gel. This method is quite simple and convenient to carry out involving minimal manipulation with the disadvantage of poor reproducibility of results. This method can be useful in circumstances where primary screening of strains is the purpose and just displays of the plasmids contained in a strain are sufficient for identification and differentiation. However, in circumstances like DNA sequencing where highly purified plasmids are required, methods such as dye-CsC1 gradient or column chromatography can be used while for recombinant DNA studies less purified plasmid can be often satisfactory. Although, the CsC1 gradient and column chromatography methods give satisfactory results, they are cumbersome, time taking, expensive, and not suitable for the preparation of templates from multiple samples.

3.1.2 Rapid Boiling Method

Rapid boiling method was developed by Holmes and Quigley [19]. In this method, bacterial cells are partially lysed so that the large chromosomal DNA remains trapped in the cell debris while the smaller plasmid DNA can escape. Thereafter, chromosomal DNA is denatured using high temperature, after which reannealing allows the plasmids to reassociate. The lysate is then centrifuge to remove the chromosomal DNA along with the cell debris while the plasmid DNA remains in the suspension. The supernatant is collected in fresh tube to which isopropanol was added to precipitate the plasmid DNA.

3.1.3 Dye-CsC1 Gradient Method

The base compositions of chromosomal and plasmid DNAs are usually so alike that their separation on the basis of difference in their density is quite unworkable. However, the buoyant density can be manipulated to produce the difference using saturating concentrations of DNA binding fluorescent dyes. Ethidium bromide (EtBr) is the most commonly used fluorescent dye which binds the DNA by intercalation between base pairs. The intercalation of EtBr dye occurs only when the double helix DNA unwinds slightly. The unwinding is unimpeded in linear or open-circular DNA but creates strain in covalently closed circular (CCC) molecules leading to binding of less dye per unit length in CCC plasmids. Consequently, the buoyant density will be less in case of linear chromosomal DNA than the CCC plasmids of similar base composition and thus can be separated using equilibrium ultracentrifugation [25]. The DNA migrates to the point at which it has density similar to that of CsCl, i.e. 1.7 g/cm^3 in the gradient and the protein molecules having lower buoyant densities remain at the top while the RNA gets pelleted at the bottom of the tube. Cesium chloride (CsCl)/EtBr ultracentrifugation is one of the traditional methods being used since 1950s for purification of nucleic acids

[26–28]. After about 72–96 h of ultracentrifugation in swinging bucket rotors using CsCl density gradient with saturated concentration of EtBr, the separated CCC plasmid DNA can be visualized under UV rays in the form of band below the linear chromosomal DNA band [29]. Like EtBr, propidium iodide can also be used for separation but it is quite expensive. Furthermore, in cases where the difference of buoyant density between plasmid and chromosomal DNA is very small, Hoechst 33258 dye can be used to augment the buoyant density difference for a possible separation. Although, the conventional equilibrium Dye-CsC1 ultracentrifugation leads to isolation of ultrapure grade of plasmid DNA suitable for almost all downstream processing [30], but requires extended hours for ultracentrifugation and additional time and labor for the removal of dye and CsCl salt from the preparation. However, with the advances in rotor design and an increased understanding of centrifugal force theory, researchers have come up with better controlled protocols with significantly reduced starting material volumes and ultracentrifugation spin time employing vertical and fixed angle rotors [31]. Two-step CsCl-EtBr discontinuous gradient method described by Garger et al. [32] is one such modified rapid method capable of isolating ultrapure DNA plasmids in about 5 h with lesser RNA contamination compared to the conventional equilibrium ultracentrifugation method. The detailed procedure of rapid two-step CsCl-EtBr discontinuous gradient single ultracentrifugation method is also described in the protocol section.

Note: This method is very sensitive to CsCl concentration and a slight change of CsCl amount may lead to a negative result; plasmid DNA is not separated as a single band in the tube.

3.1.4 Column Chromatography

Although, the extended time required for separation of plasmid DNA by density gradient ultracentrifugation can be minimized by using a vertical rotor, but still the removal of the EtBr and CsCl from the recovered plasmid preparation upholds the cumbersomeness of the method. However, the purity and yield of plasmid DNA comparable to density gradient ultracentrifugation method can also be achieved with adsorption chromatography [33] and fast protein liquid chromatography [34, 35]. Tiselius et al. [36] first developed hydroxyapatite columns for protein chromatography which was later extended to nucleic acids chromatography by Bernardi [37]. Hydroxyapatite binds with proteins and nucleic acids at low phosphate concentration while the progressive ascend in the concentration of phosphate leads to the earliest elution of proteins and RNA, trailed by smaller DNA molecules, viz. plasmid DNA and finally the high molecular weight chromosomal DNA will elute at the highest phosphate concentrations. Furthermore, under chromatography methods only the protocol for most commonly used hydroxyapatite columns chromatography will be described in detail.

3.1.5 Alkaline Lysis Method

Alkaline lysis method developed by Birnboim and Doly [13] is the most commonly used technique applicable to a wide range of bacterial species for isolation of plasmids. It fundamentally relies on differential denaturation and reannealing of plasmid DNA compared to high molecular weight chromosomal DNA and proteins. The process involves the lysis of bacterial cells using SDS and exposing the cell extract to high alkaline pH (12.0–12.6) conditions [13, 38], followed by mixing the cell extract with high concentration of low-pH potassium acetate for neutralization to selective precipitate the chromosomal DNA, or by direct extraction with unneutralized phenol [38, 39], which results in the chromosomal DNA banding at the interface. This selective precipitation occurs due to inter strand re-associations at multiple sites owing to the very high molecular weight of the chromosomal DNA leading to the formation of an insoluble DNA network. The bulk of cellular RNA and protein are also precipitated under these conditions if protein is first complexes with SDS (anionic detergent). However, the plasmid DNA remains in the soluble fraction due to its covalently closed circular (CCC) nature and much smaller size and is then precipitated using isopropanol.

Combining the different reagents appropriately, the precipitation of most of the chromosomal DNA, RNA, and protein can be accomplished in a single step and many such alterations have been applied to the original procedure, and a large number of modified and alternative rapid methods have been developed. The simplest alternative to alkaline lysis is the rapid boiling method developed by Holmes and Quigley [19]. Here, the cells are lysed partially allowing plasmids to escape, whereas the bacterial chromosomal DNA remains trapped in the cell debris. Then, the chromosomal DNA is denatured using high temperature, after which reannealing allows the plasmids to reassociate. Centrifugation removes the chromosomal DNA along with the cell debris, leaving the plasmid in suspension, from where it is recovered by isopropanol precipitation. After the initial characterization, it is possible to purify further some or all of the plasmid DNAs by RNase digestion and extraction with organic solvents. On the other hand, a kind of salts such as lithium and calcium functions to make RNA as a selective precipitate from DNA-RNA mixture [40]. This purified plasmid DNA is suitable to be used for techniques such as sub-cloning, sequencing, and construction of gene probes. However, in practice, simple rapid methods for plasmid preparation are usually more dependable and preferred for plasmid DNA isolation in most of the laboratories.

3.2 Rapid Solid Phase Extraction Methods of Plasmid Purification

In due course of time, numerous kits and systems were developed for separation of plasmid DNA from chromosomal DNA which does not demand CsCl/EtBr gradients [41]. These rapid methods used minispin column systems composed of silica matrices, glass particles or powder, magnetic beads, and diatomaceous earth or ion exchange carriers [42–45]. These methods are mainly based on partial or complete lysis of cell followed by the removal of chromosomal DNA by centrifugation and selective precipitation optimized with specific buffer and extremely precise pH and salt concentrations [46]. It should be noted that the swiftness and ease of performing plasmid isolation from multiple samples requires a high speed microcentrifuge. Based on these properties, many isolation kits were made commercially available for diagnosis and research purposes, which could isolate the plasmid within 30 min. Among many, the one using an anion-exchange resin and other employing silica membrane with chaotropic solutions for plasmid preparation are the two most commonly used kits. These kits most oftenly used diethyl aminoethyl (DEAE) resin and guanidine hydrochloride or guanidine thiocyanate as chaotropic agent for adsorption of DNA. Also, both these kits utilized the alkaline-SDS lysis principle for the separation of plasmid DNA from chromosomal DNA and require addition of RNase to digest the RNA contamination. These commercially available kits became popular because of the limited labor requirement, ease of use, and rapid and consistent preparation of high-quality plasmid DNA that can be used in PCR, sequencing, restriction enzyme digestion, and transformation. Also, it is worthy to note that these kits are rather expensive.

3.3 Protocols

3.3.1 Protocol 1

Agarose electrophoresis method for the detection and preliminary characterization of plasmid DNA in clinical isolates (Time: 3–4 h)

1. Prepare 0.75–1.2% agarose gel in Electrophoresis Buffer (**Note 7**). Use appropriate comb to form the required number of well for loading the samples.
2. Add 15 μL of Lysozyme Mixture in wells of mounted gel.
3. Pick 1 or 2 single colony (10^6 to 10^8 cells) of bacteria grown overnight on LB/BHI agar with the help of flat end of toothpick or micro tip and resuspend in Lysozyme Mixture poured in wells of mounted gel. In case of liquid culture growth, take 0.1 to 0.5 mL of overnight grown culture corresponding to 10^6 to 10^8 cells. Centrifuge at 5000 × *g* for 10 min and resuspend the cell pellet in 10 μL of Electrophoresis Buffer with 20% Ficoll 400,000 to prepare cell suspension. Add the cell suspension in Lysozyme Mixture in well of mounted gel.

4. Leave the mixture at room temperature for 2–5 min in case of Gram-negative bacteria and 30–45 min in case of Gram-positive bacteria.
5. Add carefully 30 μL of the SDS Mixture on the top of the bacteria-lysozyme mixture in agarose well. Gently mix the two layers by side to side movement of a toothpick/microtip so that the two layers should still be distinguishable. Avoid complete mixing of the two layers.
6. Now add 100 μL of Overlay Mixture on the top without disturbing the now viscous DNA lysate.
7. Seal the mouth of the wells containing lysate using agarose gel (~50 °C) and then fill the tanks of electrophoresis apparatus with Electrophoresis Buffer.
8. Perform electrophoresis for 60 min at 2 mA and then for 60–150 min at 40 mA depending on the size and the desired resolution of the plasmid(s).
9. After the completion of the electrophoresis, stain the gel for 15 min with Ethidium Bromide (0.4 pg/mL) in Electrophoresis Buffer (**Note 8**) and then observe/analyze the gel under UV rays using transilluminator or gel documentation system (**Note 9**).

3.3.2 Protocol 2

Boiling method for rapid extraction of plasmid DNA (Time: 30–45 min).

1. Culture the test bacteria in 2–3 mL BHI or LB broth with appropriate antibiotic for overnight at 37 °C in shaker incubator (120–150 rpm) (**Note 10**).
2. Harvest the bacterial cells from 1.5 mL of overnight grown culture in a microfuge tube by centrifugation at 12,000 × *g* for 1 min.
3. Discard the supernatant carefully and resuspend the obtained cells pellet in 20 μL STET by gentle pipeting or vortex.
4. Immediately place the tubes in boiling water using a floater or open-bottom stand for exactly 45 s.
5. Centrifuge the tubes for 10 min at 12,000 × *g* to obtain a loose and sticky pellet.
6. Carefully collect the pellet with the help of sterile wooden toothpick in a fresh sterile microfuge tube and add 200 μL isopropanol followed by centrifugation at 12,000 × *g* for 5 min.
7. Carefully discard the supernatant and add 500 μL of 70% ethanol for washing the pellet by centrifugation at 12,000 × *g* for 1 min.

8. Aspirate the 70% ethanol and air dry the pellets for 10 min (**Note 11**).
9. Resuspend the pellet by adding 100 μL nuclease free water (NFW) or TE buffer for analysis/use.

3.3.3 Protocol 3

CsCl-EtBr gradient method of plasmid DNA purification.

Preparation of DNA extract

1. Grow test bacteria colony harboring plasmid in 1 L of BHI/LB broth with appropriate antibiotic(s) in shaker incubator for overnight (**Note 10**).
2. Harvest the cultured bacterial cells by centrifuging at 5000 × *g* for 10 min at 4 °C and resuspended the pellet in 24 mL of Glucose buffer.
3. Add freshly prepared 4 mL Glucose Buffer containing 20 mg/mL lysozyme and keep suspension at room temperature for 10 min incubation.
4. Add 55.2 mL of 1% SDS in 0.2 N NaOH and mix by gently swirling.
5. Place the mixture immediately in ice cold water for 5 min and then add 28 mL of potassium acetate, pH 4.8 (3 M Potassium, 5 M Acetate).
6. Allow the mixture to remain in ice cold water for additional 15 min to precipitate the proteins, chromosomal DNA, and high molecular weight RNA.
7. Centrifuge at 15,000 × *g* for 10 min to remove the insoluble contaminants and collect the supernatant in fresh tube.
8. Extract the supernatant adding an equal volume of phenol, chloroform, and isoamyl alcohol (25:24:1) by centrifugation 12,000 × *g* for 15 min and collect the aqueous phase containing nucleic acids carefully in fresh tube.
9. Add 0.6 volume of isopropyl alcohol and incubate for 10 min at room temperature for precipitation.
10. Centrifuge at 15,000 × *g* for 15 min at 4 °C to collect the plasmid DNA pellet.
11. Resuspend the pellet TE Buffer and store at −20 °C for further use.

Conventional CsCl-EtBr Equilibrium Gradient Ultracentrifugation Method of Plasmid DNA Purification (Time: 24–48 h)

1. Make the final volume of DNA sample (500 μL) to 4.0 mL by adding TE buffer.
2. Add 400 μL EtBr (10 mg/mL) and 4.4 g of solid CsCl. After mixing the content the refractive index is $n = 1.3865$ (1.35 g/mL).

3. Load the sample solution into ultracentrifuge tube, overlay with mineral oil and seal strictly maintain the balance of the tubes.
4. Spin at 192,553 × *g* at 20 °C in ultraspeed centrifuge for overnight.
5. Visualize DNA bands under long wave UV-light.

Rapid Two-Step CsCl-EtBr Gradient Ultracentrifugation Method of Plasmid DNA Purification (Time: 4–5 h)

1. Prepare CsCl solution (density = 1.470 g/mL, $n = 1.3780$) in TE buffer.
2. Place 8 mL of above prepared CsCl solution in large ultracentrifuge tubes or 4.0 mL in small ultracentrifuge.
3. Adjust the density of aliquot containing 14 mg of the buffered total nucleic acids to 1.80 g/mL ($n = 1.4080$) by adding solid CsCl and ethidium bromide solution. For large tube, dissolve 4.2 g of CsCl in 2.4 mL of buffered total nucleic acid extract to obtain a weight of 6.6 g followed by the addition of 0.4 mL of EtBr solution (10 mg/mL) to arrive at a final weight of 7.0 g. For small tube, dissolve 2.1 g of CsCl in 1.2 mL of buffered total nucleic acid extract to obtain a weight of 3.3 g followed by addition of 0.2 mL EtBr solution to arrive at a final weight of 3.5 g.
4. Layered the dense nucleic acid-containing CsCl solution (4.0 mL final volume for the large tubes and 2.0 mL final volume for the small tubes) beneath the less dense CsCl solution using a glass Pasteur pipette or syringe with a long cannula without disturbing the solution interface.
5. Then fill the tube to its capacity with the less dense CsCl solution and seal it properly.
6. Run the spin in fixed titanium rotor (Type 80 Ti or Type 75 Ti) at 324,000 × *g* for 4 or 5 h at 20 °C.
7. Visualize DNA bands under long wave UV-light after completion of the run.

Recovery of Plasmid DNA from CsCl Gradients

1. Collect the plasmid DNA from the gradients by puncturing the side of the tubes with an 18-gauge needle.
2. Wash repeatedly with H_2O-saturated isoamyl alcohol to remove the EtBr.
3. Dilute the plasmid DNA with 3 volumes of TE buffer and precipitate with 2 volumes of cold ethanol.

3.3.4 Protocol 4: Small Scale Extraction of Plasmid DNA by Alkaline Lysis Method

1. Culture the test bacteria in 2–3 mL BHI or LB broth with appropriate antibiotic for overnight at 37 °C in shaker incubator.
2. Harvest the bacterial cells from 1.5 mL of overnight grown culture in a microfuge tube by centrifugation at 12,000 × *g* for 1 min.
3. Decant the supernatant carefully so as to avoid any leftover media and add 100 μL resuspension solution to dissolve the pellet by gentle pipeting or vortex.
4. Add 200 μL of lysis solution and mix by inverting the tube intermittently for at least 2–3 min to allow the lysis to take place.
5. Add 150 μL of neutralizing solution and mix by inverting the tubes gently for several times.
6. Centrifuge the tubes at 12,000 × *g* for 5 min in a microfuge for phase separation.
7. Carefully remove the tubes from the microfuge without disturbing the precipitate.
8. Transfer the liquid phase into fresh labeled tubes containing 250 μL isopropanol.
9. Mix by inverting tubes or gentle brief vortex and centrifuge at 12,000 × *g* for 30 s to precipitate the plasmid DNA as a white pellet.
10. Discard the supernatant with care not to lose the pellet. Add 750 mL 70% ethanol, vortex briefly and centrifuge at high speed for 30 s for washing of the pellets.
11. Aspirate the 70% ethanol and air dry the pellets for 5–10 min.
12. Resuspend the pellet by adding 50 μL nuclease free water (NFW) or TE buffer for analysis/use.

3.3.5 Protocol 5: Solid Phase Extraction Using Mini Column Kits for Plasmid Purification ()

There are several commercially available kits using solid phase mini column for rapid extraction of plasmid DNA provided with well standardized procedure steps to follow for using these kits. Here, we describe the protocol for mdi pDNA Miniprep Kit (mdi membrane technologies, Ambala, India) as per the manufacturer's protocol with certain modifications as used in our laboratory.

1. A colony of plasmid-containing bacterial culture was inoculated in 5 mL LB broth containing appropriate antibiotic in a glass tube and incubated at 37 °C overnight with 180 rpm in a shaker incubator.
2. Centrifuge the overnight grown culture at 6000 × *g* for 5 min to obtain the bacterial cells pellet.

3. Decant the supernatant carefully and keep the tubes in inverted position on a paper towel for 1 min to drain the last traces of the media.
4. Resuspend the pellet in 250 μL of buffer AL1. Then, add 250 μL of AL2 buffer for lysis of cells and immediately mixed by inverting the tube 4–6 times or until the solution became viscous and slightly clear.

   Note: Mixing by vortex is not advised as it may lead to shearing of genomic DNA.
5. Without allowing the lysis reaction to proceed for more than 2 min, add 350 μL of buffer AL3 for neutralization and mix immediately but gently by inverting the tube 4–6 times.
6. Centrifuged the solution at 15,000 × *g* for 10 min and collect the supernatant quickly and carefully to transfer into the spin column with collection tube.
7. Spun the column at 13,000 × *g* for 1.5 min and discard the flow through.
8. Add 750 μL buffer W and centrifuged at 13,000 × *g* for 1.5 min. Discard the flow through.
9. Run an empty spun at 13,000 × *g* for 1 min to remove the residual of buffer W.
10. Place the spin column in fresh sterile 1.5 mL microcentrifuge tube and add 50 μL prewarmed buffer E directly to the center of spin column to eluted the plasmid DNA.
11. Incubate for 5 min at 37 °C, then centrifuged at 13,000 × *g* for 2 min.
12. Store the eluted plasmid DNA at −20 °C till further use.

3.4 Analysis of Plasmids

The plasmid preparations obtained from different methods/kits can easily be checked by electrophoresis in agarose gel with the help of EtBr dye. In addition to the number and form of plasmid, agarose gel electrophoresis also reveals the size of plasmid(s) when converted into linear form by restriction endonucleases. In electrophoresis, plasmids due to their compact and small structure easily get separated from the contaminating large fragments of chromosomal DNA and/or RNA, if present and indicate about the purity of preparation. However, the separation of plasmid from smaller fragments of chromosomal DNA is not possible in routine agarose gel electrophoresis and requires other techniques such as pulse field gel electrophoresis. Generally, 0.7–1.0% agarose gel is used to observe the plasmids depending on the size. But for demonstration of larger plasmids as low as 0.3% agarose gel can be used in horizontal gel electrophoresis.

Note: The larger the plasmid, the lower the percentage of agarose gel is required to resolve them. But at lower percentage

required for demonstration of larger plasmids, the gel became sloppy and difficult to achieve satisfactory resolution. This became even more difficult with some natural isolates having both small and larger plasmid and one wants to resolve them on the same gel for plasmid profile. In such case other techniques like pulsed-field electrophoresis can be employed to obtain satisfactory resolution.

4 Notes

1. Suitable antibiotic(s) should be supplemented in media to ensure retention of required plasmid.
2. Use sterilized distilled water for preparation of solutions and buffer in DEPC treated glass bottles to minimize the risk of DNAses contamination and do not solutions stored for more than a month.
3. Always wear gloves while performing nucleic acid extraction to minimize the chances of nucleases contamination from hands and also to avoid accidental exposure to harmful solutions such as phenol, sodium hydroxide, chloroform, and ethidium bromide.
4. Prefer using freshly prepared solutions especially lysozyme and sodium hydroxide for optimizing the procedure.
5. The ribonuclease is first dissolved in 0.4 M sodium acetate buffer, pH 4.0, at 10 mg/mL and heated for 2 min at 98 °C before diluting it into the rest of the lysozyme mixture. Both lysozyme mixtures are stable for several months at room temperature.
6. TBE or TAE buffer can be used as per the resources and choice of the researcher based on the purpose as both buffers have their own advantages and limitations. TBE buffer has better buffering capacity, whereas TAE buffer has better conductivity. Agarose cross-linkage is better in case of TBE buffer thus gives better resolution of large DNA fragments while smaller DNA fragments get better resolved in TAE buffer. Also, TAE buffer are cheaper to make than TBE buffer.
7. EtBr is carcinogenic. Use it very carefully and always wear gloves while staining and handling the stained gel.
8. Prefer using gel documentation system for visualizing/analyzing the separated plasmid(s). If using UV rays transilluminator visualizing/analyzing, avoid observing for long time and never forgot to wear UV-protected goggles or face shield.
9. Wherever feasible use appropriate antibiotic(s) to achieve selective growth of plasmid-containing bacterial cells.
10. Do not decant to avoid the loss of the pellet.

11. Always wear gloves while handling solutions such as sodium hydroxide, TE-buffer-saturated phenol, chloroform: isoamyl alcohol, and ethidium bromide.

References

1. Thomas JO, Kornberg RD (1975) Octamer of histones in chromatin and free in solution. Proc Natl Acad Sci 72:2626–2630
2. Alberts B, Johnson A, Lewis J et al (2002) Molecular biology of the cell, 4th edn. Garland Science, New York
3. Lodish H, Berk A, Zipursky SL et al (2000) Molecular cell biology, 4th edn. W. H. Freeman, New York
4. Broach JR (1981) The yeast plasmid 2 micron circle. In: Strathern JN, Jones EW, Broach JR (eds) The molecular biology of the yeast saccharomyces: life cycle and inheritance. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp 445–470
5. Hayes W (1968) The genetics of bacteria and their viruses, 2nd edn. Blackwell, Oxford
6. Watanabe T (1963) Infective heredity of multiple drug resistance in bacteria. Bacteriol Rev 27(1):87–115
7. Wink M (2006) An introduction to molecular biotechnology: molecular fundamentals, methods and application in modern biotechnology. Wiley-VCH, Weinheim
8. Lesk AM (1969) Why does DNA contain thymine and RNA uracil? J Theor Biol 22(3): 537–540. https://doi.org/10.1016/0022-5193(69)90021-6
9. Ali N, Rampazzo RCP, Costa ADT, Krieger MA (2017) Current nucleic acid extraction methods and their implications to point-of-care diagnostics. Biomed Res Int 2017: 9306564. https://doi.org/10.1155/2017/9306564
10. Cheng L, Li TY, Zhang Y (2004) Rapid preparation of total nucleic acids from E. coli for multi-purpose applications. J Biochem Mol Biol 37:351–355
11. Chowdhury EH, Akaike T (2005) Rapid isolation of high quality, multimeric plasmid DNA using zwitterionic detergent. J Biotechnol 119: 343–347
12. Chowdhury K (1991) One step 'miniprep' method for the isolation of plasmid DNA. Nucleic Acids Res 19:2792
13. Birnboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 7: 1513–1523
14. Altschuler M, Heddens DK, Diveley RR Jr, Kresheck GC (1994) Plasmid DNA isolation utilizing a novel nonionic detergent. BioTechniques 17:434–436
15. Currier TC, Nester EW (1976) Isolation of covalently closed circular DNA of high molecular weight from bacteria. Anal Biochem 76: 431–441
16. Ferrus MA, Alonso JL, Amoros I, Hernandez M, Hernandez J (1999) A rapid procedure for the isolation of plasmid DNA from environmental bacteria. Int Microbiol 2: 115–117
17. Guerry P, LeBlanc DJ, Falkow S (1973) General method for the isolation of plasmid deoxyribonucleic acid. J Bacteriol 116:1064–1066
18. He M, Wilde A, Kaderbhai MA (1990) A simple single-step procedure for small-scale preparation of Escherichia coli plasmids. Nucleic Acids Res 18:1660
19. Holmes DS, Quigley M (1981) A rapid boiling method for the preparation of bacterial plasmids. Anal Biochem 114(1):193–197. https://doi.org/10.1016/0003-2697(81)90473-5
20. Klein RD, Selsing E, Wells RD (1980) A rapid microscale technique for isolation of recombinant plasmid DNA suitable for restriction enzyme analysis. Plasmid 3:88–91
21. Paul B, Cloninger C, Felton M, Khachatoorian R, Metzenberg S (2008) A non-alkaline method for isolating sequencing-ready plasmids. Anal Biochem 377:218–222
22. Serghini MA, Ritzenthaler C, Pinck L (1989) A rapid and efficient 'miniprep' for isolation of plasmid DNA. Nucleic Acids Res 17:3604
23. Song J, Geltinger C, Sun K, Kanazawa I, Yokoyama KK (1999) Direct lysis method for the rapid preparation of plasmid DNA. Anal Biochem 271:89–91
24. Eckhardt T (1978) A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. Plasmid 1:584–588
25. Brown TA (2010) Gene cloning and DNA analysis: an introduction, 6th edn. Blackwell Publishing, Hoboken, NJ
26. Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning, a laboratory manual. Cold

Spring Harbor Laboratory Press, Cold Spring Harbor, NY

27. Meselson M, Stahl FW, Vinograd J (1957) Equilibrium sedimentation of macromolecules in density gradients. Proc Natl Acad Sci U S A 43(7):581–588. https://doi.org/10.1073/pnas.43.7.581
28. Radloff R, Bauer W, Vinograd J (1967) A dye-buoyant-density method for the detection and isolation of closed circular duplex DNA: the closed circular DNA in HeLa cells. Proc Natl Acad Sci U S A 57(5):1514–1521. https://doi.org/10.1073/pnas.57.5.1514
29. Anet R, Strayer DR (1969) Density gradient relaxation: a method for preparative buoyant density separations of DNA. Biochem Biophys Res Commun 34(3):328–334
30. Green MR, Sambrook J (2018) Preparation of plasmid DNA by alkaline lysis with sodium dodecyl sulfate. Maxipreps. Cold Spring Harb Protocols 2018(1). https://doi.org/10.1101/pdb.prot093351
31. Flamm NF, Bond HE, Burr HE (1966) Density-gradient centrifugation of DNA in a fixed-angle rotor: a higher order of resolution. Biochim Biophys Acta 129:310–317
32. Garger SJ, Griffith OM, Grill LK (1983) Rapid purification of plasmid DNA by a single centrifugation in a two-step cesium chloride-ethidium bromide gradient. Biochem Biophys Res Commun 117(3):835–842
33. Colman A, Byers MJ, Primrose SB, Lyons A (1978) Rapid purification of plasmid DNAs by hydroxyapatite chromatography. Eur J Biochem 91:303–310
34. Chandra G, Patel P, Kost TA, Gray JG (1992) Large-scale purification of plasmid DNA by fast protein liquid chromatography using a hi-load Q Sepharose column. Anal Biochem 203(1):169–172
35. McClung JK, Gonzales RA (1989) Purification of plasmid DNA by fast protein liquid chromatography on superose 6 preparative grade. Anal Biochem 177(2):378–382. https://doi.org/10.1016/0003-2697(89)90069-9
36. Tiselius A, Hjerten S, Levin O (1956) Protein chromatography on calcium phosphate columns. Arch Biochem Biophys 65:132–155
37. Bernardi G (1969) Chromatography of nucleic acids on hydroxyapatite I. Chromatography of native DNA. Biochim Biophys Acta 174:423–434
38. Kado CI, Liu ST (1981) Rapid procedure for detection and isolation of large and small plasmids. J Bacteriol 145:1365–1373
39. Kieser T (1984) Factors affecting the isolation of CCC DNA from *Streptomyces lividans* and *Escherichia coli*. Plasmid 12(1):19–36. https://doi.org/10.1016/0147-619x(84)90063-5
40. Eon-Duval A, Gumbs K, Ellett C (2003) Precipitation of RNA impurities with high salt in a plasmid DNA purification process: use of experimental design to determine reaction conditions. Biotechnol Bioeng 83:544–553. https://doi.org/10.1002/bit.10704
41. Tan SC, Yiap BC (2009) DNA, RNA, and protein extraction: the past and the present. J Biomed Biotechnol 2009:574398. https://doi.org/10.1155/2009/574398
42. Azimi SM, Nixon G, Ahern J, Balachandran W (2011) A magnetic bead-based DNA extraction and purification microfluidic device. Microfluid Nanofluid 11(2):157–165. https://doi.org/10.1007/s10404-011-0782-9
43. Esser KH, Marx WH, Lisowsky T (2006) maxXbond: first regeneration system for DNA binding silica matrices. Nat Methods 3(1):2005–2006. https://doi.org/10.1038/nmeth845
44. Koo K, Foegeding PM, Swaisgood HE (1998) Isolation of RNA and DNA fragments using diatomaceous earth. Biotechnol Tech 12(7):549–552. https://doi.org/10.1023/A:1008859632378
45. Padhye V, York C, Adam B (1997) United States patent US 5658548. Promega Corporation; Nucleic acid purification on silica gel and glass mixture
46. Chacon-Cortes D, Griffiths L (2014) Methods for extracting genomic DNA from whole blood samples: current perspectives. J Biorepository Sci Appl Med 2:1–9. https://doi.org/10.2147/BSAM.S46573

Chapter 7

Recombinant Antigen-Based Diagnostic Assays of Pig Viral Diseases

Rajib Deb, Ajay Kumar Yadav, Gyanendra Singh Sengar, Seema Rani Pegu, Souvik Paul, Swaraj Rajkhowa, and Vivek Kumar Gupta

Abstract

Recombinant DNA technology or genetic engineering is one of the most explored technologies in the current era as it is one of the convincing technologies for the detection of both antigen and antibody. Recombinant DNA-based technology involves cutting of desired genes with suitable restriction enzymes, cloning, and expression of the immunodominant antigenic epitopes in the bacterial, yeast, baculovirus, or cell culture system. These expressed proteins are mostly purified from the host system in the pure form conferring its conformational structure that does not reduce its reactivity. The technology involves skill to design primer that to in the frame of the expression vector, standardization of expression and purification, and lesser cost utilization. Recombinant DNA technology is utilised to address a variety of diseases in human, but it is also widely employed in the area of animal disease diagnosis, leading to the creation of diagnostic assays like as ELISA, ELISPOT, and Lateral Flow Devices. This method is also used to create GMOs (genetically modified organisms) (GMOs). Recombinant proteins are used in a number of diagnostic tests for the detection of pig viral disease.

Key words Genetic engineering, Recombinant DNA technology, Protein, ELISA

1 Introduction

"Genetic engineering" is an important field of biotechnology having a significant impact on agricultural sciences and its application can be expected in the management of crops improvement, feed industry, improvement of livestock health and genetic manipulations, development of novel diagnostics and therapeutics or vaccines. Industrial capacity could be developed in geographic regions which do not have any vaccine production capability. In addition, the use of deoxyribonucleic acid (DNA) or proteins/enzymes in health diagnostics is impending exponentially. Uses of enzymes in food science, medicinal chemistry, leather, detergent, textile,

Rajib Deb et al. (eds.), *Protocols for the Diagnosis of Pig Viral Diseases*,
Springer Protocols Handbooks, https://doi.org/10.1007/978-1-0716-2043-4_7,

sectors are also growing rapidly. The intensifying need of therapeutic agents and other applications of enzymes or proteins could only be met by synthesis of heterologous recombinant proteins.

Discovery of the structure of the genetic material so-called as deoxyribonucleic acid (DNA), by Watson and Crick (1953) instigated an era of biochemical exploration of cellular physiology that has congregated momentum in the succeeding years, with more recent advances in molecular genetics as well as biochemistry leading to a rebellion in biological research. The so-called new biology allows investigators to polish the experimental methodologies beyond what was believed possible only a few years ago. Applications of this research and related biotechnological applications to various problems in agricultural sciences are now perceived as being possible. In the year 1973, Stanley Cohen, Herbert Boyer, and their co-workers invented a methodology for transmitting genetic information from one organism to another and this became known as "recombinant DNA technology" or "Genetic Engineering," which permitted researchers to isolate specific target genes and perpetuate them in different host organisms.

The founding stones of "Genetic Engineering" have been laid by strenuous efforts by various researchers across the globe and many of which have been awarded with the prestigious Nobel prizes. Here, we catalogued some of the milestones which molded the destiny of genetic engineering.

1663	Cells are first described by Robert Hooke
1675	Leeuwenhoek discovers protozoa and bacteria
1855	Bacterium *Escherichia coli* discovered
1869	Miescher discovers the DNA in the sperm of trout
1902	Walter Sutton coins the term gene
1919	Karl Ereky coins the term biotechnology
1928	Federick Griffith's discovers the transforming principle
1938	Edward Tatum coins the term molecular biology
1941	A. Jost coins the term genetic engineering
1943	Oswald Avery proves DNA is heritable material
1953	James Watson and Francis Crick elucidate the double helical structure of DNA
1958	Kornberg discovers the DNA polymerase.
1964	DNA ligase was isolated
1971	Arber, Smith, and Nathans discover and characterize restriction enzyme
1972–77	Cohen and Boyer successfully splice a gene
1973–78	Paulberg creates recombinant DNA

(continued)

1975	Sanger, Maxam, and Gilbert discover nucleotide sequencing
1976–80	Formation of Genentech Inc.
1977	Genome of bacteriophage φX 174, first to be sequenced
1979	Genome of first plasmid pBR 322 was sequenced
1983	Karry Mullis discovers polymerase chain reaction
1991	Human genome project initiated
1992	Genome of first chromosome, yeast chromosome III was sequenced
1995	Genome of first cellular organism, *Haemophilus influenza* was sequenced
1996	Genome of first eukaryotic organism, *Saccharomyces cerevisiae* was sequenced
1998	Human embryonic stem cells isolated by Thomson, Jones, and co-workers
1998	Nematode *C. elegans* sequenced
1999	Human genome project launched
2000	Genome of first mammalian organism, Homo sapiens was sequenced Genome of first plant genome, Arabidopsis thaliana was sequenced
2002	Malaria parasite, *Plasmodium falciparum* and mosquito, Anopheles sequenced Synthetic poliovirus created from the published sequence of the virus
2003	Human genome map project completed
2004	Laboratory rat genome sequenced
2005	Draft of Chimpanzee genome released Rice genome mapped
2006	Regulatory approval for plant-based vaccines against Newcastle disease of poultry given to Dow Agrosciences
2008	Synthesis of artificial genome of bacteria, *Mycoplasma laboratorium* derived from the genome of *Mycoplasma genitalium* termed minimal genome project
2009	Draft sequence of Maize revealed Cows and goats have been engineered to express drugs and other proteins in their milk FDA approved a drug produced in goat milk
2010	First synthetic genome of a bacterium, *Mycoplasma mycoides* JCVI-syn 1.0 capable of self-replicating revealed by Craig Venter's institute
2012	Develop the CRISPR/Cas9 system

(continued)

	Alipogene tiparvovec became the first gene therapy treatment to be approved for clinical use
2013	Entire genome of a Neanderthal, an extinct species of humans revealed
2015	CRISPR was used to edit the DNA of non-viable human embryos

The word "cloning" refers to the fact that the method involves the replication of a single DNA molecule starting from a single living cell to generate a large population of cells containing identical DNA molecules. Molecular cloning generally uses DNA sequences from two different organisms: the species that is the source of the DNA to be cloned and the species that will serve as the living host for replication of the recombinant DNA. Molecular cloning methods are central to many contemporary areas of modern biology and medicine.

DNA to be cloned is obtained from an organism of interest, the DNA is then treated with restriction enzymes to generate smaller DNA fragments. Afterward, these fragments are ligated with vector DNA which is also digested by same restriction enzyme to generate recombinant DNA molecules. The recombinant DNA is then introduced into a competent host organism (typically an easy-to-grow, benign, laboratory strain of *E. coli* bacteria). This will generate a population of organisms in which recombinant DNA molecules are replicated along with the host DNA. Because they contain foreign DNA fragments, these are transgenic or genetically modified microorganisms (GMO). This process takes the advantage of the fact that a single bacterial cell can be induced to take up and replicate a single recombinant DNA molecule. This single cell can then be expanded exponentially to generate a large amount of bacteria, each of which contain copies of the original recombinant molecule. Thus, both the resulting bacterial population and the recombinant DNA molecule are commonly referred to as "clones." Strictly speaking, recombinant DNA refers to DNA molecules, while molecular cloning refers to the experimental methods used to assemble them.

Competence is the ability of a cell to take up extracellular naked DNA from its environment. Competence may be differentiated between natural competence, a genetically specified ability of bacteria which is thought to occur under natural conditions as well as in the laboratory, and induced or artificial competence, which arises when cells in laboratory cultures are treated to make them transiently permeable to DNA.

1.1 Principle

Logarithmically growing *E. coli* strains (e.g. DH5α cells) when treated with chilled $CaCl_2$ become able to take up exogenous DNA during transformation.

Transformation is a genetic alteration of a cell resulting from the direct uptake, incorporation and expression of exogenous DNA from its surrounding and taken up through the cell membrane.

When a brief heat shock is given to *E. coli* strains (e.g. DH5α) in a media containing exogenous DNA (plasmids), the *E. coli* strains take up the DNA. During the brief period of heat shock the pores in the bacterial membrane widens facilitating intake of the DNA.

Bacteria, such as *Escheria coli*, has its own chromosomal DNA, however, also carries an extrachromosomal DNA, known as "plasmid" which can replicate by its own capabilities. Plasmid can carry the foreign DNA fragment into the bacteria, ultimately made it possible to make multiple copies of it, and thus act as "cloning vector." With the advancement of genetic manipulation tools, now a days it is possible to insert foreign genes in such a way that *E. coli*'s cellular machinery will be used not only for making multiple copies of the plasmid, but also to generate mRNAs from the cloned genes to translate into functional proteins and the so-called as expression vector. Proteins obtained by expressing of cloned genes in bacteria can be used in a variety of ways, such as, for investigating the structural and biochemical functions of the protein, raising antibodies, and so on. Preferably, an expression vector developed in such a fashion that it should contain multiple cloning sites, ribosomal binding site, transcriptional termination site, and most importantly a strong inducible promoter under which the foreign gene can be expressed. With these suitable conditions, the foreign gene thus can be transcribed, and translated, in the bacterial host system to produce the functional proteins encoded by the target genes. Commonly expression vector used for production of recombinanat protein is a pET system, which is under the controlling power of T7 RNA polymerase promoter. pET vector is transformed into an *E. coli* host strain (DE3), which contain a copy of the gene for T7 RNA polymerase (T7 gene 1) under the control of the lac promoter. Furthermore, the promoter for both target gene and T7 gene 1 also contain the lacO operator sequence and are, therefore, inhibited by the lac repressor (lacI). IPTG (Isopropyl-beta-D thiogalactoside) induction allows the transcription of the T7 RNA polymerase gene whose protein product subsequently activates the expression of target gene.

Plasmids expressed foreign proteins make it possible to obtain relatively large amounts of proteins that might otherwise be very difficult to purify by traditional methods such as gel filtration/ion-exchange/hydrophobic interaction. These methods for purification of heterologous recombinant proteins from *E. coli* are difficult as well as time consuming. Therefore, what is required is the ability to impart the target protein with a unique property that can be used to

separate it from all other host proteins. To facilitate the purification of the expressed protein, "affinity tags" are mostly using. These are a short DNA sequences that are fused to the coding sequence of the gene, either at the 5′ end or the 3′ end. These sequences are transcribed and translated together with the gene, resulting in the formation of what is known as a "fusion protein." The purification of tagged proteins from host cell consists of four steps (1) Cell lysis; (2) Binding of the tagged protein to an affinity column; (3) Washing the column to remove the untagged ones; and (4) elution of the tagged protein itself. Most commonly used tag is his-tag. When histidine are used as affinity tag, the fusion protein (target gene plus 6 his residues) expressed in bacteria, bind non-covalently to a nickel-NTA column. In a technique called immobilized metal ion affinity chromatography (IMAC), metal nickel are bound to a resin matrix (nitriloacetic acid or NTA) and used to capture his-tagged proteins. NTA has four coordinated sites that bind a single nickel ion very tightly. The charging of NTA with Ni^{+2} leaves two of six possible coordination sites of the ion free, which can strongly bind to imidazole rings of consecutive histidine residues on a polypeptide chain. At least six histidine residues are required to provide the necessary binding affinity to firmly adhere the tagged proteins to the column. The majority of the host protein will not be able to bind to the column and thus the contaminating proteins will washed off and fusion his-tag protein will elute using imidazole.

1.2 Lowry's Method

Lowry protein assay is a biochemical assay for determining the total level of protein in a solution which invented by the biochemist Oliver H. Lowry in the 1940s. The total protein concentration is exhibited by a color change of the sample solution in proportion to protein concentration, which can then be measured using colorimetric techniques. The principle behind the Lowry method of determining protein concentrations lies in the reactivity of the peptide nitrogen with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin-Ciocalteayphosphomolybdicphosphotungstic acid to heteropolymolybdenumblue by the copper-catalyzed oxidation of aromatic acids. The Lowry method is sensitive to pH changes and therefore the pH of assay solution should be maintained at 10–10.5. The Lowry method is sensitive to low concentrations of protein ranging from 0.10 to 2 mg of protein per mL. The major disadvantage of the Lowry method is the narrow pH range within which it is accurate. However, we will be using very small volumes of sample, which will have little or no effect on pH of the reaction mixture. A variety of compounds will interfere with the Lowry procedure. These include some amino acid derivatives, certain buffers, drugs, lipids, sugars, salts, nucleic acids, and sulfhydryl reagents. It should be noted that ammonium ions, zwitter ionic buffers, nonionic buffers, and thiolcompounds may also interfere with the Lowry

reaction. These substances should be removed or diluted before running Lowry assays.

Various methods have been implemented for introducing cloned eukaryotic DNAs into cultured mammalian cells such as calcium phosphate, DEAE-dextran mediated, protoplast fusion, polybrene coating, electroporation, microinjection in nuclei, and liposome driven delivery system. However, liposome mediated transfection are most commonly used technique for delivery of plasmids in mammalian cells. "Liposome" is an artificial membrane vesicles described by Mannino and Gould-Fogerite (1988), which involve encapsulation of DNA/RNA within liposome followed by fusion of the liposome with the cell membrane and inside cell DNA/RNA released. Liposomes are cationic charges and thus bind with DNA. After entering inside mammalian cells, the foreign DNA will replicate, transcribed, and followed by translated using cellular machinery. Copy number of RNA and concentration of protein formed inside the cell depend on cell density as well as live: dead cell ratio. Another issue is how to extract the protein from mammalian cells. For extraction of recombinant proteins from mammalian cells, the crucial step is lysis. Cells need to be lysed such a way that proteins remain in its immunoreactive state should be undegradable and remain biologically active. In view of a wide range of physical and biological properties of mammalian proteins, it is not surprising that no single method of lysis is sufficient for every purpose. The efficiency of solubilization and subsequent immunoprecipitation depends on ionic strength and pH of lysis buffer, concentration and or type of detergents, presence of divalent cations/cofactors/stabilizing ligands. Although there are exception, many soluble nuclear and cytoplasmic proteins can be solubilized by lysis buffer that contains the nonionic detergents such as Nonidet P-40 (NP-40) and either no salt at all or relatively high concentration of salts (for example, 0.5 M Nacl). Extraction of membrane bound/hydrophobic proteins are less effected by the ionic strength of the lysis buffer but often requires a mixture of ionic and nonionic detergent. When attempting to solubilize a protein for the first time there are two different strategies can be employed, at one extreme, harsh conditions can be maintained to ensure that protein is released quantitatively from cells and on the other hand, gentle conditions can be used to help preserve the proteins in a native confirmatory state. Denatured proteins are much more likely to be degraded than native ones; therefore, it is advisable to take steps to minimize proteolytic activity in cell extracts, when harsh conditions are applied. It is necessary to keep the extracts in cold conditions and inhibitors of protease need to be supplemented in lysis buffer.

2 Materials

1. Gene of interest (GOI).
2. Primers with restriction enzyme sites.
3. Taq DNA polymerase.
4. Restriction enzymes.
5. PCR Machine.
6. Gel Documentation system.
7. Horizontal gel electrophoresis.
8. Vertical gel electrophoresis.
9. SDS PAGE buffers (Tris Buffers, TEMED, Tris-Glycine buffer, ammonium per sulfate (APS).
10. Acrylamide/Bis-acrylamide.
11. Western blot machine.
12. Ni-NTA agarose.
13. His-tag antibody.
14. Protein purification buffers.
15. Urea.
16. Imidazole.
17. Western blot buffers.
18. Nitro-cellulose membrane or PVDF membrane.
19. Horseradish Peroxidase (HRPO).
20. Anti-species antibody with HRPO conjugate.
21. Chemidoc system.

3 Methods

3.1 Cloning

3.1.1 Purification of Amplified PCR Product

It is carried out to remove non-specific amplified products, contaminants, un-used primers, reagents, etc., and separate only the target specific PCR product for further processing and cloning. There are several commercial kits available.

By Using Gel Extraction Kit

Gel purification of amplified PCR product for cloning may be done using any gel extraction kit. For example, QIAquick® Gel Extraction Kit (Qiagen, USA).

1. The DNA fragment was excised from the agarose gel using a clean sharp scalpel and kept in a colorless tube.
2. The gel slice was weighed and 3 volume of buffer QG was added to one volume of gel.

3. Incubated at 50 °C for 10 min until the gel slice was completely dissolved.
4. One gel volume of isopropanol was added to the sample and mixed.
5. QIAquick spin column was placed in the two mL collection tube.
6. The sample was applied to the QIAquick column and centrifuged for 1 min at 16,128 × *g*.
7. The flow through was discarded and the column was placed in the same collection tube.
8. 0.5 mL of buffer QG was added to the column and centrifuged for 1 min at 17,900 × *g*.
9. 0.75 mL of buffer PE was added to the column and centrifuged for 1 min.
10. The flow through was discarded and centrifuged at 16,128 × *g* for additional 1 min.
11. The QIAquick spin column was placed in a clean 1.5 mL microcentrifuge tube.
12. 50 μL of buffer EB was added into the center of QIAquick membrane and centrifuged for 1 min.
13. The DNA was stored at −20 °C.

By Using PCR Cleaning Kit

The PCR cleaning can be done with any PCR clean up kit such as *QIAquick PCR purification kit (Qiagen)*. The protocol was designed to purify single or double stranded DNA fragments from PCR and other enzymatic reactions.

1. Add 5 vol. of Buffer PB to 1 volume of the PCR sample and mix.
2. Place a QIAquick spin column in a provided 2 mL collection tube.
3. To bind DNA apply the sample to the QIAquick column and centrifuge for 30–60 s.
4. Discard flow through and place QIAquick column back in the same tube.
5. To wash add 0.75 mL Buffer PE to the column and centrifuge for 30–60 s.
6. Discard flow through and place the QIAquick column back in the same tube. Centrifuge the column for another minute at maximum centrifugation speed.
7. Place QIAquick column in a clean 1.5 mL microcentrifuge tube.

8. To elute DNA add 50 μL buffer EB (10 mM Tris-Cl, pH 8.5) or H_2O to the center of the QIAquick membrane and centrifuge the column for 1 min.

3.1.2 Preparation of Gene of Interest and Plasmid and Ligation

Both the vector (e.g. pET32a) and the purified PCR product need to be double digested with respective restriction endonuclease (RE) enzymes in separate reaction conditions. For example, RE with *Bam*HI and *Xho*I as in the following.

Preparation of *Bam*HI/*Xho*I Cut Insert DNA (PCR Product)

Components	Volume (μL)
PCR product	10
10× RE buffer	2
*Bam*HI	5
*Xho*I	3
Total volume	20

Incubate the reaction mixture at 37 °C for 3–4 h. After digestion inactivate the enzyme at 65 °C for 10 min.

Preparation of *Bam*HI/*Xho*I Cut Vector DNA (PCR Product)

Set up the reaction as follows:

1. 5 μL pET32ab DNA (2 μg) + 4 μL 10× buffer+1 μL *Bam*HI + 1 μL *Xho*I+ 27 μL MQ water.
2. Set up 10 tubes (total volume 40 μL).
3. Incubate at 37 °C for 1 h.
4. Add 1 μL *Bam*HI and 1 μL *Xho*I (second addition).
5. Incubate at 37 °C for 1 h.
6. Run 3 μL sample on gel to check the extent of digestion.
7. Incubate at 67 °C for 20 min.
8. Pool all tubes into 2 tubes of 200 μL each.
9. Ammonium acetate precipitation.
10. Resuspend in 30 μL of water.
11. Add 50 μL MQ water +9 μL 10× CIAP buffer +1 μL 10× diluted Alk phosphatise (diluted in 1× buffer).
12. Incubate at 37 °C for 1 h.
13. Add 1 μL 10× freshly diluted Alkaline phosphatise.
14. Incubate for 1 h.
15. Heat inactivation is done by heating at 67 °C for 20 min.

Gel Purification of Digested Insert DNA and Plasmid

Following complete digestion load the RE digested products into separate wells of 1% agarose gel. Run the gel and follow the procedure of gel extraction/purification of products as described earlier. Quantify the product by UV spectrophotometer. Store the gel eluted products at −20 °C until further use.

3.1.3 Ligation of Insert and Vector

Ligation is the process of covalent linking of two ends of insert DNA/gene molecule with ends of vector/plasmid DNA using T4 DNA ligase enzyme.

The amount of Vector and insert required for the ligation were calculated as per the following formula:

$$\frac{\text{Size of the insert (bps)}}{\text{Size of the vector (bps)}} \times \text{amount of vector(ng)} \times 3$$

For efficient ligation set up the ligation reaction as follows:

Component	Volume
Vector pET32a	1 μL
10× ligation buffer	1 μL
PCR product	3–4 μL
T4 DNA ligase	1 μL
Water, nuclease- free	To 10 μL
Total volume	10 μL

1. Vortexed briefly and centrifuged for 3–5 s.
2. Incubated at 22 °C for 2.5 h followed by 4 °C for overnight.
3. 5 μL of ligation reaction was used directly for transformation.

3.1.4 Preparation of Competent Cells

1. A single bacterial colony (e.g. DH5α) was picked up and inoculated in a fresh 15 mL autoclaved falcon tube containing 3 mL LB media for incubation at 37 °C and 180 rpm in a shaker incubator for overnight.
2. 0.5 mL of overnight culture was inoculated in to 50 mL of LB broth and incubated as above until the bacteria reach log phase (for around 3 hrs.) or until the OD reach 0.35–0.4.
3. After that keep the flask and the Oak Ridge tube on ice for 30 min to 1 h.
4. Pour the contents of the conical flask in four Oak Ridge tube and centrifuge at 4032 × *g* for 10 min at 4 °C.
5. The supernatant was decanted under laminar flow and 10 mL of ice cold 100 mM $CaCl_2$ was added to the tube and kept on ice for 30 min.

6. Tubes were centrifuged at 4032 × *g* for 10 min, supernatant was decanted under laminar flow.
7. 2 mL of $CaCl_2$ (containing 15% glycerol) in each tube.
8. Aliquot the contents in 1.5 mL tubes and kept at −20 °C for overnight then kept at −80 °C until further use.

3.1.5 Transformation and Plating

1. 50 μL of competent DH5α from −80 °C was thawed on ice.
2. 5 μL of ligated PCR product was mixed with 50 μL competent cell and kept on ice for 30 min.
3. The mixture was subjected to shock at 42 °C for 1 min, followed by 2 min on ice.
4. 1 mL of LB broth was added to the tube and mixed by inversion, incubated at 37 °C in a shaker incubator for 2 h.
5. Centrifuge at 2800 × *g* for 2 min.
6. 50 μL of supernatant was taken and rest discarded and the pellet was reconstituted in 50 μL supernatant.
7. This 50 μL was spreaded over LB agar plate with ampicillin (50 μg/mL), X-gal (30 μg/mL), and IPTG (40 μg/mL).
8. The Petridish was incubated at 37 °C for overnight.

3.1.6 Screening of Recombinant Clones

Screening of recombinant clones is done by the following methods.

1. Observing growth of bacteria containing the recombinant plasmid in the presence of particular antibiotic (antibiotic selection)—the vector has the property of resistance to a particular antibiotic and it gives the antibiotic resistance to the bacteria which is otherwise susceptible to that antibiotic. Therefore, the bacteria containing the recombinant plasmid will grow but the others will not.
2. Amplification of insert DNA from recombinant plasmid construct/colony PCR using specific primers.
3. Restriction analysis of the plasmid/ PCR amplicon in agarose gel for identification of the insert.

3.2 Expression in Prokaryotic Host System

3.2.1 Small Scale Production of Recombinant Protein

1. Transform the plasmid into competent *E. coli* cells and plate on LB (antibiotic added) plates. Incubate overnight at 37 °C and then re-streak a single colony.
2. Inoculate 5 mL of LB media containing antibiotics with a single colony. Let the tube stand overnight at 37 °C.
3. Inoculate 10 mL of LB containing antibiotics with 1/20th (500 μL) of overnight growth culture. Incubate with aeration at 37 °C until the culture reaches 0.5–0.6 OD_{600}.
4. Remove a 1 mL sample to a 1.5 mL microcentrifuge tube and centrifuge for 1 min. Discard the supernatant and resuspend

the pellet in 150 μL of 2× sample buffer. This is the uninduced protein samples which may be stored at −20 °C. Immediately induced the remaining culture by adding IPTG to a final concentration of 1 mM and resume incubation.

5. After ~13 h, remove a sample and process it as in **step 4**. If performing a time course optimization, remove and process samples at several intervals after induction.
6. Mix the protein sample with equal volume of Lamelli buffer. Heat all samples to 95 °C for 5 min and clarify by centrifugation for 1 min in a microcentrifuge. Load 10–20 μL of each samples on an SDS-Polyacrylamide gel. Apply protein molecular weight standards in adjoining lanes. Electrophoresis until the bromophenol blue dye migrates to the end of the gel.
7. Fix and stain the gel with Coomassie Brilliant Blue (CBB) dye after electrophoresis. Induced proteins are identified by comparison with uninduced protein control lane.

3.2.2 Large Scale Production of Recombinant Protein

1. Transform the plasmid into competent *E. coli* cells and plate on LB (antibiotic added) plates. Incubate overnight at 37 °C and then re-streak a single colony.
2. Inoculate 5 mL of LB media containing antibiotics with a single colony. Let the tube stand overnight at 37 °C.
3. Use the entire 5 mL overnight culture to inoculate 500 mL LB containing antibiotics and incubate with shaking until $OD_{600} = 0.5$.
4. Remove a 1 mL of the culture (uninduced sample) to a 1.5 mL microcentrifuge tube and centrifuge for 1 min. Discard the supernatant and resuspend the pellet in 150 μL of 2× sample buffer.
5. Immediately induced the remaining culture by adding IPTG to a final concentration of 1 mM.
6. Grow for 2 h at 37 °C with shaking (the length of induction depends upon the previous optimized times).
7. Remove a 1 mL of the culture (induced sample) to a 1.5 mL microcentrifuge tube and centrifuge for 1 min. Discard the supernatant and resuspend the pellet in 150 μL of 2× sample buffer.
8. Mix the protein sample with equal volume of Lamelli buffer. Heat all samples to 95 °C for 5 min and clarify by centrifugation for 1 min in a microcentrifuge. Load 10–20 μL of each samples on an SDS-Polyacrylamide gel. Apply protein molecular weight standards in adjoining lanes. Electrophoresis until the bromophenol blue dye migrates to the end of the gel.

9. Fix and stain the gel with Coomassie Brilliant Blue (CBB-R250/G-250) dye after electrophoresis. Induced proteins are identified by comparison with uninduced protein control lane.

3.3 Recombinant Protein Purification

E. coli cells containing an inducible expression vector need to grow and induced to produce the tagged fusion protein.

- The cells will be lysed and insoluble debris will be removed by centrifugation.
- The supernatant from **step 2** will be applied to a Ni^{+2}-NTA column.
- The column will be washed with a low concentration (20 mM) of imidazole, which will compete with low-affinity histidine-column interactions to remove from the column any, perhaps histidine-rich, proteins that are non-specifically bound.
- Finally, the tagged protein itself is removed from the column by various ways.
  - Increasing the concentration of the imidazole to a high level (250 mM).
  - Alternatively, elution conditions with lowering the pH from 8 to 4.5, which will alter the protonated state of histidine residues and results in the dissociation of the protein from metal complex.
  - The tagged proteins also can be removed by adding chelating agents for instance, EDTA, to strip nickel ions from the column and consequently removed the tagged protein.
- Proteins will be visualized by staining of SDS gel with Coomassie blue dye.

Ni-NTA Column-Based Purification of Recombinant Protein

1. Take 10 mL of prewarmed media and add 1/20th (500 μL) of overnight grown culture.
2. Keep at 37 °C for 30 min at orbital shaker and check the OD_{600} should reach around 0.5–0.7.
3. Induced with 1 mM IPTG and grow the culture for an additional 4–5 h.
4. Harvest the cells after centrifugation at 15,000 rpm for 1 min and discard the supernatant.
5. Resuspend the cell pellet in 400 μL of lysis buffer (pH 8.0).
6. Lyse the cell by gentle vortexing.
7. Centrifuge the lysate for 20–30 min at 15,000 rpm.
8. Collect the supernatant in fresh tube.

9. Equilibrate the Ni-NTA spin column with 600 μL lysis buffer (pH 8.0).
10. Centrifuge for 2 min at 448 × *g*.
11. Load the clear lysate supernatant on equilibrate spin column.
12. Centrifuge the spin column for 2 min at 448 × *g*.
13. Collect the flow through.
14. Wash the spin column twice with 600 μL wash buffer(pH 6.3).
15. Centrifuge for 2 min at 448 × *g*.
16. Elute the protein with 100–200 μL elution buffer(pH 4.5) after centrifugation for 2 min at 448 × *g*.
17. Collect the elute and check in SDS PAGE.

3.4 Western Blot Analysis Using Anti-His Conjugate

1. After electrophoresis, the gel was washed three times in transfer buffer at a 5 min interval. The nitrocellulose membrane (NCM) to be utilised for transfer was pre-wetted for at least 30 min in transfer buffer.
2. Three Whatman No.3 filter papers were cut to the size of the gel and soaked in the transfer buffer and were placed on the anode plate over which the pre-wetted membrane was kept making an orientation marks on it. Transfer buffer was poured over the gel and pressed carefully to exclude the excess buffer and air bubbles.
3. Three Whatman No.3 filter papers soaked in ice-cold transfer buffer was placed over the gel.
4. The cathode plate was replaced in position and a constant current of 35 mA was applied for 60 min.
5. After the transfer, the gel was removed and washed twice for 10 min with TBS-T at room temperature.
6. The membrane was blocked overnight at 4oC with blocking buffer (5% skim milk powder in TBS-T).
7. The membrane was washed twice with TBS-T for 10 min followed by TBS wash twice for 10 min at room temperature.
8. Post-wash the membrane was incubated with Ni-NTA HRPO conjugate (1:1000 dilution) for 1 h at 37 °C.
9. After 1 h the membrane was washed with TBS-T and TBS as mentioned in the previous step.
10. Then the membrane was developed by dissolving 0.5 mg/mL of 3, 3′-diaminobenzidine tetra hydrochloride (DAB)(M/s BIO BASIC INC.) and 1 μL per mL of 3% H_2O_2 (M/s Sigma-Aldrich, St. Louis, USA) solution in 20 mL of TBS. The membrane was soaked in the developer solution for 15 min at 37 °C and then the color reaction was stopped by

addition of excess distilled water. After stopping the reaction, the membrane was air dried and documented.

3.5 Protein Estimation

3.5.1 Reagents

1. 2% Na_2CO_3 in 0.1 N NaOH.
2. 1% NaK Tartrate in H_2O.
3. 0.5% $CuSO_4.5\ H_2O$ in H_2O.
4. Reagent I: 48 mL of A, 1 mL of B, 1 mL C.
5. Reagent II- 1 part Folin-Phenol [2 N]: 1 part water.
6. BSA Standard—1 mg/ mL.

3.5.2 Protocol

1. 0.2 mL of BSA working standard in five test tubes and make up to 1 mL using distilled water.
2. The test tube with 1 mL distilled water serves as blank.
3. Add 4.5 mL of Reagent I and incubate for 10 min.
4. After incubation add 0.5 mL of reagent II and incubate for 30 min.
5. Measure the absorbance at 660 nm and plot the standard graph.
6. Estimate the amount of protein present in the given sample from the standard graph.

3.5.3 Bradford Method

The protein in solution can be measured quantitatively by different methods. The methods described by Bradford uses a different concept-the protein's capacity to bind to a dye, quantitatively. The assay is based on the ability of proteins to bind to Coomassie brilliant blue and form a complex whose extinction coefficient is much greater than that of free dye.

3.5.4 Reagents

- Dissolve 100 mg of Coomassie Brilliant blue G250 in 50 mL of 95% Ethanol.
- Add 100 mL of 85% phosphoric acid and make up to 600 mL with distilled water.
- Filter the solution and add 100 mL of glycerol, then make up to 1000 mL. The solution can be used after 24 h.
- Bovine serum albumin (BSA).

3.5.5 Protocol

1. Prepare various concentration of standard protein solutions from the stock solution (say 0.2, 0.4, 0.6, 0.8, and 1.0 mL) into series of test tubes and make up the volume to 1 mL.
2. Pipette out 0.2 mL of the sample in two other test tubes and make up the volume to 1 mL.
3. A tube with 1 mL of water serves as blank.

4. Add 5.0 mL of Coomassie brilliant blue to each tube and mix by vortex or inversion.
5. Wait for 10–30 min and read each of the standards and each of the samples at 595 nm.
6. Plot the absorbance of the standards verses their concentration.
7. Plot graph of optical density versus concentration. From graph find the amount of protein in unknown sample.

3.6 Expression of Recombinant Protein in Eukaryotic System

3.6.1 Lipofectamine-Based Transfection in Mammalian Cells

1. Take a 45 mL sterile media in a 50 mL conical flask after passing through 0.22 μm filter.
2. Add 5 mL (10%) FBS in the media.
3. Add 1× (500 μL) of desired antibiotics tin the media.
4. Now remove the pre media from the parent flask after rigorous shaking.
5. Treat with 2–3 mL of Trypsin Versene Glucose (TVG) for 2–3 min at 37 °C.
6. Now when cells become rounded in shape, add 2–3 mL of media, shake rigorously for several times.
7. Add 15 mL of growth media and distribute in 6 well plates, keep at 37 °C in 5% CO_2 incubator till the monolayer formed.
8. Next day, remove the media and supplement the cells with 2–3 mL OptiMEM (without serum and antibiotics)/well after 2–3 time washing with the same media and keep at CO_2 incubator at 37 °C.
9. Prepare the DNA-lipofectamine complex as follows.
   - Dilute the plasmid DNA (500–750 ng) in 500 μL Opti-MEM solution, mix thoroughly.
   - Mix lipofectamine solution gently before use.
   - Add 3.0–4.5 μL of lipofectamine directly to the diluted DNA, mix thoroughly.
   - Incubate for 1 h at room temperature.
10. Add 500 μL of DNA-lipid complex dropwise to the well containing cells and mix gently by rocking the plate back and forth.
11. Keep at 37 °C in 5% CO_2 incubator for 4–6 h and remove the media and supplement with fresh OptiMEM.

3.6.2 Cell Lysis and Protein Extraction

1. Add the lysis buffer of choice (pre-cooled at 0 °C) to wash the cell monolayer. Incubate for 20 min on a flat aluminum tray on a bed of crushed ice.

Volume of lysis buffer (mL)	Size of wells (mm)
1.0	90
0.5	60
0.25	35
0.25	30

2. Scrap the cells to one side of the dish with a cell scrapper.
3. Centrifuge the lysate at 12,000 $\times$ *g* for 2 min at 4 °C.
4. Transfer the supernatant to a fresh microfuge tube and store it on ice or at −70 °C depending on the sensitivity of the target antigen for freezing and thawing.
5. Now the supernatant will be mixed with PAGE loading dye and run the sample in SDS PAGE as described earlier,
6. Furthermore, the presence of protein can be determined by Western blot/Immune fluorescent assay/Immune peroxidase test.

Chapter 8

RNA-PAGE-Based Diagnosis of Viral Diseases

Naveen Kumar, Geetika Kaur, Shubhankar Sircar, Zunjar Dubbal, R. S. Sethi, and Yashpal Singh Malik

Abstract

Regardless of low resources, many of the techniques have proven indispensable in every laboratory around the world, particularly in developing countries. Polyacrylamide gel electrophoresis (PAGE) is one of them, and it is a useful tool for studying RNA from biological samples. PAGE can provide information on the size, content, and quality of RNA, as well as resolving RNA-protein complexes, depending on the type of PAGE used. In the presence of an electric current, RNAs move toward the anode because they are negatively charged. The polyacrylamide gel works as a sieve, preventing RNA from migrating in proportion to its mass, assuming that its mass is primarily proportionate to its charge. Furthermore, because the chain length is almost proportional to its mass, the length of an RNA is usually governed by its migration. This chapter outlines the detailed step-by-step procedures for setting up instruments, preparing samples, loading and running samples on gels, staining gels, and lastly visualizing stained gels.

Key words Electrophoresis, AGE, RNA-PAGE, Genomic Segmentation, Virus Detection, Silver staining

1 Introduction

Polyacrylamide gel electrophoresis (PAGE) is a widely accepted important technique in Ribonucleic acid (RNA) analysis and has been in use in several laboratories across the world for diverse applications such as RNA detection, purification by size, assessment of genomic quality, and nature [1]. In principle, PAGE can be classified as denaturing or non-denaturing PAGE. In denaturing PAGE, the sample composition as well as the structural integrity of individual RNA species can be assessed whereas the separation of conformers and alternatively folded RNA species can be determined using non-denaturing gel electrophoresis [2]. Denaturing PAGE is more appropriate for various electrophoretic procedures and can resolve RNAs from ~600 to $\leq$20 nucleotides [3]. It distinguishes macromolecules based on linear length and mass-to-charge ratio. In addition, by assessing changes in the

Rajib Deb et al. (eds.), *Protocols for the Diagnosis of Pig Viral Diseases*,
Springer Protocols Handbooks, https://doi.org/10.1007/978-1-0716-2043-4_8,

electrophoretic mobility of the RNA samples, this method can be employed to resolve protein-RNA complexes as well as predict the formation of RNA complexes. However, non-denaturing or native PAGE is more useful in differentiating RNA conformations and RNA-protein complexes. The only limitation with PAGE is that it cannot analyze RNAs of large sizes consisting more than 600 nucleotides. In such cases of large size RNA analysis, agarose gel is the method of choice as it is easy to perform, less laborious, economical, and time-saving compared to the RNA-PAGE method which is complicated, laborious and needs an expert to perform [4]. Similar to PAGE, acrylamide gel electrophoresis (AGE) can be used for the differentiation of the rotavirus dsRNA not only within a single animal species but also between various species.

PAGE utilizes the key properties of movement of charged biomolecules (nucleic acids) under the electric fields and therefore, RNA analysis can easily be achieved due to the RNA biomolecules migration across the polyacrylamide meshes according to their charge and the strength of the electric field. The negatively charged RNA molecules migrate towards the anode under the influence of an electric field. The sieving effect of the agarose gel allows the RNA to pass through the gel based on the mass proportion of the RNA segment, which is directly proportional to the possessed charge. As the mass is invariably related to the length of RNA, it can be principally determined through migration analysis. Furthermore, morphological characteristics such as topology also influences migration behavior, causing the RNA to appear longer than it is. Moreover, in denaturing PAGE, the separation occurs largely according to the size of the RNA biomolecules, whereas in non-denaturing PAGE, RNAs mobility is determined by both the size and conformation [5]. Usually, 0.4–1.5-mm-thick gels are used for RNA-PAGE analysis. Depending on the type of the detection reagent, RNA can be stained and visualized in gels by using a variety of chemicals such as Silver nitrate, Toluidine blue, SYBR green, and ethidium bromide. Autoradiography can be used to see radioactively labeled RNA molecules, and a fluorescence scanner can see fluorescently labeled RNA molecules. However, we have limited our protocol to the silver staining method, which is frequently used for the visualization of RNA migration patterns with the naked eye.

2 RNA-PAGE Principle

Poly Acrylamide Gel Electrophoresis (PAGE) is one of the best methods in protein identification, molecular weight determination, protein–protein or protein–DNA interaction, etc. It can be also used for the separation of nucleic acid (RNA) from various viruses. Protein to be analyzed needs to be mixed with a reducing agent like

Dithiothreitol (DTT) and Sodium Dodecyl Sulfate (SDS). Before loading the samples, heating of the samples is pre-requisite that precipitates the proteins and binding of SDS to the backbone of denatured proteins provides a negative charge to make them soluble. SDS has a hydrophobic tail and a charged polar ion, thus it gets binds to the hydrophobic backbone of the protein. On electrophoresis, these proteins get migrated based on the molecular weight.

Polyacrylamide gels are formed by the polymerization of the monomer acrylamide crosslinked to the co-monomer, *N,N′*-methylenebis-acrylamide (BIS). In this process, free radicals are generated during polymerization by ammonium persulfate, and a catalyst such as *N,N,N′,N′*-tetraethylmethylenediamine (TEMED). The advantage of PAGE is that the pore size of the gel can be controlled by using different concentrations of acrylamide during gel formation.

3 Sample Preparation: RNA Extraction from Clinical or Biological Samples

Phenol-chloroform method

1. Add pre-warmed solution of 1 M sodium acetate with 1% SDS in a microcentrifuge tube containing about 100–200 μl of sample (fecal sample) to make 10% fecal suspension and centrifuge at 12,000 g for 20 min to remove coarse particles and cellular debris.
2. Vortex for 10 s and incubate for 15 min at 37 °C.
3. Add an equal amount of phenol: chloroform: Isoamyl alcohol (25:24:1).
4. Vortex again for 1 min followed by incubation at 56 °C for 15 min.
5. Vortex the mixture and centrifuge it for 3 min at 16,900 × *g*.
6. Take a fresh 1.5 mL micro centrifuge tube and transfer the upper aqueous phase.
7. Add chloroform: Isoamyl alcohol (24:1) solution to the same tube and repeat the **steps 4–6**.
8. Further, to this, add 1/10 volume of 3 M sodium acetate (pH 5.0) and an equal volume of isopropanol.
9. Invert tube to mix gently and incubate at room temperature for overnight.
10. Finally, centrifuge for 15 min at 12,000 rpm (4 °C) for RNA pelleting and wash it with 70% chilled ethanol followed by centrifuging at 16,900 × *g* for 5 min. Immediately, decant the ethanol and air-dry the pellet.
11. Re-suspend the RNA pellet in 30 μl RNA loading buffer using a pipette.

4 Procedure: From Setting Up PAGE Apparatus to Stained Gels Visualization

4.1 Electrophoresis in Cylindrical Gels Using Vertical Apparatus

Several commercial companies sell electrophoresis equipment and reagents suitable for cylindrical gel electrophoresis. Davis [6] identified a low-cost simple setup consisting of tubes where samples stack up in contiguous discs in a cylindrical gel of low concentration before entering the higher concentration gel, in which the separation actually occurs. A distinct advantage of this set up is that the reservoir located upwards is not adjoined/locked to that of the reservoir located downward, preventing the entire apparatus from being dismantled if the user wants to remove a specific tube at a different time period during the ongoing run. The apparatus with the rectangular boxes arranged in a straight line is considered to be more convenient because it allows for easier monitoring of the tracking dye during the gel electrophoresis process [7].

4.2 Apparatus Setup for Gel Electrophoresis in Rectangle-Shaped Gel Slabs

There are several advantages of using rectangle-shaped gel slabs as electrophoresis matrices rather than cylinder-shaped gel columns. In single gel slab methodology, the user can easily separate and compare the samples by maintaining identical conditions such as temperature, pH, voltage gradient, and current [8].

4.3 Power Supply

The best and most appropriate power supply for electrophoresis has several constraints while facilitating and providing sufficient and desirable current and voltage. As catalysts pass ion boundaries and final products move out of the gel, the resistance potential of the polyacrylamide gel gradually increases during overall electrophoresis. There is a detectable difference in the gel when these ion boundaries pass out of the gel at various regions. A certain and constant amount of current supply is needed to promote a constant voltage gradient in the region of migrating RNA molecules. This mechanism would also ensure that the distance traveled by these molecules changes linearly over time. There is about a quarter of the current shift during the overall run.

5 Method

Before starting the RNA-PAGE procedure, make sure of all of the required equipment and reagents. To reduce the background for direct ultraviolet scanning of gels, impurities that absorb ultraviolet radiation should be removed. Highly purified acrylamide and bis-acrylamide are used to prepare both diluted and concentrated gels.

5.1 Preparation of Gel Plates

1. Assemble the glass plates for gel casting and wash the gel plates with lab grade detergent and distilled water before each use. Glass plates must be thoroughly dried, cleaned with ethanol on the inside, and labeled on the outside. Furthermore, any residual dried acrylamide or any other substance on glass plates should be properly cleaned with a fresh single-edge razor blade so that the glass plates are not scratched.
2. A siliconizing agent or ideally a commercial alternative such as Rainex must be applied on the inner surface of notched plates. Cover the gel plates uniformly with Rainex, taking care not to have any Rainex on the edges or the other surface of the gel plate. After applying Rainex, plates should be air-dried and washed with a lint-free tissue, such as Kim Wipes®. Furthermore, plates must be rinsed with water and then dried before use.

5.2 Preparation of Solutions Used in Gel

Mark the top level of the resolving gel on the plate with a marker pen while ensuring to leave space above the resolving gel for the stacking gel. Make different buffers as follows:

Buffers	Methods of preparation
30% acrylamide stock	Dissolve 29.2 g of acrylamide and 0.8 g of *N,N′* methylene bis-acrylamide in 100 mL of distilled water. Filter before use. Place the solution in a dark or foil-covered bottle, and store at 4 °C
Resolving gel buffer (1.5 M, pH 8.8)	Dissolve 18.15 g of Tris base in 60 mL of distilled water. Adjust the pH to 8.8 with 1 N HCl. Make up to 100 mL with distilled water. Filter through Whatman no. 1 filter paper and store at 4 °C
Stacking gel buffer (0.5 M, pH 6.8)	Dissolve 6.0 g of Tris base in 60 mL of distilled water. Adjust the pH to 6.8 with 1 N HCl. Make up to 100 mL with distilled water. Filter through Whatman no. 1 filter paper and store at 4 °C
10% (w/v) ammonium per sulfate (APS)	Dissolve 0.1 g of APS in 1 mL of distilled water just before use. Store at 4 °C for a maximum of 3 days
1× Tris-glycine running buffer	Dissolve 3.0 g of Tris base and 14.4 g of glycine in distilled water, and make up to 1000 mL with distilled water
2× RNA-PAGE sample loading buffer	Dissolve 400 μL of 0.05% bromophenol blue, 200 μL of 10% SDS, and 3 mL of glycerolin 5 mL of stacking gel buffer. Make up to 20 mL with distilled water

5.3 Assembling of the Gel

1. Place spacers on the inside edges of the PAGE plate (10 × 8 cm × 1.0 mm) and cover it with the other/second plate (treated) side down; do not move the spacers while fixing the plates and keep the plates in place with the help of binder clips attached on one side of the plates.
2. Fix a piece of tape over the unclipped side and the bottom part; smoothen the tape with a single edged razor by applying the no-sharp side over the tape. Extend the tape over gel spacer tabs if the small plate does not have "ears." One-third of the way up from the bottom, clip the tape-covered side. It is necessary to ensure that the clip end is placed over the spacer and tape.
3. Remove the clip from the other side and tape the plates up using the same method as before. Apply tape to the bottom of the plate, covering a small area next to it by a few inches. Combs should be checked regularly to ensure that they are fixed properly.

5.4 Prepare the 10% Resolving and 5% Stacking Gels

Prepare the resolving and stacking gels as follows:

Stock solution	Resolving gel (10%)				Stacking gel (5%)			
	5 mL	10 mL	15 mL	20 mL	1 mL	2 mL	3 mL	4 mL
Distilled water	1.9	4	5.9	7.9	0.68	1.4	2.1	2.7
30% acrylamide	1.7	3.3	5.0	6.7	0.17	0.33	0.5	0.67
1.5 M Tris HCL (pH 8.8)	1.3	2.5	3.8	5.0	–	–	–	–
0.5 M Tris HCL (pH 6.8)	–	–	–	–	0.13	0.25	0.38	0.5
10% APS	0.05	0.1	0.15	0.2	0.01	0.02	0.03	0.04
TEMED	0.002	0.004	0.006	0.008	0.001	0.002	0.003	0.004

1. Pour 10% resolving gel solution down the side of the balanced plates by gently moving the plate to the other corner side when the gel hits the bottom corner. This will allow the solution to spread evenly over the bottom surface. Maintain a steady supply of gel solution to fill the remaining space in the plates, and gently move the plates to ensure that they are fully filled from the bottom. Allow the separating/resolving gel to solidify. Then apply a layer of water-saturated iso-butanol to the gel

(to ensure the formation of an even interface and for the exclusion of oxygen).

2. After solidification of the resolving gel, gently pour the stacking gel over the resolving gel, and when the gel solution reaches the top of the plates, immediately insert the comb in the gel and clamp the two corners of the gel plates to keep the comb in place. Most importantly, make sure that there are no bubbles around the wells in the gel; if there are bubbles, eliminate them by tightening the comb again. Otherwise, a thin film of polymerized acrylamide will form between the plates and wells which will subsequently interfere with the loading of RNA samples.
3. Additionally, leave a small amount of gel solution in the flask to confirm the polymerization of gel. Polymerization would take about 15–20 min, and the partition between the comb's teeth would need to be formed. Furthermore, the refractive index of the gel changes during the polymerization, which can be visualized as a "Schlieren line" near the edge of the gel plate and comb. The difference in refractive index between the polymerized and un-polymerized acrylamide causes this effect.

5.5 Set Up the Gel After Polymerization

1. To begin, remove all tapes and clips from the bottom side of the gel, and rinse the top side of the gel near the comb with distilled water. The comb should then be carefully removed, and the wells could be rinsed with the distilled water using a syringe or pipette.
2. Place the shorter side of the gel plates inwards on the gel apparatus. Maintain equal pressure on both sides of the clamps to secure them in place.
3. Fill the bottom and top chambers with an appropriate amount of 1× Tris-glycine running buffer. Rinse the wells with TBE using a syringe to prevent the formation of air bubbles in the wells as well as in the bottom step of the gel.
4. Close the lids gently and attach the electrodes before starting the pre-run at 45 mA for 30–45 min. A pre-run helps in reducing excess of persulfates and eliminates hyper focusing. For the analysis of short RNA with a size of lesser than or equal to 50 nucleotides, longer pre-runs of nearly 45 min are usually recommended.

5.6 Loading the Gel

1. Firstly, turn off the power supply.
2. Add 2× RNA-PAGE sample loading buffer to RNA samples and heat them between 90 and 95 °C before snap chilling them on ice for 5 min.

3. Rinse the wells repeatedly with the help of a syringe. This practice will make it easier for the sample to settle and disperse upward.
4. Load the RNA samples quickly with a RNAse and DNAse free micropipette. Continue to prevent the bubble formation, which may cause sample disturbance. Fix the sample loading volume in accordance with the width and thickness of the well.

5.7 Running the Gel

1. A gel running should be carried at a stable voltage (80 V) till the dye comes out of the gel. To monitor the heat in the apparatus, use silicone grease to attach a thermometer to the front plate. The average temperature range must be between 57 and 58 °C. An increase in voltage and current could cause the gel to overheat or develop cracks. This activity can result in band distortion in the gel as well as other unusual activities within the gel matrix. To avoid the risk of overheating, high percentage gels should be run at a constant current rate. The milliamps or current capacity could be determined by taking into account the gel percentage and size.
2. Run the gel at a suitable distance to allow a better band resolution. The dyes comprising xylene cyanol and bromophenol blue in the loading buffers still migrate to similar positions, but they migrate along with the variable sized nucleic acids depending on the gel percentage. To achieve higher reproducibility, gels should be run under identical conditions, and it is therefore advised to become familiar with the gel to be used for further work to prevent any complications or work odds.

5.8 Disassembling the Gel

1. Turn off the power supply and remove/drain the buffer from the gel apparatus. If the RNA samples were radioactively labeled, use caution when handling the apparatus.
2. After that, detach the plates and remove the yellow tape by pulling it off or with a razor blade. Afterward, place the plate on a flat surface.
3. Carefully remove the gel remnants from the gel surfaces.
4. Gels may also be processed by wrapping them in plastic wrap and drying them thoroughly with a vacuum-driven gel drier.

5.9 Silver Staining

The RNA–PAGE in combination with silver staining is a sensitive and time-saving technique for the diagnosis of various viral diseases. After electrophoretic separation on polyacrylamide gels, nucleic acids are detected using silver staining [9]. It combines high sensitivity with easy and inexpensive equipment and chemicals. RNA fixation, sensitization, silver impregnation, and image development are the stages of silver staining in order. Silver staining can be done in a variety of ways and takes anywhere from 2 h to a day

after the electrophoretic separation is completed. Since the method of silver staining is a delicate and temperature-dependent, so some protocols perform poorly when the temperature is either below 20 °C or above 30 °C [10].

After electrophoresis, the first step in gel staining is fixation which results in RNA binding to the gel matrix and removal of buffer components from gels. Fixation is performed in fixing solution (10% ethanol and 0.5% acetic acid) for around 30 min [11]. After that, rinse the gels twice in distilled water for 1 min each time. Silver Nitrate solution (0.185 g $AgNO_3$ in 100 mL distilled water) is prepared just before use and the gel is dipped in it for 30 min at room temperature on the gel rocker. After staining with silver nitrate, the gel is treated with developing solution (add 1.5 mL of 36% formaldehyde to 6.0 g of NaOH, and make up to 200 mL with distilled water, prepare fresh before use) and kept for about 5 min at room temperature or until RNA bands are visible. The developing step should be carried out in dark place. After that, drain off the developing solution, and add the stopping solution (add 10 mL of acetic acid to 190 mL of distilled water) to prevent further color development. Furthermore, keep the gel for 5–10 min at room temperature in the stopping solution before rinsing in distilled water. Finally, dry the gel in a standard vacuum gel dryer; the gel may also be temporarily stored in a 20% ethanol/1% glycerol mixture or in a 5% acetic acid solution [10].

RNA molecules inside gels can be visualized using a variety of methods depending on the detection reagents. There are a varieties of dyes such as SYBR green, toluidine blue, and ethidium bromide, which can be used to stain RNAs. Autoradiography can be used to observe radioactively labeled RNA molecules, while a fluorescence scanner can be used to visualize fluorescently labeled RNA molecules [12].

Many RNA viruses have genetic material in the form of segmented genomes, which can be visualized using RNA-PAGE, such as Influenza A virus (eight segments), Bluetongue virus (ten segments), Coltivirus (Twelve segments), Rotavirus (11 segments), Picobirnavirus (two segments), Bunyavirus (tripartite genomes), Arenavirus (two segments), Birnavirus (two segments), and Infectious Pancreatic Necrosis Virus (IPNV, two segments). RNA-PAGE is unquestionably the most effective technique for RNA analysis, including viral RNA identification, quantification, size purification, and even mixed infection detection in gel electrophoresis, and it will continue to be an indispensable tool for RNA biology research.

References

1. Duesberg PH, Cardiff RD (1968) Structural relationships between the RNA of mammary tumor virus and those of other RNA tumor viruses. Virology 36:696
2. Rio DC, Ares M Jr, Hannon GJ, Nilsen TW (2010a) Nondenaturing agarose gel electrophoresis of RNA. Cold Spring Harb Protoc 2010(6):pdb.prot5445. https://doi.org/10.1101/pdb.prot5445
3. Rio DC, Ares M, Hannon GJ, Nilsen TW (2010b) Polyacrylamide gel electrophoresis of RNA. Cold Spring Harb Protoc 2010(6):pdb-prot5444
4. Dubal ZB, Mawlong M, Susngi B, Sanjukta R, Puro K, Ghatak S, Sen A, Shakuntala I, Barbuddhe SB, Ahuja A, Bhattacharjee U (2015) Comparison of agarose gel electrophoresis and RNA-PAGE for rapid detection of rotavirus from faecal samples. J Appl Anim Res 43(1): 77–82
5. Stellwagen NC (2009) Electrophoresis of DNA in agarose gels, polyacrylamide gels and in free solution. Electrophoresis 30(supplement 1):S188–S195
6. Davis BJ (1964) Disc electrophoresis II: method and application to human serum proteins. Ann N Y Acad Sci 121:321–349
7. Adesnik M (1971) Polyacrylamide gel electrophoresis of viral RNA. In: Methods in virology, vol 5. Elsevier, pp 125–177
8. Adams JM, Jepessen PGN, Sanger F, Barrel BG (1969) Nature (London) 223:1009
9. Whitton JL, Hundley F, O'donnell B, Desselberger U (1983) Silver staining of nucleic acids. Applications in virus research and in diagnostic virology. J Virol Methods 7(4): 185–198
10. Chevallet M, Luche S, Rabilloud T (2006) Silver staining of proteins in polyacrylamide gels. Nat Protoc 1(4):1852
11. Minakshi P, Ranjan K, Kumar P, Prasad G (2013) A novel method of staining of RNA in polyacrylamide gel electrophoresis. Adv Anim Vet Sci 1(4S):20–23
12. Petrov A, Tsa A, Puglisi JD (2013) Analysis of RNA by analytical polyacrylamide gel electrophoresis. Methods Enzymol 530:301–313

Chapter 9

Peptide Nucleic Acid (PNA): A Diagnostic Molecule for Infectious Diseases

Vinay G. Joshi, Anu Kumari, Sushila Maan, Tarun Kumar, and Satish Kumar

Abstract

Disease diagnosis performs an important role in the epidemiology of the disease. Genetic material identification is the definite way of identification of the pathogen. Available techniques involved in genetic material identification are economically as well as time consuming. Both these aspects are important in disease diagnosis, and particularly very crucial for infectious diseases. The distinctive physiochemical properties of PNA molecules have opened the path to use them for the development of rapid diagnostic methods. Here, we have briefly discussed about the designing and synthesis of PNA, its two types clamp PCR and PNA and gold nanoparticles agglomeration science in disease diagnosis.

Key words PNA, PNA-clamping PCR, Gold nanoparticles, Diagnosis

1 Introduction

Emerging infectious diseases are a serious threat to both human and animal life. An increase in the risk of infectious diseases particularly zoonotic diseases, due to the human population explosion, congested wet markets, climate change, globalization, pollution has brought back health care at the center of concern. Certainly, disease diagnosis is one of the crucial steps in the process of disease control, eradication, and treatment. Globalization has broadened the economic opportunities but also increases the chances of infection (contagion) spread. A localized outbreak can easily be converted into a pandemic like in swine flu, SARS (severe acute respiratory syndrome), avian influenza, and COVID 19. The confirmatory diagnosis helps in monitoring the disease, betters its prognosis and treatment, and ultimately helps in disease eradication. Therefore, advancement in disease diagnosis is an exigency. Diagnostic tests can be broadly of two types depending on the site of testing: conventional laboratory-based and point of care (POC)

Rajib Deb et al. (eds.), *Protocols for the Diagnosis of Pig Viral Diseases*,
Springer Protocols Handbooks, https://doi.org/10.1007/978-1-0716-2043-4_9,

test. POC tests are those which can be performed near, or at, the point of patient care [1]. Disease diagnosis is a rapidly evolving discipline with the current scenario. Standard tests such as antibody neutralization or identification, virus/bacteria isolation, conventional PCR, RT-PCR, and real-time quantitative RT—PCR (RT—qPCR) are being commonly used. These lab-based tests have some constraints as they require sophisticated instrumentation, skilled personnel, poor stability of reagents, high cost, and time. The ideal diagnostic test should fulfill the proposed "ASSURED criteria, i.e. Affordable, Sensitive, Specific, User friendly, Rapid and robust, Equipment-free, and Deliverable to end-users" [2].

2 Peptide Nucleic Acid (PNA)

PNA was first reported by Nielson et al. in 1991 [3]. In PNA, the four naturally occurring nucleobases are connected by a peptide backbone. The backbone is made up of repetitive units of *N*-(2-aminoethyl) glycine Fig. 1. The purine and pyrimidine bases are attached via a methyl carbonyl linker to the backbone of PNA [4]. The PNA, nucleic acid mimetic is devoid of phosphate and glycosidic linkages [5]. This modification changes the polarity of the molecule and makes it charge neutral. The PNA-RNA/DNA duplex demonstrates higher binding/hybridization efficiency and thermal stability compared to native DNA/DNA, RNA/RNA, or DNA/RNA duplexes. The PNA being achiral and charge-neutral molecule shows strong and specific binding to the gene target [6]. Additionally, PNA is a chimeric molecule making it resistant to cleavage by hydrolytic enzymes such as DNase and Proteases [3]. The PNA is proved to be a robust molecular probe that can be used for disease diagnosis in both conventional PCR diagnostics and point of care diagnosis (Table 1). The use of PNA for PCR clamping based assay and colorimetric visual detection are the most useful applications. PNA can be synthesized by automated peptide synthesizing platform or solid-phase peptide synthesis (SPPS). SPPS is the standard method for PNA synthesis. Both *tert*-butyloxycarbonyl (Boc) and 9-fluorenylmethoxycarbonyl (Fmoc) chemistry can be used for PNA synthesis. They have their advantages and disadvantages.

3 Materials and Methods

3.1 List of Materials

The Fmoc PNA monomers, Rink amide-MBHA resin, 4-(2′,4′-Dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl-MBHA resin (100–200 mesh), (1-[Bis (dimethylamino) methylene]-1*H*- 1,2,3- triazolo [4,5-*b*] pyridinium 3-oxide

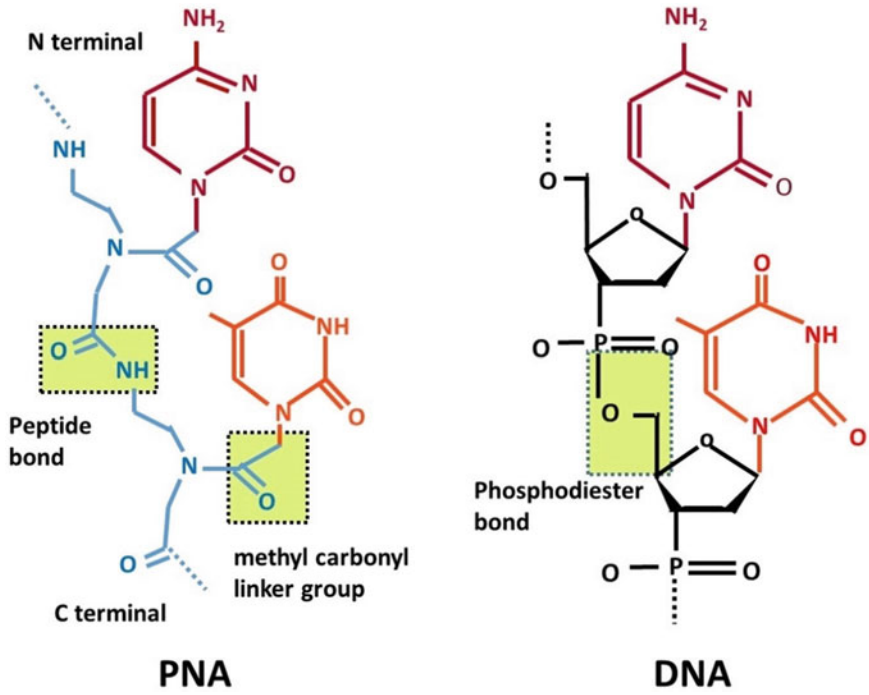


Fig. 1 Chemical structures of PNA and DNA

hexafluorophosphate) (HATU), Pyridine, m-cresol, and triisopropylsilane (TIS), Specially dried analytical grade, *N*,*N*-dimethylformamide (DMF), dichloromethane (DCM), piperidine, diethyl ether and *N*,*N*-diisopropylethylamine (DIEA) and HPLC grade acetonitrile and water. All the chemicals and biologicals should be of analytical, molecular biology, and cell culture grade.

3.2 Designing PNA Probes

For the designing of the PNA probe, there is no set frame of rules; however, a general probe composition guideline can help to design a successful PNA probe. The length of PNA can be varying from 6 mer to 30 mer. Synthesis of longer PNA probe results in significantly higher Tm values. The Tm for PNA is higher than its contemporary DNA probes. Varying reports have claimed that the PNA probe as small as 6 mer can inhibit RNA or sufficient to work as a clamp in clamping PCR. Ideally, PNA should not contain loner purine-rich sequences it may reduce the product yield in solid-phase peptide synthesis. A PNA probe should have purine contains below 50%. More than 5–6 complementing positions should be avoided while designing the PNA probe. Due to its hydrophobic nature, PNA probes may have poor water solubility generally it is observed when the probe has higher G contents. For chemical modification PNA [2-(2-(Fmoc-amino) ethoxy) ethoxy] acetic acid (AEEA) linker can be used. Swada et al. in 2017 reported the utility of azobenzene linker in the PNA probe for detecting

Table 1
PNA-based disease diagnosis of pathogenic organisms

Sr. no	Diagnosis for	Spp/things affected	Method	References
1	Porcine reproductive and respiratory syndrome virus genotypes	Porcine	PNA probe-mediated one-step real-time RT-PCR	[29]
2	Antigenic discrimination of canine parvovirus	Canine	PNA array	[30]
3	*Salmonella enterica* serovar Enteritidis	Milk, eggs and mayonnaise samples	PNA fluorescence in situ hybridization (PNA FISH)	[31]
4	*Megalocytivirus*	Aquatic animal diseases	PNA-based real-time PCR assay	[32]
5	Multiple strains of influenza A virus	Avian	PNA biosensor	[21]
6	*Stenotrophomonas maltophilia*	Respiratory tract infections in humans and animals	PNA FISH	[33]
7	Newcastle disease virus (NDV)	Avian	PNA and gold nanoparticles (AuNPs)	[20]
8	*Mycobacterium tuberculosis*	Human	PNA biosensor based on reduced graphene oxide/water-soluble quantum dots	[34]
9	*Dengue virus*	Human	PNA and gold nanoparticles (AuNPs)	[35]
10	Human papillomavirus	Human	PNA probes	[36]

homopurine sequences in a target gene [7]. In some cases, to improve the water solubility of the PNA molecule the probe can be labeled with a basic amino acid such as Lys at C terminus. PNA probes can be labeled with suitable fluorescent labels for real-time diagnostic studies. For PNA probe designing reader can refer to an online PNA design tool "PNA tool." It provides a general guideline for probe designing.

3.3 Synthesis of PNA

The PNAs are synthesized by the standard method of SPPS using Fmoc chemistry on Rink amide-MBHA resin. Briefly, swell the Rink amide-MBHA resin in the DMF overnight. De-blocking of the resin-bound Fmoc protecting group is done by treating with 20% (v/v) piperidine solution in DMF. Wash the beads five times with DMF and then DCM and followed by two DMF wash. For the first coupling, pre-activation of amino acids is done to activate their carboxyl group so that amide linkage will establish during the

coupling reaction. Pre-activation is done by incubating the PNA monomers in especially dried DMF and activated using HATU in DIEA and pyridine solution at 4 °C for 20 min. Add the pre-activated PNA into the beads and incubate it for 2 h under shaking conditions at room temperature. After the first complete coupling reaction, all other remaining activated sites are capped by adding a mixture of DMF, DIEA, and acetic anhydride. De-blocking, pre-activation, coupling, and capping reactions are repeated until the desired length of PNA is achieved. The final product is de-protected and cleaved from the resin by treating the resin with TFA/m-cresol/TIS/water (85/10/2.5/2.5, v/v) for 5 h at room temperature. The cleaved PNA in the TFA extracts is then precipitated by dried and chilled diethyl ether. At each step progress of PNA, monomer coupling can be monitored by the Kaiser test.

3.4 Procedure for the Kaiser Test

The standard protocol for the Kaiser test [8].

3.4.1 Reagents for Kaiser Test

1. Dissolve 8.25 mg of KCN in 12.5 mL of double-distilled water.
2. Make 1:50 dilution of the above solution in pyridine label it as Solution A.

Solution A

Solution B

1. Dissolve 500 mg of ninhydrin in 10 mL of 1-butanol and label it as solution B.

Solutions C

1. Dissolve 20 g of phenol in 10 mL of 1-butanol and label it as solution C.

3.4.2 Kaiser Test

1. Take 30–40 beads and wash it three times with excessive ethanol.
2. Remove the ethanol and two drops of Solution A, B, and C serially.
3. Heat this solution in boiling water for 5 min.

3.4.3 Interpretations

1. The colorless or faint blue color of beads indicates the successful coupling.
2. The beads are dark blue indicates that coupling incomplete and coupling step can be repeated.

3.5 Characterization and Quantification of PNA Concentration

The PNA probes can be purified by RP-HPLC using the C-8 column. For RP-HPLC preferred buffer in PNA purification can be Buffer A-0.08% TFA in HPLC grade water, Buffer B-0.08% TFA in acetonitrile. A linear gradient of 95% water to 30–70% of buffer A and buffer B for 40 min with 0.8 mL/min flow rate be used. After the fraction collection dry lyophilized PNA probe can be sent for

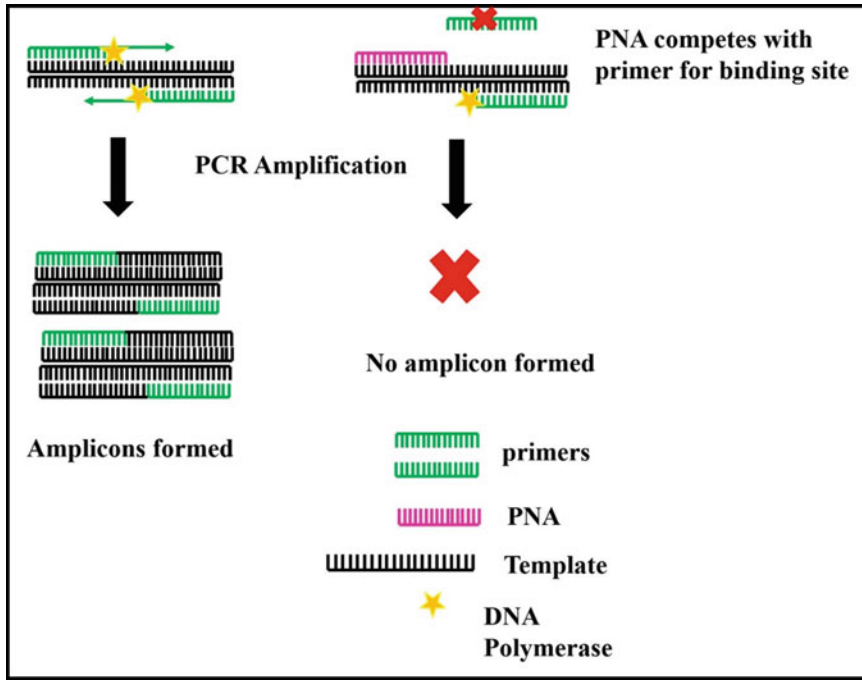


Fig. 2 PNA—Clamping PCR.

the molecular weight analysis using MALDI-TOF. After confirmation of desired molecular weight PNA probes can be used for the diagnostic application. Absorbance at 260 nm can be used to determine the concentration of PNA probe.

3.6 PNA Clamping PCR

Clamping PCR is based on the principle of competitive binding between the primer and PNA. When PNA clamps to complementary target gene sequence it does not allow DNA polymerase to extend the sequence, hence there will be no amplification in PCR (Fig. 2). PNA mediated clamping specifically blocks amplification of a given gene template while allowing amplification of other templates that differ by as little as one nucleotides. PCR clamping can be achieved by two basic methods. First competition at the primer binding site between the primer and the PNA. Second, by blocking the elongation, the PNA binds near the primer binding site and arrest the elongation [9]. This phenomenon was widely being used for diagnostic detection of SNP in genetic diseases, oncogenic point mutations, mitochondrial DNA mutation in degenerative diseases, microbial mixed community analysis, analysis of mRNA editing, cloning IgG variable regions, finding of allelic allocations using PNA clamping PCR [10–16]. A few studies have developed variants of clamping PCR for the detection of point mutations in the overwhelming concentration of wild-type sequence templates. Such a situation is generally observed in cancer

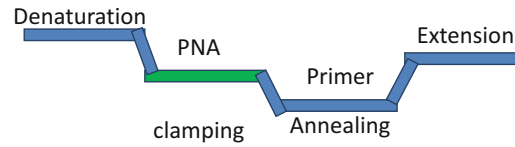


Fig. 3 Various steps of typical clamping PCR assay

genetics where early cancer detection can be possible by detecting a low level of point mutations using PNA clamp PCR. Real-time assay-based detection of mutation can be achieved using these variations of clamping PCR. Oh et al. 2010 explain the use of unlabeled PNA probe for mutation detection using LC Green dye [6]. While Han et al. 2016 explain the use of fluorescently labelled PNA probes for mutation detection in clamping PCR experiments [17]. The simplest method for the use of clamping PCR is to use the PNA probe as a clamp in classical PCR and identify the presence or absence of target gene.

3.6.1 Methods Clamping PCR

For a general guideline, reaction condition can be as follows 5–10 pmol of forward and reverse primers. 2× PCR master mix. Initially, the experiment should be set with variable PNA concentration from say 0–2 μM PNA with a fixed amount of template to understand the required concentrations of PNA for inhibition of PCR amplification. For PCR clamping identifying PNA, the annealing temperature is a crucial step, and it can be performed with addition PNA clamping step before primer annealing steps Fig. 3. As PNA probes have higher Tm pre-anneal clamping step should be preferred. After standardization of protocol PCR reaction can be prepared with 2× master mix, 5–10 pmol forward and reverse primers, 1 μL PNA probe (predetermined suitable concentration) and nuclease-free water to compensate the reaction volume.

3.7 Peptide Nucleic Acid Visual Diagnostics

The PNA based label-free biosensors can be an important candidate by fulfilling the ASSURED criteria. Kanjanawrut and Su first put forth the idea of using PNA probes to detect a specific DNA sequence using unmodified metallic nanoparticles [18]. This group discovered a unique PNA metallic nanoparticle behavior of the citrate anions-protected gold nanoparticles. These nanoparticles undergo immediate agglomeration in the presence of PNA. This agglomeration of nanoparticles is retarded when a complementary nucleic acid is present to form the PNA-nucleic acid complex. This PNA: nucleic acid complex interaction is specific and can be used to discriminate the presence or absence of target nucleic acid. It suggested that the induced particle aggregation originates from the strong PNA gold interactions with the involvement of both nucleobases and peptide backbone of PNA [19]. In

addition to these factors, the presence of positive charges of the N-terminal amines of the PNA at neutral pH may contribute to the immediate aggregation by electrostatic interaction [18]. With the use of this agglomeration phenomenon, label-free biosensors having the ability of viral RNA detection could be devised. Reports on label-free detection of Newcastle disease virus (NDV) using PNA and gold nanoparticles based agglomeration visual assay [20] and label-free PNA biosensor for identification of multiple strains of influenza A virus [21], showed the way forward for application of these techniques in POC, viral disease diagnosis. Biosensors, according to IUPAC are integrated receptor-transducer devices, which are capable of providing selective quantitative or semi-quantitative analytical information using a biological recognition element [22]. Biosensors may be classified into two broad groups depending on the methods of detection: with and without labels (markers). The label-free biosensor utilizes biological or chemical receptors for direct detection of an analyte in the sample, without the use of enzyme and radioactive or fluorescent labels [23].

3.7.1 Gold Nanoparticle Synthesis

Gold nanoparticles (AuNPs) assembly and disassembly cause a visual color change, this property is due to surface plasmon resonance (SPR) which is utilized by scientists to develop colorimetric biosensor. Here, analytes such as DNA, PNA, RNA can cause aggregation or dispersion of AuNP directly or indirectly and leads to a huge absorption band shift (up to ~300 nm), which can be visualized by naked eyes [24] For AuNPs synthesis, all the glassware should be thoroughly cleaned with freshly prepared Aqua Regia (1: 3 HNO_3/HCl) and washed twice with triple-distilled water and dried overnight. Citrate-stabilized gold nanoparticles are synthesized by the reduction of gold (III) chloride trihydrate ($HAuCl_4 \cdot 3H_2O$) by sodium citrate [25] Briefly, 50 mL of a 1 mM tetrachloroauric (III) acid ($HAuCl_4 \cdot 3H_2O$) solution is heated with continuous stirring on a magnetic stirrer till boiling. Then, add a 5 mL solution of 38.8 mM trisodium citrate quickly to the boiling solution. Let the solution boil for 10 min with constant stirring. Stop the heat and allow the solution to stir for another 15 min until a brick-red wine color appear. The colloidal gold preparation is cooled to room temperature and store at 4 °C until further use, and it is characterized by UV–vis spectrophotometry for its characteristic absorbance at 520 nm.

3.7.2 Visual Viral RNA Detection

The procedure for visual detection of viral using metallic nanoparticles (Silver or gold nanoparticles) and PNA is simple and can be performed on the field. For the actual diagnostic assay, various combinations should be tested to arrive at identifying required PNA concentration which can induce the visual color change in gold/silver nanoparticles. The researchers can use either gold nanoparticles or silver nanoparticles as an indicator for color change

but the use of gold nanoparticles is more common; therefore, we will describe the gold nanoparticle-based assay. Interested readers can refer the article by Kanjanawaru and Su in 2009 which in detail explanation about the use of gold/silver or combination of gold and silver nanoparticles for PNA based visual sensing [18]. The concentration of PNA required for induction of color change can vary with length of PNA, a smaller size PNA can induce better color change as it can easily cap citrate core [18]. Once the actual PNA concentration required for the visual color change is identified, the second step involves experiments to determine the suitable temperature for PNA: RNA hybridization. If there is a shortage of target RNA or reference RNA synthetic complementary target can also be used to standardize the PNA: target nucleic acid complementation. Such initial experimentations will provide baseline information required to optimize the visual detection assay. After this step PNA: RNA complementation can be confirmed with the addition of gold nanoparticles to check inhibition of PNA induced gold nanoparticle agglomeration due to its complementation with RNA Fig. 4. Usually in the absence of complementary target color change appears rapidly within a minute but if the PNA probe of a larger size of about 20 mer and above wait for 2–5 min is essential. The color retention or color change in gold nanoparticles can be visually monitored. The phenomenal changes in gold nanoparticle color can also be monitored using spectrum scanning with recording absorption spectra of solution from 400 to 750 nm. The stable gold nanoparticle solution of 13–18 nm particle size gives maximum absorption at 520 nm and blue colored agglomerated gold nanoparticles give maximum absorption at around 600–650 nm with a significant reduction in the pick at 520 nm. The test has specificity to identify a single base mismatch [20]. However, to improve the visual color discrimination and to identify single base mismatches, one needs to optimize the reaction with higher salt concentrations. Various combinations from 50 to150 mM NaCl can be tried.

Interpretations of visual RNA detection assay using PNA and gold nanoparticles can be summarized as follows.

1. The red color of gold nanoparticle turns in to blue after the addition of preincubated PNA: RNA complex indicates the absence of a PNA complementary strand.
2. Retention of gold nanoparticles red colour after the addition of preincubated PNA: RNA complex, indicates the presence PNA complementary strand.

3.7.3 Essentials of Visual RNA Detection Experiments

1. PNA: metallic nanoparticle assay could be useful for the detection, identification of point mutation at target sequence, and quantification of viral RNA.

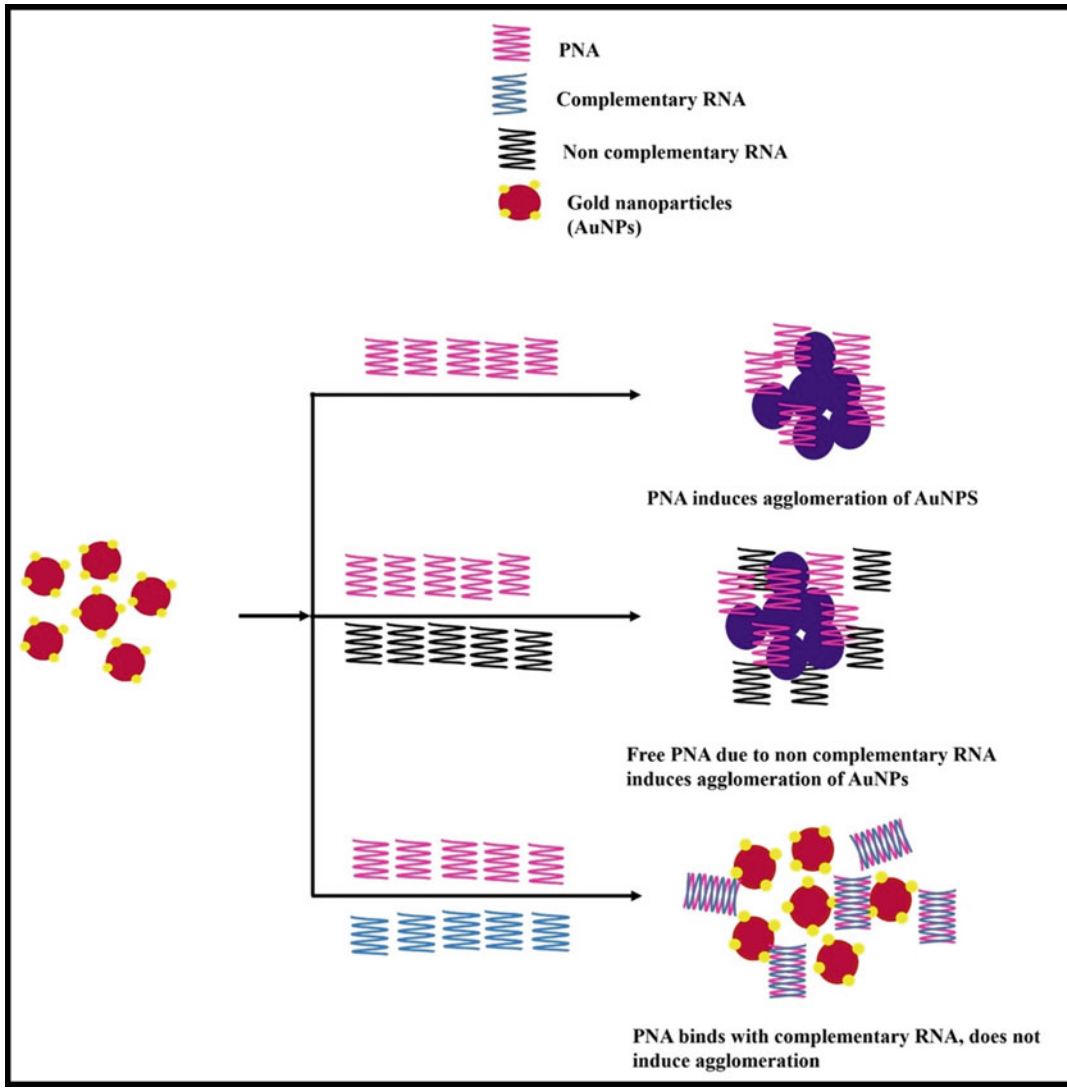


Fig. 4 Principal of colorimetric assay based on agglomerative nature of AuNPs with PNA in presence and absence of complementary RNA sequence

2. From the description and procedure, it is amply clear that the color change phenomenon depends on charge interactions. It also underlines some of the specific points to consider before establishing an actual diagnostic assay. The importance of which includes maintaining the no-template PNA and no PNA, RNA controls with gold nanoparticles.
3. At the time of standardization of the assay, it is advisable to do nonspecific RNA spiking to understand the interference of the overwhelming negatively charged RNA molecule in stabilizing the gold nanoparticle solution. Such RNA charge induced stabilization of nanoparticle may result in false-positive interpretations of results.

3.7.4 Viral RNA Quantification Using PNA Gold Nanoparticle Interactions

Induction of change in the color of gold nanoparticles is a function of free PNA available in the solution. The gradual changes with increasing concentration of PNA can be appreciated using difference spectra analysis. The difference spectra can be generated using a gold nanoparticle as reference spectra. Incremental addition of PNA in gold nanoparticles provides a correlation in induced color change vs concentration of PNA. These concomitant changes in the spectra of the gold nanoparticle on difference spectra analysis will provide a common crossover point, refereed as the isosbestic point, indicating equilibrium between the PNA and the gold nanoparticles color change. Such spectra analysis will provide two absorption maximum one at 520 nm for gold nanoparticle showing no color change and other at 630–640 nm for PNA induced blue colored gold nanoparticles. The ratio of OD640/OD520 can be used as a function of available complementary template which prevents PNA from inducing color change. After identification PNA concentration required for stable spectral change with induction of absorbance maxima at 640 passing through an isosbestic point, a standard curve can be prepared with the ratio of OD640/OD520. For standard curve two-fold serial dilutions of complementary RNA, templates can be used. With the increase in RNA template gold nanoparticle stability will be retained and ratio OD640/OD520 will be minimal. This will generate a standard curve as a function of complementary RNA concentration present in the solution that is preventing PNA from inducing color changes in the solution. Joshi et al. have shown quantification of viral RNA using this method. This approach can give relative estimates of the presence of viral RNA in test samples. The method is simple and does not require sophisticated lab equipment.

3.7.5 PNA and Other Nanoparticles for Diagnostic Applications

In the previous sections, we mostly discussed the colorimetric which is based on the aggregation of unmodified metallic nanoparticles [18, 20]. The PNA can also be used for the diagnosis of viral diseases using SPR imaging. The SPR imaging method enhanced with ultrasensitive nanoparticles and uses PNA probes showed greater selectivity toward the target template with single-nucleotide mismatches detection abilities [26]. Kerman et al. 2008 demonstrated the electrochemical biosensor for the rapid analysis of nucleic acids and nuclease activity [27]. Wang et al., 2006 prepared PNA-modified magnetic nanoparticles from the 4-pyridyldithiol derivative of PNA [28]. The PNA was attached to 3-mercapropropyloxysilane coated magnetic nanoparticles. This nanoparticle conjugated PNA was found to hybridize with the ssDNA target efficiently. This approach also provides a simple and cost-effective label-free assay for diagnosis.

References

1. Kosack CS, Page AL, Klatser PR (2017) A guide to aid the selection of diagnostic tests. Bull World Health Organ 95(9):639–645. https://doi.org/10.2471/BLT.16.187468
2. Peeling RW, Holmes KK, Mabey D, Ronald A (2006) Rapid tests for sexually transmitted infections (STIs): the way forward. Sex Transm Infect 82(suppl 5):v1. https://doi.org/10.1136/sti.2006.024265
3. Nielsen PE, Egholm M, Berg RH, Buchardt O (1991) Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. Science (New York, NY) 254(5037):1497–1500. https://doi.org/10.1126/science.1962210
4. Pellestor F, Paulasova P (2004) The peptide nucleic acids (PNAs), powerful tools for molecular genetics and cytogenetics. Eur J Hum Genet 12(9):694–700. https://doi.org/10.1038/sj.ejhg.5201226
5. Orum H, Nielsen PE, Egholm M, Berg RH, Buchardt O, Stanley C (1993) Single base pair mutation analysis by PNA directed PCR clamping. Nucleic Acids Res 21(23):5332–5336. https://doi.org/10.1093/nar/21.23.5332
6. Oh JE, Lim HS, An CH, Jeong EG, Han JY, Lee SH, Yoo NJ (2010) Detection of low-level KRAS mutations using PNA-mediated asymmetric PCR clamping and melting curve analysis with unlabeled probes. J Mol Diagn 12(4):418–424. https://doi.org/10.2353/jmoldx.2010.090146
7. Sawada S, Takao T, Kato N, Kaihatsu K (2017) Design of Tail-Clamp Peptide Nucleic Acid Tethered with Azobenzene linker for sequence-specific detection of Homopurine DNA. Molecules 22(11):1840. https://doi.org/10.3390/molecules22111840
8. Wellings DAA, E. (1997) Solid-phase peptide synthesis. In: Methods in enzymology, vol 289. Academic Press, San Diego
9. Orum H (2000) PCR clamping. Curr Issues Mol Biol 2(1):27–30
10. Mrozikiewicz PM, Landt O, Cascorbi I, Roots I (1997) Peptide nucleic acid-mediated polymerase chain reaction clamping allows allelic allocation of CYP1A1 mutations. Anal Biochem 250(2):256–257. https://doi.org/10.1006/abio.1997.2246
11. von Wintzingerode F, Landt O, Ehrlich A, Göbel UB (2000) Peptide nucleic acid-mediated PCR clamping as a useful supplement in the determination of microbial diversity. Appl Environ Microbiol 66(2):549–557. https://doi.org/10.1128/aem.66.2.549-557.2000
12. Nanthakumar T, Tiwari AK, Kataria RS, Butchaiah G, Kataria JM, Goswami PP (2000) Sequence analysis of the cleavage site-encoding region of the fusion protein gene of Newcastle disease viruses from India and Nepal. Avian Pathol 29(6):603–607. https://doi.org/10.1080/713651205
13. Zhong S, Nguyen NY, Eggerman TL (1999) Detection of apolipoprotein B mRNA editing by peptide nucleic acid mediated PCR clamping. Biochem Biophys Res Commun 259(2):311–313. https://doi.org/10.1006/bbrc.1999.0687
14. Murdock DG, Wallace DC (2002) PNA-mediated PCR clamping. Applications and methods. Methods Mol Biol 208:145–164. https://doi.org/10.1385/1-59259-290-2:145
15. Thiede C, Bayerdörffer E, Blasczyk R, Wittig B, Neubauer A (1996) Simple and sensitive detection of mutations in the ras proto-oncogenes using PNA-mediated PCR clamping. Nucleic Acids Res 24(5):983–984. https://doi.org/10.1093/nar/24.5.983
16. Nanthakumar T, Kataria RS, Tiwari AK, Butchaiah G, Kataria JM (2000) Pathotyping of Newcastle disease viruses by RT-PCR and restriction enzyme analysis. Vet Res Commun 24(4):275–286. https://doi.org/10.1023/a:1006403017578
17. Han JY, Choi JJ, Kim JY, Han YL, Lee GK (2016) PNA clamping-assisted fluorescence melting curve analysis for detecting EGFR and KRAS mutations in the circulating tumor DNA of patients with advanced non-small cell lung cancer. BMC Cancer 16:627. https://doi.org/10.1186/s12885-016-2678-2
18. Kanjanawarut R, Su X (2009) Colorimetric detection of DNA using unmodified metallic nanoparticles and peptide nucleic acid probes. Anal Chem 81(15):6122–6129. https://doi.org/10.1021/ac900525k
19. Gourishankar A, Shukla S, Ganesh KN, Sastry M (2004) Isothermal titration calorimetry studies on the binding of DNA bases and PNA base monomers to gold nanoparticles. J Am Chem Soc 126(41):13186–13187. https://doi.org/10.1021/ja046785g
20. Joshi VG, Chindera K, Singh AK, Sahoo AP, Dighe VD, Thakuria D, Tiwari AK, Kumar S (2013) Rapid label-free visual assay for the detection and quantification of viral RNA using peptide nucleic acid (PNA) and gold

nanoparticles (AuNPs). Anal Chim Acta 795: 1–7. https://doi.org/10.1016/j.aca.2013.06.037

21. Kumar N, Bhatia S, Pateriya AK, Sood R, Nagarajan S, Murugkar HV, Kumar S, Singh P, Singh VP (2020) Label-free peptide nucleic acid biosensor for visual detection of multiple strains of influenza a virus suitable for field applications. Anal Chim Acta 1093:123–130. https://doi.org/10.1016/j.aca.2019.09.060
22. Thévenot DR, Toth K, Durst RA, Wilson GS (2001) Electrochemical biosensors: recommended definitions and classification1International union of pure and applied chemistry: physical chemistry division, commission I.7 (biophysical chemistry); analytical chemistry division, commission V.5 (electroanalytical chemistry).1. Biosens Bioelectron 16(1): 121–131. https://doi.org/10.1016/S0956-5663(01)00115-4
23. Andryukov BG, Besednova NN, Romashko RV, Zaporozhets TS, Efimov TA (2020) Label-free biosensors for laboratory-based diagnostics of infections: current achievements and new trends. Biosensors 10(2):11. https://doi.org/10.3390/bios10020011
24. Zhao W, Brook MA, Li Y (2008) Design of gold nanoparticle-based colorimetric biosensing assays. Chembiochem 9(15):2363–2371. https://doi.org/10.1002/cbic.200800282
25. Grabar KC, Freeman RG, Hommer MB, Natan MJ (1995) Preparation and characterization of au colloid monolayers. Anal Chem 67(4): 735–743. https://doi.org/10.1021/ac00100a008
26. D'Agata R, Corradini R, Ferretti C, Zanoli L, Gatti M, Marchelli R, Spoto G (2010) Ultrasensitive detection of non-amplified genomic DNA by nanoparticle-enhanced surface plasmon resonance imaging. Biosens Bioelectron 25(9):2095–2100. https://doi.org/10.1016/j.bios.2010.02.008
27. Kerman K, Saito M, Tamiya E (2008) Electroactive chitosan nanoparticles for the detection of single-nucleotide polymorphisms using peptide nucleic acids. Anal Bioanal Chem 391(8): 2759–2767. https://doi.org/10.1007/s00216-008-2204-8
28. Wang F, Shen H, Feng J, Yang H (2006) PNA-modified magnetic nanoparticles and their hybridization with single-stranded DNA target: surface enhanced Raman scatterings study. Microchim Acta 153(1):15–20. https://doi.org/10.1007/s00604-005-0460-2
29. Park J-Y, Kim S-H, Lee K-K, Kim Y-H, Moon B-Y, So B, Park C-K (2019) Differential detection of porcine reproductive and respiratory syndrome virus genotypes by a fluorescence melting curve analysis using peptide nucleic acid probe-mediated one-step real-time RT-PCR. J Virol Methods 267:29–34. https://doi.org/10.1016/j.jviromet.2019.02.008
30. An D-J, Jeong W, Jeoung H-Y, Lee M-H, Park J-Y, Lim J-A, Park B-K (2012) Peptide nucleic acid-based (PNA) array for the antigenic discrimination of canine parvovirus. Res Vet Sci 93(1):515–519. https://doi.org/10.1016/j.rvsc.2011.06.003
31. Almeida C, Cerqueira L, Azevedo NF, Vieira MJ (2013) Detection of salmonella enterica serovar Enteritidis using real time PCR, immunocapture assay, PNA FISH and standard culture methods in different types of food samples. Int J Food Microbiol 161(1):16–22. https://doi.org/10.1016/j.ijfoodmicro.2012.11.014
32. Lee ES, Cho M, Min EY, Jung SH, Kim KI (2020) Novel peptide nucleic acid-based real-time PCR assay for detection and genotyping of Megalocytivirus. Aquaculture 518:734818. https://doi.org/10.1016/j.aquaculture.2019.734818
33. Hansen N, Rasmussen AKI, Fiandaca MJ, Kragh KN, Bjarnsholt T, Høiby N, Stender H, Guardabassi L (2014) Rapid identification of Stenotrophomonas maltophilia by peptide nucleic acid fluorescence in situ hybridization. New Microbes New Infect 2(3): 79–81. https://doi.org/10.1002/nmi2.38
34. Mat Zaid MH, Abdullah J, Yusof NA, Sulaiman Y, Wasoh H, Md Noh MF, Issa R (2017) PNA biosensor based on reduced graphene oxide/water soluble quantum dots for the detection of mycobacterium tuberculosis. Sensors Actuators B Chem 241:1024–1034. https://doi.org/10.1016/j.snb.2016.10.045
35. Abdul Rahman S, Saadun R, Azmi NE, Ariffin N, Abdullah J, Yusof NA, Sidek H, Hajian R (2014) Label-free dengue detection utilizing PNA/DNA hybridization based on the aggregation process of unmodified gold nanoparticles. J Nanomater 2014:839286. https://doi.org/10.1155/2014/839286
36. Menashi A, Cohenford WW, Brian Lentrichia A, (2005) Detection and typing of human papllomavirus using pna probes US 6,936,443 B2 Aug. 30, 2005

Chapter 10

Nucleic Acid Sequence-Based Amplification (NASBA) Methods and CRISPR/Cas13 System to Detect Pig Viral Diseases

Ajay Kumar Singh, Soumen Naskar, Pramod W. Ramteke, and Rohit Kumar

Abstract

For the diagnosis of pathogens, amplification and detection of their genetic material is an essential step, but the traditional method of amplification and detection requires sophisticated equipment or complex experimental procedures and is not portable, precluding their deployment in the field. The distinctive physiochemical amplification of nucleic acid sequence-based amplification (NASBA) and detection through CRISPR/Cas13 based system has opened the path to use them for the development of rapid diagnostic procedures in the field. Here, we have briefly discussed the principle and protocol of NASBA and CRISPR/Cas13 based detection system in pig viral disease diagnosis.

Key words Cas13, Isothermal amplification, CRISPR associated proteins, Nucleic acid amplification, Disease diagnosis

1 Introduction

In the present scenario, the pig industry contributes a majority share to the ever-increasing demand of quality food fuelled by the increasing human population, urbanization, and social mobility. However, the intensive production systems have made them vulnerable to various transboundary diseases such as African swine fever (ASF), Classical swine fever (CSF), porcine reproductive and respiratory syndrome (PRRS), Foot-and-mouth disease (FMD), and Porcine epidemic diarrhea (PED).

Briefly, the virus responsible for classic swine fever (CSF) or hog cholera is classic swine fever virus (CSFV), which causes a highly contagious disease in domestic pigs and wild boar. It is a member of the genus Pestivirus in the family Flaviviridae [1, 2,]. Outbreaks of CSF lead to considerable economic losses in many countries

Rajib Deb et al. (eds.), *Protocols for the Diagnosis of Pig Viral Diseases*,
Springer Protocols Handbooks, https://doi.org/10.1007/978-1-0716-2043-4_10,

worldwide [3]. Although, in European countries, CSFV has been successfully eradicated by a stamping-out policy, and the China-manufactured attenuated vaccine is being safely and efficiently employed as prophylactics worldwide [4].

African swine fever virus, a member of genus Asfivirus, family Asfarviridae, is a causative agent of ASF. It is a large enveloped virus with a double-stranded DNA genome [5]. The ASF is a hemorrhagic viral disease that affects not only domestic and wild boars but also ticks. The current outbreak of ASF has once again drawn attention to the potential threat of the virus and is quite worrisome. For ASF, as of now, no vaccine is commercially available.

PRRS virus is the etiologic agent of PPRS, a disease that causes breathing problems in pigs and kills piglets and unborn litters. It is a small enveloped positive-strand RNA virus that belongs to the family Arteriviridae in the genus Arterivirus [6]. At present, due to virus variation and unresolved issues of transmission, there is no single successful strategy available to cure the PRRS.

FMD, it is also one of the most devastating diseases of porcine caused by infection with a picornavirus, generically referred as FMD virus (FMDV) [7]. Vaccination of susceptible pigs against FMD is a well-established strategy for helping to combat the disease [8].

PED is also a highly contagious disease of porcine caused by the PED virus, an enveloped, ss RNA virus belonging to the Alphacoronavirus genus of the Coronaviridae family [9]. It causes high mortality in neonatal piglets; however, effective and safe vaccines are still not available [10].

The above diseases are some of the biggest threats to the pig industry and global security. In addition, the great majority of globally circulating pathogens go undetected, hindering outbreak preparedness and response. To enable routine surveillance and comprehensive diagnostic applications, there is a need for detection technologies that can scale to test many samples while simultaneously testing for many pathogens [11].

Nucleic acid amplification is a key process in molecular biology and has been widely used to detect the virus at scale—that causes viral diseases—for surveillance and diagnostic purposes. Polymerase chain reaction (PCR) was the first DNA amplification method, developed in the early 1980s [12] and until now has been the method of choice. However, it has a good number of limitations, including high cost of equipment, contamination chances, sensitivity to certain classes of contaminants and inhibitors, requirement of thermal cycling, etc. [13]. These limitations insisted on alternative methods such as loop-mediated isothermal amplification (LAMP), nucleic acid sequence-based amplification (NASBA), and most recent clustered regularly interspaced short palindromic repeats (CRISPR) based assay. These methods offer potential advantages

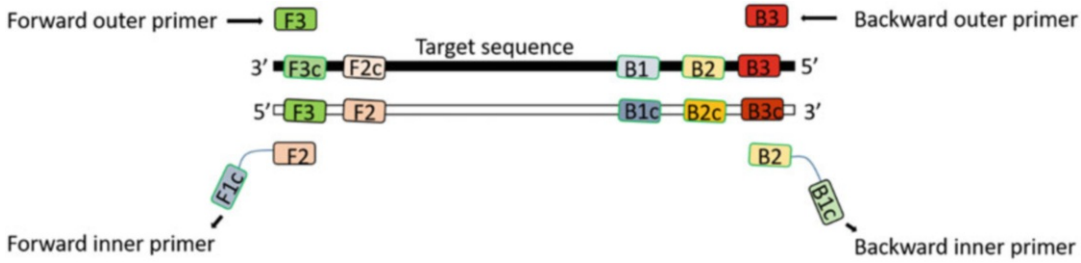


Fig. 1 Schematic representation of the position of primers

over PCR for speed, cost, scale or portability. In this chapter, we will focus on NASBA- and CRISPR-based assay to detect pig viral diseases.

2 Loop-Mediated Isothermal Amplification (LAMP)

It is a simple, rapid, specific, and cost-effective nucleic acid amplification method in comparison to PCR and nucleic acid sequence-based amplification. It was developed by Eiken Chemical Co., Ltd. In this method specifically designed four different primers are employed to recognize the six distinct regions on the target gene (Fig. 1).

Amplification and detection of gene are completed in a single step, by incubating the mixture of samples, primers, DNA polymerase with strand displacement activity and substrates at a constant temperature (about 65 °C). Combination with reverse transcription, it can amplify RNA sequences with high efficiency [14]. It is comparable to PCR in terms of sensitivity, but is less affected by presence of non-targeted DNA and inhibitory molecules [15]. Therefore, because of its simplicity, rapid amplification, and easy detection, LAMP has been used for diagnosis of several important emerging and re-emerging diseases such as CSF, ASF, PPRS, and FMD. However, through this method, we cannot detect the amplified product on a sequence basis.

3 Nucleic Acid Sequence-Based Amplification (NASBA)

It is a robust technology, amplifies nucleic acid continuously in a single mixture at one temperature. A detailed process of amplification was described by J. Compton in 1991. Briefly, it includes three enzymes, namely reverse transcriptase, T7 RNA polymerase, RNase H, two specific primers and appropriate buffer components to amplify single-stranded RNA with opposite polarity of the target. The amplified RNA product can be detected through the use of a target-specific capture probe bound to magnetic particles in

conjunction with a ruthenium-labeled detector probe and an instrument called NUCLISENS capable of measuring electrochemiluminescence (ECL) [16]. Alternatively, it can also detect in real-time through the use of molecular beacon probes included in the amplification reaction [17]. Molecular beacon probes possess a 5′ fluorescent dye and a 3′ quencher molecule and are designed to form stem-loop structures that bring into close proximity the 5′ and 3′ ends of the probe, resulting in minimal fluorescence. In the presence of a complementary target sequence, the probe is hybridized to the target, resulting in a measurable increase in fluorescence. The template is mainly RNA, can also be DNA. It can also be applied to single nucleotide polymorphism (SNP) analysis using genomic DNA as a template. The combination of DNA NASBA with multiplex hybridization of specific molecular beacons makes it possible to unambiguously discriminate the presence of the SNP of interest. The G-quadruplex/hemin complex can catalyze the oxidation of 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS2−) by H_2O_2 to produce a colorimetric signal that can be visualized by the naked eye. Recently, Lu et al. [18] developed a novel CSFV detection approach by combining this with NASBA technique (Fig. 2).

4 Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)

In 2012, Charpentier and Doudna reported "that the Cas9 endonuclease can be programmed with guide RNA engineered as a single transcript to cleave any double-stranded DNA sequence" [19]. Their discovery has led to widespread applications of the CRISPR-Cas9 system as a powerful and versatile tool in genome editing. CRISPR-Cas systems provide microbes with RNA guided adaptive immunity to foreign genetic elements by directing nucleases to bind and cut specific nucleic acid sequences. Through a process termed adaptation, microbes capture snippets of foreign genetic elements and incorporate them into their genomic CRISPR array. Transcription of CRISPR arrays creates CRISPR RNAs (crRNAs) that bind to Cas nucleases and provide specificity by base-pairing with target nucleic acids [20]. Presently, on the basis of Cas protein it is majorly divided into two classes. Class 1 CRISPR–Cas systems have effector modules composed of multiple Cas proteins that form a crRNA-binding complex and function together in binding and processing of the target. Class 2 systems have a single, multi-domain crRNA-binding protein that is functionally analogous to the entire effector complex of class 1 (Fig. 3) [21].

Recently, it was found that several Cas such as Cas12a/b, Cas13a/b, and Cas14 protein from the CRISPR-Cas system have collateral cleavage activity that can degrade single-stranded DNA or

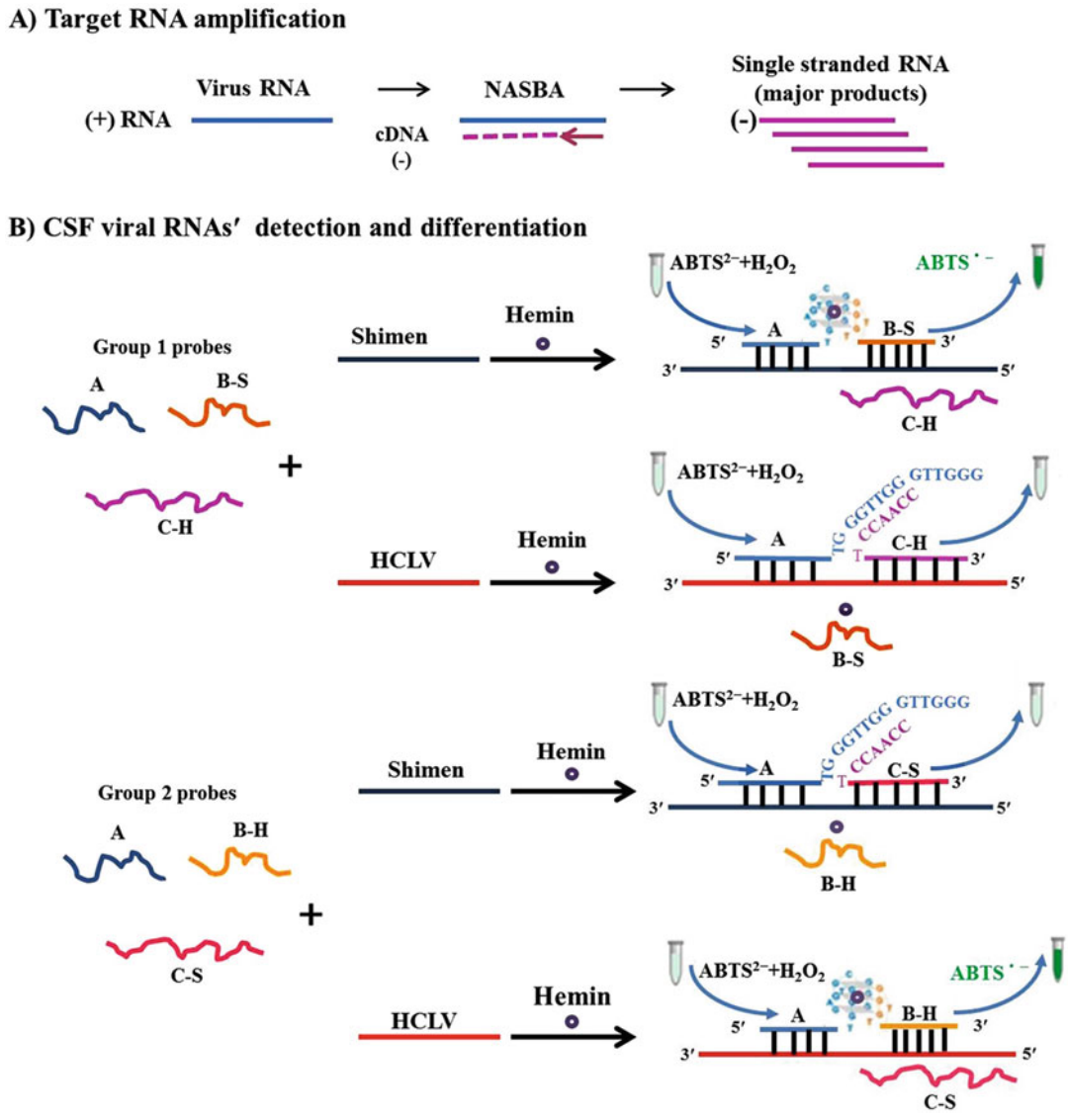


Fig. 2 Schematic representation of the NASBA-DNAzyme approach for detection and differentiation of CSF viral RNAs

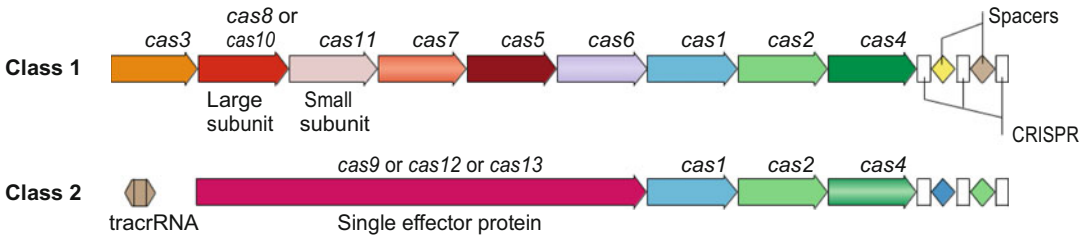


Fig. 3 Schematic illustration of the generic organizations of class 1 and class 2

RNA non-specifically upon binding to their target site. This ability can be used to detect pathogens when a short reporter sequence is also added to the cells. This discovery has led to the emergence of many CRISPR-Cas based virus detection protocols. This technology could be an ideal tool in diagnostics as a result of its excellent performance in several important areas including high sensitivity, specificity, and accuracy as well as the fact that this technology is rapid and easy to use [22]. Recently, Ackerman et al. develop Combinatorial Arrayed Reactions for Multiplexed Evaluation of Nucleic acids (CARMEN), a platform for scalable, multiplexed pathogen detection. In the CARMEN platform, nanolitre droplets containing CRISPR-based nucleic acid detection reagents self-organize in a microwell array to pair with droplets of amplified samples, testing each sample against each CRISPR RNA (crRNA) in replicate. The combination of CARMEN and Cas13 detection (CARMEN–Cas13) enables robust testing of more than 4500 crRNA–target pairs on a single array.

As a summary, these emerging CRISPR/Cas detection tool shows great potentials in the detection of virally derived nucleotides. Therefore, CRISPR-based methods would be more suitable for molecular diagnosis of major epidemic outbreaks than conventional techniques.

References

1. Thiel H-J, Collett MS, Gould EA, Heinz FX, Houghton M, Meyers G, Purcell RH, Rice CM (2005) Family Flaviviridae. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA (eds) Virus Taxonomy. VIIIth Report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego, pp 979–996
2. Edwards S, Fukusho A, Lefevre PC, Lipowski A, Pejsak Z, Roehe P, Westergaard J (2000) Classical swine fever: the global situation. Vet Microbiol 73:103–119. https://doi.org/10.1016/S0378-1135(00)00138-3
3. Moennig V, Floegel-Niesmann G, Greiser-Wilke I (2003) Clinical signs and epidemiology of classical swine fever: a review of new knowledge. Vet J 165:11–20
4. van Oirschot JT (2003) Emergency vaccination against classical swine fever. Dev Biol 114:259–267
5. Murphy FA, Gibbs EPJ, Horzinek MA, Studdert MJ (1999) Asfarviridae and Iridoviridae. In: Murphy FA, Gibbs EPJ, Horzinek MA, Studdert MJ (eds) Veterinary virology. Academic Press, San Diego, CA, pp 293–300
6. Conzelmann KK, Vissner N, van Woensel P, Thiel HJ (1993) Molecular characterization of porcine reproductive and respiratory syndrome virus, a member of the Arterivirus group. Virology 193:329–339
7. OIE Technical Disease Cards. Foot-and-Mouth Disease (2017). Available from. http://www.oie.int/fileadmin/Home/eng/Animal_Health_in_the_World/docs/pdf/Disease_cards/FOOT_AND_MOUTH_DISEASE.pdf
8. Kahn S, Geale DW, Kitching PR, Bouffard A, Allard DG, Duncan JR (2002) Vaccination against foot-and-mouth disease: the implications for Canada. Can Vet J 43(5):349
9. Antas M, Woźniakowski G (2019) Current status of porcine epidemic diarrhoea (PED) in European pigs. J Vet Res 63(4):465–470
10. Hou Y, Ke H, Kim J, Yoo D, Su Y, Boley P, Chepngeno J, Vlasova AN, Saif LJ, Wang Q (2019) Engineering a live attenuated porcine epidemic diarrhea virus vaccine candidate via inactivation of the viral 2'-O-methyltransferase and the endocytosis signal of the spike protein. J Virol 93(15):e00406–e00419
11. Ackerman CM, Myhrvold C, Thakku SG, Freije CA, Metsky HC, Yang DK, Simon HY,

Boehm CK, Kosoko-Thoroddsen TSF, Kehe J, Nguyen TG (2020) Massively multiplexed nucleic acid detection with Cas13. Nature 582:277–282

12. Mullis K, Faloona F, Scharf S, Saiki RK, Horn GT, Erlich H (1986) Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. In: Cold Spring Harbor symposia on quantitative biology, vol 51. Cold Spring Harbor laboratory Press, Cold Spring Harbor, New York, pp 263–273
13. Fakruddin M (2011) Loop mediated isothermal amplification-An alternative to polymerase chain reaction (PCR). Bangladesh Res Pub J 5: 425–439
14. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T (2000) Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 28:E63. https://doi.org/10.1093/nar/2
15. Kaneko, H, Kawana T, Fukushima E, Suzutani T (2007) Tolerance of loop-mediated isothermal amplification to a culture medium and biological substances. J Biochem Biophys Methods 70(3):499–501
16. Chan AB, Fox JD (1999) NASBA and other transcription-based amplification methods for research and diagnostic microbiology. Rev Med Microbiol 10:185–196
17. Romano JW, van Gemen B, Kievits T (1996) NASBA: a novel, isothermal detection technology for qualitative and quantitative HIV-1 RNA measurements. Clin Lab Med 16:89–103
18. Lu X, Shi X, Wu G et al (2017) Visual detection and differentiation of classic swine fever virus strains using nucleic acid sequence-based amplification (NASBA) and G-quadruplex DNAzyme assay. Sci Rep 7:44211. https://doi.org/10.1038/srep44211
19. Jinek M et al (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337(6096): 816–821
20. Knott GJ, Doudna JA (2018) CRISPR-Cas guides the future of genetic engineering. Science 361(6405):866–869
21. Makarova KS et al (2020a) Evolutionary classification of CRISPR-Cas systems: a burst of class 2 and derived variants. Nat Rev Microbiol 18(2):67–83
22. Wang X, He S, Zhao N et al (2020) Development and clinical application of a novel CRISPR-Cas12a based assay for the detection of African swine fever virus. BMC Microbiol 20:282. https://doi.org/10.1186/s12866-020-01966-6

Chapter 11

Aptamers as Diagnostic Markers for Viral Infections of Veterinary Importance

Victoria C. Khangembam and Dimpal Thakuria

Abstract

Viral infections can cause serious diseases and remain one of the biggest challenges in animal healthcare. Early and accurate diagnosis is crucial to prevent further spread of infections and for effective treatment. It is also essential in case of emerging diseases to adopt correct control measures such as containment, confinement, antimicrobials, and vaccines. Aptamers are promising molecules for developing biosensors to detect infectious agents. They are a special class of small molecules consisting of single-stranded nucleic acids, peptides, and peptide nucleic acids which can bind to a broad range of target molecules with high affinity and specificity. Owing to its equal specificity to antibody based technologies and better sensitivity with lower manufacturing cost, many aptamer based detection systems are being developed. Another added advantage of aptamer based technology is its suitability for point-of-care testing in remote or less equipped areas. This chapter aims to provide an overview of aptamers, its types and summarize the aptamer based technologies in detection of viral pathogens of veterinary importance.

Key words Aptamers, Viral infection, Diagnostic, Nucleic acid, Peptide, Peptide nucleic acid

1 Introduction

Aptamers are a special class of single-stranded nucleic acids, peptides, and peptide nucleic acids which can bind to a variety of target molecules with high affinity and specificity [1, 2]. Nucleic acid aptamers are 10–100 nucleotides long and form secondary and tertiary structures ensuring the specific binding to its target molecules such as amino acids, nucleotides, antibiotics, proteins, viruses, bacteria, and even cells [3–8]. Peptide aptamers consists of 5–20 amino acids usually embedded as a loop within a stable protein scaffold [9]. They can be regarded as smaller versions of immunoglobulin T-cell receptors which are highly soluble, stable, and exhibit fast folding kinetics. Both peptide and nucleic acid aptamers are highly similar in terms of conformational flexibility and variability in functional groups [10]. An additional class of aptamers is peptide nucleic acids (PNAs) which has high similarity to nucleic

Rajib Deb et al. (eds.), *Protocols for the Diagnosis of Pig Viral Diseases*,
Springer Protocols Handbooks, https://doi.org/10.1007/978-1-0716-2043-4_11,

acids [2]. Structurally, PNA consists of a pseudopeptide backbone where nucleobases are attached as side chains as in case of nucleic acid. Since the PNA backbone is not charged unlike DNA and RNA, there is no electrostatic repulsion during hybridization with complementary nucleic acid sequence resulting in a more stable PNA–DNA or PNA–RNA duplexes than the DNA–DNA or DNA–RNA duplexes [11, 12]. Because of its non-natural polyamide backbone, PNA is resistant to nucleases and proteases digestion thereby extending their lifetime both in vivo and in vitro [13].

The term "aptamer" is derived from two words, "*aptus*" (Latin) meaning "fit" and "*meros*" (Greek) meaning "part." These molecules particularly the nucleic acid aptamers have gained interests due to their low production cost, easy chemical modification, high chemical stability and binding affinity, repeatability, and reusability. They may form secondary and tertiary structures which gives them high specificity and affinity to a range of targets of varying size and complexities [14]. They are also considered as strong chemical competitors of antibodies due to their characteristic features such as small size, low immunogenicity, cheaper, and easier chemical synthesis and modification [15]. In fact, aptamers are more amendable for on-site detection methods owing to their stability at higher temperature and ability to recover their conformation and activity upon re-annealing [14, 16]. Considering its advantageous unique characteristics, technologies based on aptamers for detection of infectious diseases have achieved tremendous progress in recent years. In addition to detection, aptamers are also applicable in therapy, purification of target molecules, biosensors, in vivo imaging [17–20]. Owing to its wide applicability, research on aptamers is increasing and related information of several existing aptamers can be accessed at the special database (http://aptamer.icmb.utexas.edu) [1].

2 Selection of Aptamers

The method of aptamer selection called SELEX (Systematic Evolution of Ligands by EXponential enrichment) is an in vitro process. The method was invented by two groups independently in 1990 for selection of RNA ligands against T4 DNA polymerase and various organic dyes [5, 21]. In the same year, another group has devised techniques for the mutation, selection, and amplification of catalytic RNA, a process that can be performed rapidly in vitro [22]. SELEX is based on repeating cycles of binding, separation, and amplification of nucleotides. Briefly, the first step of SELEX is to incubate a sequence pool or nucleic acid library with targets. The nucleic acids have a random internal region flanked by constant sequences at both ends. The random region is tested for high specificity and affinity to the target. The second step involves

removal of unbound nucleic acids. In the third step, the bound sequences are purified and amplified that forms a new sequence pool for the next cycle. Normally, the cycle is repeated 8–15 times to achieve the desired aptamer sequence pool [7, 23]. Another step of negative selection can be included to obtain aptamers with higher specificity for the target. This is done by passing the sequence pool over analogs or a supporting matrix in the absence of the target which will eliminate the nucleic acids that bind to competing analogs or matrix. The oligos selected from the final round are then amplified and sequenced to identify aptamer. Alternatively, sequence data from the oligos present in the pool after selection rounds can be obtained by next generation sequencing. This is followed by synthesis and characterization of most potential sequences. Frequently, aptamers with nanomolar dissociation constants are identified but aptamers with picomolar dissociation constants have also been isolated [24].

For peptide aptamer selection, there are different in vivo and in vitro methods are available. The yeast two-hybrid (Y2H) strategy and phage display are examples of in vivo and in vitro methods, respectively [25, 26]. Initially, the method of phage display was introduced to map epitope binding sites of immunoglobulins by panning large phage libraries of random peptides [9, 26]. In this, external gene is inserted into the gene of appropriate coat protein of the bacteriophage most commonly M13 phage. The resulting phage is amplified in *E. coli* and during assembly; the translated fusion protein gets displayed on the phage surface. Then the phage population exhibiting strong binding with the target of interest are identified. The Y2H strategy is based on a system of two hybrid proteins containing parts of GAL4 of *Saccharomyces cerevisiae*. The hybrid proteins are GAL4 DNA-binding domain and GAL4 activating region, fused with Snf1 and Snf4 yeast proteins. The presence of both the hybrids results in high transcriptional activity in a cell [26]. Notably, in both selection processes, peptide aptamers are isolated together with their coding sequences [27]. Other in vitro display based selection methods of peptide aptamer includes cell surface display, ribosome display, mRNA display, DNA display, and in vitro compartmentalization [9].

3 Application of Aptamer in Detection of Viral Diseases

Several viral infections can cause serious disease in animals and some may passed to human. Therefore, rapid and accurate identification of a causative agent is critical to ensure correct, well timed treatment of the patient but also to prevent the spread of the infectious agent. Technologies based on aptamer have great potential in virus detection and therapeutics. In diagnosis of viral diseases, virus isolation is regarded as gold standard which is always performed

in a designated virology laboratory [28, 29]. However, it may take a longer period depending on the types of viruses and susceptibility of the cell lines and may not be possible to detect the non culturable or slow growing (non-cytopathic) viruses [28]. Hence, viral nucleic acids or proteins involved in adsorption, penetration, and replication and finally release of virus may be targeted for aptamer selection and development of biosensor. A biosensor is an analytical device that typically consists of a bioreceptor (enzyme/antibody/cell/nucleic acid/aptamer) which recognizes and binds the target with high sensitivity and selectivity and a transducer component which translates and outputs biological signals resulting from the interaction [30, 31]. Aptamers based biosensor also called aptasensors, uses aptamers as either bioreceptor or transducer [32, 33]. Aptasensors, which are cheap, user friendly, quick, and specific for the target, stable and most importantly applicable at field condition may receive wider acceptance.

4 Nucleic Acid Aptamers

Nucleic acid aptamers (DNA, RNA, oligos with modified nucleotides) have high affinity and specificity for a broad range of potential targets. They are more preferred than antibodies for use in biosensor because of their flexible structure, lower cost, stability, smaller size, and non-immunogenecity [24]. DNA aptamers have been employed in several detection methods of animal viral diseases and many are reported for H5N1 avian influenza virus. Highly pathogenic avian influenza virus (HPAIV) subtype H5N1 virus can cause severe disease in poultry often leading to huge mortality and can also infect mammalian species including humans, rats, and mice, weasels and ferrets, pigs, cats, tigers, and dogs. In severe form of H5N1 infection in birds, a mortality rate up to 100% within 48 h can be observed (OIE).

Shiratori et al. [34] have used DNA aptamers that bind to HA1 (hemagglutinin 1) proteins of multiple influenza A virus subtypes to develop novel aptamer based sandwich detection method. DNA aptamers were selected through a modified protocol of SELEX and amplified by PCR. The aptamer based assay successfully detected the H5N1, H1N1, and H3N2 subtypes of influenza A virus with almost equal sensitivities. In another study, Bai et al. [35] have used a DNA aptamer as the specific recognition element in a portable Surface Plasmon Resonance (SPR) biosensor for rapid detection of highly pathogenic avian influenza subtype H5N1 in poultry swab samples. The aptasensor could detect 0.128–1.28 hemagglutination units (HAUs) of the virus within 1.5 h. In the similar line, a pair of aptamers was successfully applied to develop the sandwich-type SPR-based biosensor for detection of H5N1 whole virus which could detect a virus concentration of 200 EID_{50}/ml (50%

embryo infective dose/ml) in fecal samples [36]. Fu et al. [37] proposed a method for detecting H5N1 based on exploitation of enzymatic catalysis in ultra-low ion strength media to induce ion strength increase for developing a novel impedance biosensing method. The method used a bionanocomposites formed with magnetic beads were modified with H5N1-specific aptamer to capture the H5N1 virus, concanavalin A (ConA), glucose oxidase (GOx), and gold nanoparticles. The biosensor showed high sensitivity with a detection limit of 8×10^{-4} HAU in 200 μl sample. A highly sensitive quartz crystal microbalance (QCM) aptasensor based on ssDNA crosslinked polymeric hydrogel was also developed for rapid, sensitive, and specific detection of avian influenza H5N1 virus which has a detection limit of 0.0128 HAU (HA unit) and time required from sampling to detection was only 30 min [38]. Lum et al. [39]used a biotinylated DNA aptamer to develop impedance biosensor using microfluidics flow cell and an interdigitated microelectrode which can detect 0.0128 hemagglutinin units (HAU) of H5N1 AIV specifically in 30 min. Another impedance aptasensor developed using specific H5N1 aptamer and a gold interdigitated microelectrode could detect 0.25 HAU of pure virus and 1 HAU for the H5N1 virus spiked tracheal chicken swab samples [40].

In poultry industry, another economically important viral disease is Newcastle disease (ND) caused by a virus in the family of paramyxoviruses. It is a highly contagious and in severe cases variable mortality as high as 100% can occur (OIE). Based on the pathogenicity of the ND virus (NDV) strains in chickens, they are divided into three pathotypes; velogenic (very virulent), mesogenic (moderate virulent), and lentogenic strains (mild virulent) [41]. A sandwich enzymatic linked aptamer assay (ELAA) using ssDNA have been developed for rapid and sensitive detection of NDV in farm samples [42]. The aptamers showed high specificity towards NDV with no cross-reactivity towards other avian viruses. The aptamers had affinity within the nanomolar range and the accuracy of the assay was comparable with standard qRT-PCR method.

Among the economically important diseases of ruminants, bovine viral diarrhea is a significant disease of cattle. It is caused by bovine viral diarrhea virus (BVDV), a member of the genus Pestivirus of the family, Flaviviridae. In bull, infection leads to fall in semen quality and the infectious virus can be isolated from the ejaculate. In cow, infection leads to poor conception rates, abortions, and congenital defects [43]. For detection of BVDV type 1, an aptamer based sandwich-typed assay had been developed. The ultrasensitive detection method used a ssDNA aptamer conjugated gold nanoparticle in an aptamer–aptamer sandwich-type sensing format which had detection limit of 800 copies/ml [44].

In pigs, porcine reproductive and respiratory syndrome (PRRS) is one of the most important swine diseases worldwide. It is caused

by a porcine reproductive and respiratory syndrome virus (PRRSV) of the genus, Arterivirus. The symptoms include reproductive failure, pneumonia, high levels of neonatal mortality, and increased susceptibility to secondary bacterial infection [45]. A biosensor for detection of PRRSV using DNA aptamer had been developed. The aptamer could bind specifically to the PRSSV when presented with the classical swine fever virus and a pseudo rabies virus. The developed platform was more rapid, accurate as compared to ELISA, PCR, and RT-PCR and had a detection limit of 1.87×10^{10} particles [46].

5 Peptide Aptamers

Peptide aptamers resemble antibodies with a variable antigen-binding domain with more advantageous features such as smaller size, higher stability and solubility, high yield bacterial expression, possibility of chemical synthesis, rapid folding properties, and in some cases absence of disulfide bonds and free cysteine residues [27]. The concept of peptide aptamers was originally proposed as a short amino acid sequence fixed in a small and stable protein backbone [9, 47]. Peptide aptamers are basically a "loop on a frame" strategy where a peptide of around 5–20 amino acids is embedded (constrained) in a neutral scaffold. Scaffold is rigid, compact, stable protein core preferably monomeric, and capable of displaying variable sites of interaction with target molecule as in case of complementarity determining region (CDR) of immunoglobulin molecule [9]. The embedded peptide imparts variability to select high affinity binders to a target molecule. The binding affinity of this constrained aptamers is much higher than the free peptide which can be up to 1000 times [9, 48, 49].

As peptide aptamer based platforms are flexible providing binding surfaces capable of accommodating large flat protein–protein interfaces as well as small molecules in the clefts and pockets, its applications in biomedical and bioanalytical field are on the rise [50]. Peptide aptamers having potentials to bind to Newcastle disease virus (NDV) have been identified by biopanning method using phage display technology [51]. The peptides were synthesized and their binding specificities were also confirmed by competitive phagemid ELISA using chicken anti-NDV antiserum as competitor.

6 Peptide Nucleic Acid Aptamers

Peptide nucleic acids (PNAs) are chemically stable and resistant to hydrolytic (enzymatic) cleavage and capable of sequence specific binding to complementary DNA and RNA through Watson–Crick

hydrogen bonding with high affinity due to their uncharged and flexible polyamide backbone [12, 52]. PNA is considered as a DNA analogue but its chemical structure is more like a peptide or protein with N-terminal at left and C-terminal at right side. PNAs can be synthesized by the standard method of solid-phase peptide synthesis using Fmoc-chemistry. Owing to their unique chemical, physical, and biological properties, PNA have been used to develop powerful biomolecular tools, molecular probes, and biosensors [53].

Using PNA and gold nanoparticles (AuNPs), a rapid label-free visual assay have been developed for the detection, genotyping/pathotyping, and quantification of Newcastle disease (ND) viral RNA [54]. The developed visual assay exploited the ability of free PNA to induce agglomeration in AuNPs leading change in color from red to blue and prevention of aggregation by hybridization of PNA to specific DNA [55]. In the assay, PNA probes complementary to the cleavage site of the ND virus F gene were used to detect viral RNA. The probes could detect 5–10 ng of viral RNA in 100 μl of biological samples, such as allantoic fluids, cell culture fluids, and vaccines. The end result of the assay could be observed with naked eye as plasmon color changes in the AuNPs solution and confirmed spectrophotometrically. The assay could also detect difference in single nucleotide thus enabling a visual viral genotyping/pathotyping. Another label-free visual assay using PNA was also developed for multiple strains of Influenza A Virus. The method provides accurate quantification of viral RNA on a spectrophotometer with visual limit of detection of 2.3 ng of Influenza A viral RNA [56].

7 Advantages of Aptamers

Aptamers are considered as a promising substitute for antibodies since their unique features can overcome some of the scientific challenges associated with antibodies. Though antibodies have wider range of applications, aptamers have a number of advantages as compared to antibodies. Nucleic acid aptamers are small, 20–60 nucleotides long, single-stranded RNA or DNA that can be arranged with a higher density on the biosensor surface. Nucleic acid aptamers are more thermally stable and maintain their structures over repeated cycles of denaturation/renaturation. It can recover their native conformation and can bind to targets upon re-annealing [10]. High resistance of aptamers to degradation by blood nucleases may be due to the formation of specific three-dimensional structures that protect the 3′- and 5′-termini of aptamers against exonucleases [1, 57]. Peptide nucleic acids, due to its non-natural polyamide backbone, are extremely stable in acidic environment, resistant to degradation by nucleases and proteases [13, 58]. Aptamer production is significantly easier and cheaper

than the production of antibodies [59, 60]. Furthermore, modifications of aptamers can be done during or after synthesis to increase their stability and nuclease resistance or to introduce signal moieties, such as fluorophores and quenchers to facilitate the development of biosensors [16, 61, 62]. The most common and effective type of modifications to increase resistance to nucleases without affecting their binding to target molecules is the modification of 3′- and 5′-nucleotides [63]. Another modification to make aptamer highly resistant to degradation by nucleases is ligation of the 3′- and 5′-termini of the same [64, 65]. Unlike antibodies which are significantly immunogenic, nucleic acid aptamers are low-immunogenic and low-toxic molecules. Aptamers has high affinity and specificity for their targets and even for those ligands which cannot be recognized by antibodies, such as ions or small molecules [16, 62].

8 Future Prospects

A rapid, user friendly, and accurate identification of causative agent of economically important animal diseases is highly required for mass screening of a population. Aptamer based biosensors have enabled to detect biomarkers at a very low concentration which can be applied for detection of infection at early stage. This will facilitate to segregate the infected animal in time to prevent the spread of the disease in the flock. The advantages of aptamers such as lower production cost, easy modification to improve stability, high sensitivity, reproducibility, specificity, and less detection time may be exploited to develop diagnostic methods for use at the point-of-care and simple laboratories. In addition, the ability of aptamers to detect even those small ligands which cannot be recognized by antibodies makes it a strong and versatile alternative to antibodies. Aptamer based techniques such as electrochemical, colorimetric, optical using aptamers are being studied worldwide. In therapeutic application, pegaptanib (brand name Macugen), an anti-vascular endothelial growth factor (VEGF) aptamer was approved by the U.S. Food and Drug Administration (FDA) in December 2004 for the treatment of neovascular age related macular degeneration. This has created confidence among aptamer developers and may lead to expansion of markets for aptamer based technologies. Furthermore, low immunogenicity, low-toxicity, and small size of these molecules make them more versatile for different biomedical applications and may receive a wider acceptance in the future.

References

1. Lakhin AV, Tarantul VZ, Gening LV (2013) Aptamers: problems, solutions and prospects. Actanaturae 5(4):34–43
2. Lee EJ, Lim HK, Cho YS, Hah SS (2013) Peptide nucleic acids are an additional class of aptamers. RSC Adv 3:5828
3. Zou X, Wu J, Gu J, Shen L, Mao L (2019) Application of aptamers in virus detection and antiviral therapy. Front Microbiol 10:1462
4. Szpechciński A, Grzanka A (2006) Aptamery w diagnostyceklinicznej [aptamers in clinical diagnostics]. Postepy Biochem 52(3):260–270
5. Ellington AD, Szostak JW (1990) In vitro selection of RNA molecules that bind specific ligands. Nature 346(6287):818–822
6. Schneider D, Tuerk C, Gold L (1992) Selection of high affinity RNA ligands to the bacteriophage R17 coat protein. J Mol Biol 228: 862–869
7. Torres-Chavolla E, Alocilja EC (2009) Aptasensors for detection of microbial and viral pathogens. Biosens Bioelectron 24:3175–3182
8. Ku TH, Zhang T, Luo H, Yen TM, Chen PW, Han Y, Lo YH (2015) Nucleic acid aptamers: an emerging tool for biotechnology and biomedical sensing. Sensors 15:16281–16313
9. Reverdatto S, Burz DS, Shekhtman A (2015) Peptide aptamers: development and applications. Curr Top Med Chem 15(12): 1082–1101
10. Mascini M, Palchetti I, Tombelli S (2012) Nucleic acid and peptide aptamers: fundamentals and bioanalytical aspects. Angew Chem Int Ed Engl 51:1316–1332
11. Jensen KK, Orum H, Nielsen PE, Nordén B (1997) Kinetics for hybridization of peptide nucleic acids (PNA) with DNA and RNA studied with the BIAcore technique. Biochemistry 36(16):5072–5077
12. Paulasova P, Pellestor F (2004) The peptide nucleic acids (PNAs): a new generation of probes for genetic and cytogenetic analyses. Ann Genet 47(4):349–358
13. Demidov VV, Potaman VN, Frank-Kamenetskil MD, Egholm M, Buchard O, Sönnichsen SH, NielsenP E (1994) Stability of peptide nucleic acids in human serum and cellular extracts. Biochem Pharmacol 48(6): 1310–1313
14. Li HY, Jia WN, Li XY, Zhang L, Liu C, Wu J (2020) Advances in detection of infectious agents by aptamer-based technologies. Emerg Microbes Infect 9(1):1671–1681
15. Keefe AD, Pai S, Ellington A (2010) Aptamers as therapeutics. Nat Rev Drug Discov 9:537–550
16. Song KM, Lee S, Ban C (2012) Aptamers and their biological applications. Sensors 12(1): 612–631
17. Yang L, Zhang X, Ye M, Jiang J, Yang R, Fu T, Chen Y, Wang K, Liu C, Tan W (2011) Aptamer-conjugated nanomaterials and their applications. Adv Drug Deliv Rev 63(14–15): 1361–1370
18. Cibiel A, Pestourie C, Ducongé F (2012) In vivo uses of aptamers selected against cell surface biomarkers for therapy and molecular imaging. Biochimie 94(7):1595–1606
19. Wang K, Fan D, Liu Y, Wang E (2015) Highly sensitive and specific colorimetric detection of cancer cells viadual-aptamer target binding strategy. Biosens Bioelectron 73:1–6
20. Zhang Y, Lai, B.S. andJuhas, M. (2019) Recent advances in aptamer discovery and applications. Molecules 24(5):941
21. Tuerk C, Gold L (1990) Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science 249(4968):505–510
22. Robertson DL, Joyce GF (1990) Selection in vitro of an RNA enzyme that specifically cleaves single-stranded DNA. Nature 344(6265):467–468
23. Davydova A, Vorobjeva M, Pyshnyi D, Altman S, Vlassov V, Venyaminova A (2016) Aptamers against pathogenic microorganisms. Crit Rev Microbiol 42:847–865
24. Ilgu M, Fazlioglu R, Ozturk M, Ozsurekci Y, Nilsen-Hamilton M (2019) Aptamers for diagnostics with applications for infectious diseases. In: Muharrem Ince, Olcay Kaplan Ince (Eds.), Recent advances in analytical chemistry, Intech Open. https://doi.org/10.5772/intechopen.84867
25. Fields S, Song O (1989) A novel genetic system to detect protein-protein interactions. Nature 340:245–246
26. Smith GP (1985) Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science 228: 1315–1317
27. Colombo M, Mizzotti C, Masiero S, Kater MM, Pesaresi P (2015) Peptide aptamers: The versatile role of specific protein function inhibitors in plant biotechnology. J Integr Plant Biol 57:892–901

28. Leland DS, Ginocchio CC (2007) Role of cell culture for virus detection in the age of technology. Clin Microbiol Rev 20(1):49–78
29. Storch GA (2000) Diagnostic virology. Clin Infect Dis 31(3):739–751
30. Hong P, Li W, Li J (2012) Applications of aptasensors in clinical diagnostics. Sensors 12: 1181–1193
31. Han K, Liang Z, Zhou N (2010) Design strategies for aptamer-based biosensors. Sensors 10(5):4541–4557
32. Cheng AK, Sen D, Yu HZ (2009) Design and testing of aptamer-based electrochemical biosensors for proteins and small molecules. Bioelectrochemistry 77:1–12
33. Hianik T, Porfireva A, Grman I, Evtugyn G (2009) EQCM biosensors based on DNA aptamers and antibodies for rapid detection of prions. Protein Pept Lett 16:363–367
34. Shiratori I, Akitomi J, Boltz DA, Horii K, Furuichi M, Waga I (2014) Selection of DNA aptamers that bind to influenza A viruses with high affinity and broad subtype specificity. Biochem Biophys Res Commun 443:37–41
35. Bai H, Wang R, Hargis B, Lu H, Li Y (2012) A SPR aptasensor for detection of avian influenza virus H5N1. Sensors 12(9):12506–12518
36. Nguyen VT, Seo HB, Kim BC, Kim SK, Song CS, Gu MB (2016) Highly sensitive sandwich-type SPR based detection of whole H5Nx viruses using a pair of aptamers. Biosens Bioelectron 86:293–300
37. Fu Y, Callaway Z, Lum J, Wang R, Lin J, Li Y (2014) Exploiting enzyme catalysis in ultra-low ion strength media for impedance biosensing of avian influenza virus using a bare interdigitated electrode. Anal Chem 86:1965–1971
38. Wang R, Li Y (2013) Hydrogel based QCM aptasensor for detection of avian influenza virus. Biosens Bioelectron 42:148–155
39. Lum J, Wang R, Hargis B, Tung S, Bottje W, Lu H, Li Y (2015) An impedance aptasensor with microfluidic chips for specific detection of H5N1 avian influenza virus. Sensors 15: 18565–18578
40. Karash S, Wang R, Kelso L, Lu H, Huang TJ, Li Y (2016) Rapid detection of avian influenza virus H5N1 in chicken tracheal samples using an impedance aptasensor with gold nanoparticles for signal amplification. J Virol Methods 236:147–156
41. Brown C, King DJ, Seal BS (1999) Pathogenesis of Newcastle disease in chickens experimentally infected with viruses of different virulence. Vet Pathol 36(2):125–132
42. Marnissi B, Kamali-Moghaddam M, Ghram A, Hmila I (2020) Generation of ssDNAaptamers as diagnostic tool for Newcastle avian virus. PLoS One 15(8):e0237253
43. Fray MD, Paton DJ, Alenius S (2000) The effects of bovine viral diarrhoea virus on cattle reproduction in relation to disease control. Anim Reprod Sci 60–61:615–627
44. Park JW, Jin Lee S, Choi EJ, Kim J, Song JY, Bock Gu M (2014) An ultra-sensitive detection of a whole virus using dual aptamers developed by immobilization-free screening. Biosens Bioelectron 51:324–329
45. Meulenberg JJ (2000) PRRSV, the virus. Vet Res 31(1):11–21
46. Kuitio C, Rasri N, Kiriwan D, Unajak S, Choowongkomon K (2020) Development of a biosensor from aptamers for detection of the porcine reproductive and respiratory syndrome virus. J Vet Sci 21(5):e79
47. Colas P, Cohen B, Jessen T, Grishina I, McCoy J, Brent R (1996) Genetic selection of peptide aptamers that recognize and inhibit cyclin-dependent kinase 2. Nature 380:548–550
48. Ladner RC (1995) Constrained peptides as binding entities. Trends Biotechnol 13:426–430
49. Cohen BA, Colas P, Brent R (1998) An artificial cell-cycle inhibitor isolated from a combinatorial library. Proc Natl Acad Sci U S A 95: 14272–14277
50. Vanhee P, van der Sloot AM, Verschueren E, Serrano L, Rousseau F, Schymkowitz J (2011) Computational design of peptide ligands. Trends Biotechnol 29:231–239
51. Ozawa M, Ohashi K, Onuma M (2005) Identification and characterization of peptides binding to Newcastle disease virus by phage display. J Vet Med Sci 67(12):1237–1241
52. Ray A, Norden B (2000) Peptide nucleic acid (PNA): its medical and biotechnical applications and promise for the future. FASEB J 14: 1041–1060
53. Nielsen PE, Egholm M (1999) An introduction to peptide nucleic acid. Curr Issues Mol Biol 1(1–2):89–104
54. Joshi VG, Chindera K, Singh AK, Sahoo AP, Dighe VD, Thakuria D, Tiwari AK, Kumar S (2013) Rapid label-free visual assay for the detection and quantification of viral RNA using peptide nucleic acid (PNA) and gold nanoparticles (AuNPs). Anal Chim Acta 795: 1–7
55. Su X, Kanjanawarut R (2009) Control of metal nanoparticles aggregation and dispersion by PNA and PNA−DNA complexes, and its application for colorimetric DNA detection. ACS Nano 3(9):2751–2759

56. Kumar N, Bhatia S, Pateriya AK, Sood R, Nagarajan S, Murugkar HV, Kumar S, Singh P, Singh VP (2020) Label-free peptide nucleic acid biosensor for visual detection of multiple strains of influenza A virus suitable for field applications. Anal Chim Acta 1093:123–130
57. Nitsche A, Kurth A, Dunkhorst A, Pänke O, Sielaff H, Junge W, Muth D, Scheller F, Stöcklein W, Dahmen C, Pauli G, Kage A (2007) One-step selection of vaccinia virus-binding DNA aptamers by MonoLEX. BMC Biotechnol 7:48
58. Zhang N, Appella DH (2010) Advantages of peptide nucleic acids as diagnostic platforms for detection of nucleic acids in resource-limited settings. J Infect Dis 201(1):S42–S45
59. Conrad RC, Giver L, Tian Y, Ellington AD (1996) In vitro selection of nucleic acid aptamers that bind proteins. Methods Enzymol 267:336–367
60. Kulbachinskiy AV (2007) Methods for selection of aptamers to protein targets. Biochemistry (Mosc) 72(13):1505–1518
61. Ferreira CS, Missailidis S (2007) Aptamer-based therapeutics and their potential in radiopharmaceutical design. Braz Arch Biol Technol 50:63–76
62. Jayasena SD (1999) Aptamers: an emerging class of molecules that rival antibodies in diagnostics. Clin Chem 45(9):1628–1650
63. Mayer G (2009) The chemical biology of aptamers. Angew Chem Int Ed Engl 48(15): 2672–2689
64. Mori Y, Nakamura Y, Ohuchi S (2012) Inhibitory RNA aptamer against SP6 RNA polymerase. Biochem Biophys Res Commun 420:440–443
65. Di Giusto DA, Knox SM, Lai Y, Tyrelle GD, Aung MT, King GC (2006) Multitasking by multivalent circular DNA aptamers. Chembiochem 7:535–544

Chapter 12

Antibody-Based Sensors for Pathogen Detection

Nirmita Dutta, Akhil Kumar, Anu Kumari, Sushila Maan, Gorachand Dutta, and Vinay G. Joshi

Abstract

Antibodies are soluble biomolecules of the Immunoglobulin family found in serum, which can specifically bind to and neutralize diverse antigens. Since their discovery, antibodies have been utilized for diagnostic, therapeutic, and research purposes. The development of genetic engineering and recombinant technology has made it possible to modify antibodies in structure and composition. Antibodies have found utility in the field of diagnostics with the incorporation of native or recombinant antibodies in biosensing platforms, capable of transducing the information of an antigen-antibody binding event into a measurable signal. This platform is termed as an immunosensor. Several approaches are available for the immobilization of antibodies on the surface of the transducer. These approaches include either a covalent or non-covalent attachment of the active form of antibodies in proper orientation, while retaining the conductivity of the transducing elements at the same time. The generated signal can be an electrical, optical, shear strain, or temperature change. Accordingly, immunosensors can be broadly divided into electrochemical, optical, piezoelectric or thermometric immunosensors. Each type has its own set of advantages and challenges in the context of design and sensing efficiency.

Key words Antibody, Biosensor, Immobilization, Electrochemical, SPR, Piezoelectric

1 Introduction

Since the dawn of Immunology during the 1880s, Emil von Behring and Shibasaburo Kitasato first reported the presence of antibodies (Abs) as anti-toxin factors (Fig. 1). Because of their high affinity and specificity towards a target molecule, antibodies against diverse antigens (Ags) have been produced by immunizing model vertebrate animals. Antisera and purified antibodies have been traditionally used for diagnosis, prevention, treatment, and epidemiology of animal and human diseases, and for research in life sciences [1].

During 1975, the advent of in vitro hybridoma technology by G. Kohler and C. Milstein marked the beginning of the modern era in antibody production. Monoclonal Antibody (mAb) technology

Rajib Deb et al. (eds.), *Protocols for the Diagnosis of Pig Viral Diseases*,
Springer Protocols Handbooks, https://doi.org/10.1007/978-1-0716-2043-4_12,

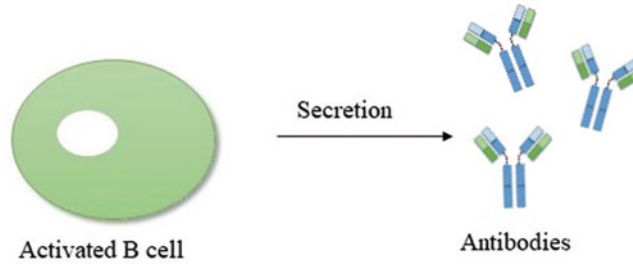


Fig. 1 Antibody production from activated B cells/plasma cells

or hybridoma technology has revolutionized the use of antibody (Ab) as a tool for research for the prevention, detection, and treatment of diseases (Fig. 2) [2, 3]. Because of hybridoma technology, a large number of immunological assays were developed and are still being used for the diagnosis of different diseases around the world. With the annual turnover of 115.2 billion US$ in 2018, the mAbs market is the fastest growing of all therapeutic proteins [4].

In the 1980s, novel recombinant DNA (rDNA)-based technologies, including heterologous antibody expression systems, such as bacteria, yeast, insects, and plants, as well as plant and mammalian cell culture systems, were developed. New technologies have enabled the production of bio-engineered Abs for application in modern science and medicine such as diagnostics, theranostics, therapeutics, proteomics, biosensors, etc. Desirable features can be introduced in the recombinant Ab (rAb) formats, and various rAbs against several clinically and biotechnologically important antigens have already been produced [5]. Murine monoclonal antibodies produced by hybridoma cultures were modified (chimerized or humanized) using rDNA techniques for in vivo application in humans (Fig. 3). The bio-engineered Abs have been projected as the "Abs of the future," which are going to replace antisera and most, if not all mAbs in the coming decades. In fact, the engineered Abs are making it possible to solve the problems that have defied traditional approaches. Moreover, such antibody constructs do not require the use of animals, thereby contributing to saving the lives of thousands of animals and promoting animal welfare [6].

Ever since their recognition more than a century ago, antibodies continue to be a class of fascinating bio-molecules globally for researchers, clinicians, and industrialists. Advancement in molecular biology and biotechnology open the path for the production of customized antibodies with desired size and specificities. Ab-based immunoassays are the most commonly used diagnostic assays and remain one of the fastest growing technologies for the analysis of bio-molecules. But traditional in vitro diagnostics have some limitations. They are time consuming, require trained person, specific equipment, sophisticated laboratories, pragmatic usage under field

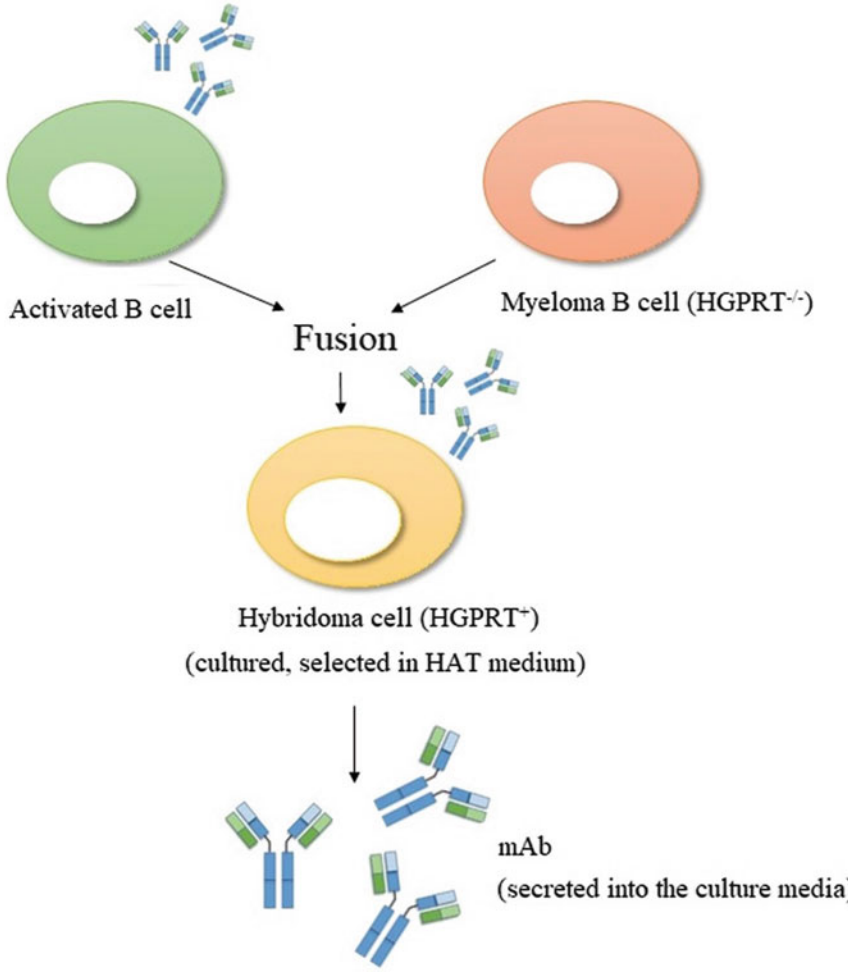


Fig. 2 Hybridoma technology for monoclonal antibody production (*HGPRT* Hypoxanthine-guanine phosphoribosyltransferase enzyme, *HAT medium* Hypoxanthine-aminopterin-thymidine medium)

conditions etc. [7] However, recent advances in biosensor technology have paved the way for the development of point-of-care diagnostics which are more accurate, less time consuming, applicable under field conditions, economical, etc. Antibodies, which are among the most exquisitely designed and engineered molecules in nature, are the ideal recognition elements for incorporation into sensors. A huge number of Ab-based biosensors (Immunosensors) are utilized clinically for the detection of a variety of analytes.

2 General Structure of an Antibody: An Overview

Antibodies are serum soluble, Y shaped glycoproteins produced by activated B cells, also known as plasma cells, as a result of the interaction between the antigen-specific surface receptor of naïve

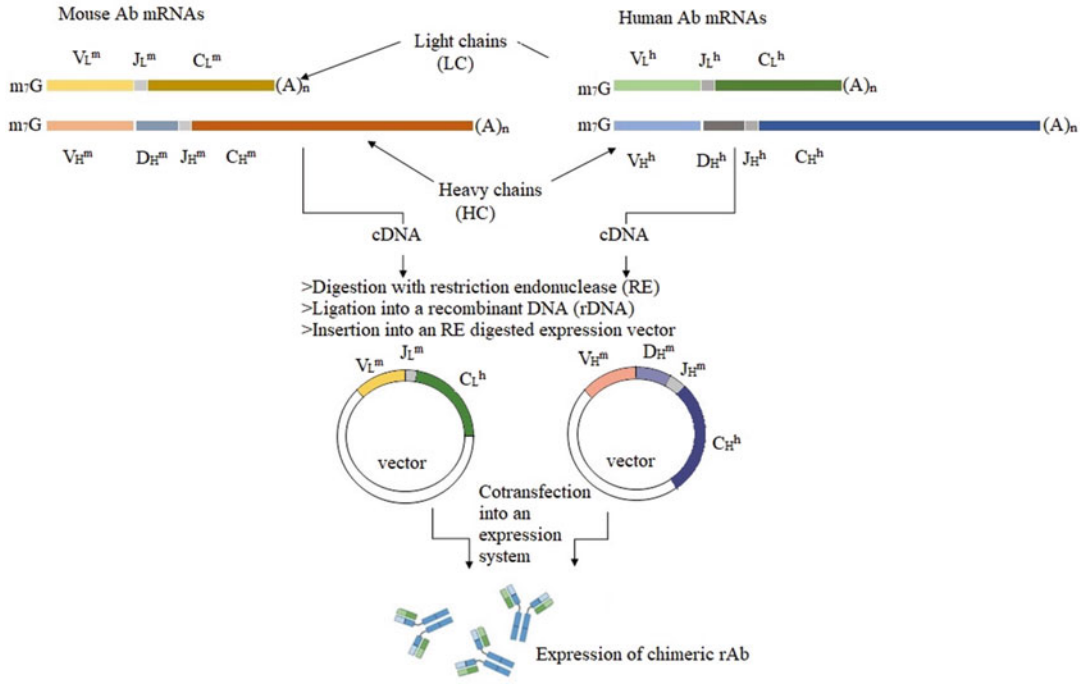


Fig. 3 Recombinant DNA technology for chimeric recombinant antibody (rAb) production

B lymphocytes and a specific antigen (Fig. 4). Antibodies have the property of combining specifically with the antigen that induced their formation. Like other proteins, antibody molecules may be classified physio-chemically on the basis of their solubility in salt solutions, electrostatic charge, molecular weight, and structure [8].

In the immune system, the best characterized molecules are antibodies, which occur mainly in the globulin fraction of serum and are known as immunoglobulins(Igs). The antibody molecule plays the central role in humoral immunity by attaching to micro-organisms and neutralizing it. This event leads to the activation of one of the many killingmechanisms, such as opsonization, complement fixation, and the antibody-dependent cellular cytotoxicity (ADCC), which involve the recruitment of effectors of the cellular immune system. Effectors of the immune system are cells that are activated and differentiate in response to an immune trigger. Effector B cells or plasma cells are responsible for producing antigen-specific antibodies and mediate humoral immunity, while effector T cells, namely helper T cells and cytotoxic T cells, help in the activation of immune cells and directly target infected cells, respectively, contributing to cell-mediated immunity. Gerald Edelman (USA) and Rodney R. Porter (UK) elucidated the structure of Igs and were awarded the Nobel Prize in 1972. They studied the chemical structure of γ (gamma)-globulin fraction of serum containing IgG

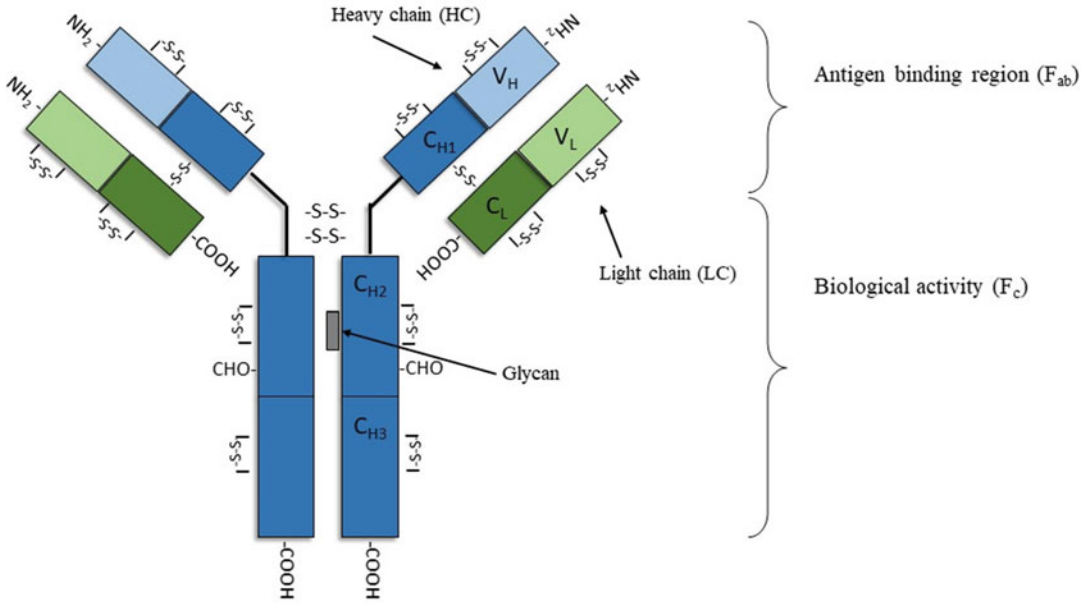


Fig. 4 General structure of an antibody.

of rabbit, using chemical solvents and proteolytic enzymes. Under electron microscope, the Ig was observed to be a typical "Y" shaped molecule [9].

Each IgG has two identical pairs of polypeptide chains: a light chain (LC) about 25–30 kDa size and a heavy chain (HC) of 50–70 kDa size(Fig. 4). The LC and HC are joined together by interchain disulphide (–S–S–) bonds. Variable (*V*) sequence and constant (*C*) sequence regions of LC and HC are folded into distinct domain structures, called "Ig domain" [10]. LC has two "Ig domains," i.e., one N-terminal V_L (110 AAs) and one C-terminal C_L (110 AAs), whereas HC has 4 or 5 "Ig domains," i.e., one N-terminal V_H (110 AAs) and three or four C_H (C_H1-C_H3 or C_H4). Each Ig domain contains a loop ("Ig fold") of about 60–70 AAs enclosed by intrachain (–S–S–) bonds. Three "hypervariable" (HV1-HV3) regions, also called complementarity determining regions (CDR1-CDR3), interspersed within four less-variable "framework" regions (FR1-FR4) exist within each V_L and V_H. The six CDRs, three each from V_L and V_H segments, fold to make one antigen-binding site or "paratope" (Fig. 5) [11]. Thus, each Ab molecule is at least bivalent, having two identical paratopes to bind the two identical epitopes in a multivalent Ag. Each paratope has a unique shape and chemical complementarity to that of the epitope for the best fit. In an immunoassay method, the most important function of antibody molecules is to combine with their specific antigens to form an antibody–antigen complex. The antigen-binding sites are responsible for the specific binding of

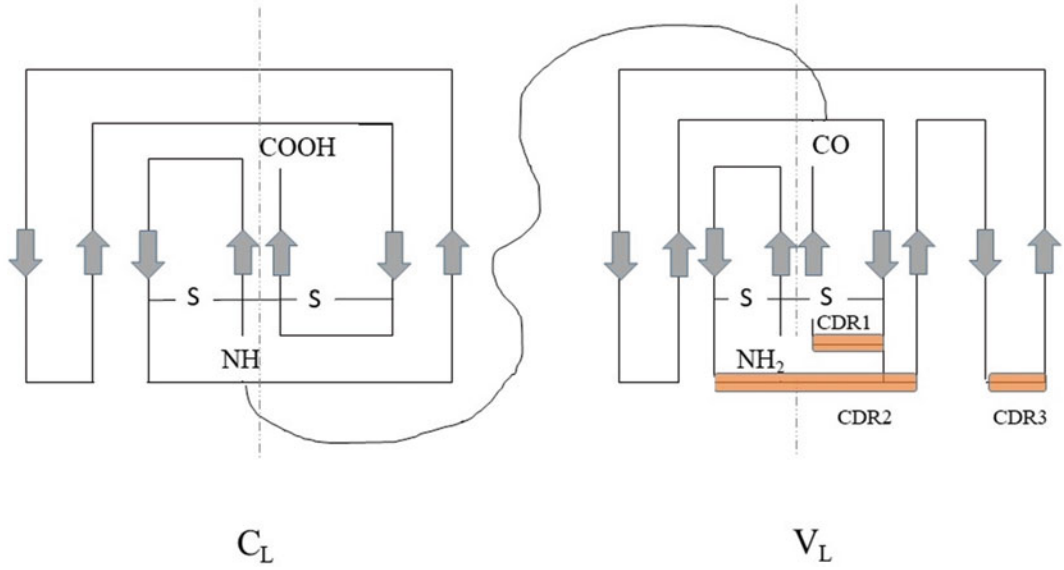


Fig. 5 Representation of a light chain showing antiparallel β-sheets of V_L and C_L regions, connected by α-helix linker, and complementarity determining regions (CDRs)

Ab to their targets. The intermolecular forces that contribute to the stabilization of the antibody–antigen complex are hydrogen bonding, electrostatic forces, hydrophobic interactions, van der Waals forces, and stearic repulsive pulses [12].

3 Biosensor/Immunosensor

Our immune system recognizes all cells and molecules in the body system and can differentiate between self and non-self-bio-molecules. When our body encounters some foreign substances (antigens), specialized immune cells get activated and produce antibodies which are specific to these antigens. This antigen-antibody interaction has been used by scientists to develop various formats of immunoassays including the development of sensors. A sensor that is based on the concept of immunology is known as an immunosensor. This antigen-antibody (immune-complex) thus formed in an immunosensor is measured by coupling this reaction to the surface of a transducer. The transducer detects and converts the reaction to an electrical signal where it can be processed, recorded, and viewed [13]. Ideally, an immunosensor should be designed with the following specifications: (1) the ability to identify target antigens quickly; (2) the ability to generate immunocomplexes without the need to add supplementary reagents; (3) the ability to give results with high reproducibility; and (4) the ability to easily detect the target in real samples [14].

The term Biosensor was first defined by the International Union of Pure and Applied Chemistry, 1992. The first biosensor was developed by Clark and Lyons in 1962 to measure glucose in biological samples. This biosensor coupled the biological specificity of enzymes with an electrode and transducer [15]. The concept of using immunological components (antibody or antigen) as sensing agents was first described within an immunoassay for plasma insulin in human subjects [16]. The journal *Biosensors and Bioelectronics* in the abstracts of the Fifth World Conference on Biosensors define a biosensor as an: "analytical device incorporating a biological material, a biologically derived material, or a biomimic, intimately associated with or integrated within a physico-chemical transducer or transducing microsystem.". The aim of a biosensor is to produce an electronic signal proportional to the specific interaction of analytes with the sensing element [17]. Biosensors can be used for the detection of analytes ranging from small molecules to intact pathogenic microorganisms. In the case of immunosensors, antibodies/antibody fragments or antigens are used for detection. Immunosensors employ the high Ab/Ag specificity to detect the presence of its analyte [18]. The generated signal is proportional to the amount of target analyte in a specific reaction. A biosensor consists of the following constituents:

Analyte: A component or substance of interest to be detected.

Bioreceptor: A molecule that specifically recognizes the analyte. Bioreceptors can be in the form of enzymes, cells, and antibodies. Once bioreceptors interact with the analyte, a signal will be produced, such as heat, charge, mass change or pH, etc., and this is called bio-recognition (or recognition receptor).

Transducer: The function of a transducer is to use the information from the bio-recognition episode between the bioreceptor and analyte, and convert it into a measurable signal.

Biosensors are useful and have a number of advantages over current analytical instruments based on these two features: (1) the proximity of the recognition receptors, e.g. antibodies, DNA, aptamers with the transducer; and (2) its practical size, suitable for fieldwork. Only a minute amount of sample is required for detection, as the sensitive part of a biosensor is normally small [14].

Basically, a biosensor has two components: a selective receptor (enzyme, Abs, etc.) and a detector (a transducer to sense the chemical or physical change upon interaction of the analyte with the receptor, and converts the signal into electrical/electronic). Depending on the method of signal transduction detection, biosensors can be classified as optical, electrochemical, thermometric, piezoelectric or magnetic. Surface plasmon resistance (SPR)-based optical biosensors are the most common. The optical biosensors allow real time, cost-effective, sensitive, and selective detection of a wide range of analytes including viruses, toxins, drugs, antibodies, tumor biomarkers, and tumor cells. Biosensors using various

transducer platforms have been developed to detect various bacterial pathogens and toxins of veterinary importance, such as *E. coli*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* strains, Mycoplasma biomarkers, Salmonella, epsilon toxin of *Clostridium perfringens*, pathogenic *S. aureus* and toxins in clinical, environmental, and food samples. The interaction of immobilized Abs with their corresponding Ags makes possible the development of Ab-based immunosensors, which have Ab immobilized onto a biosensor chip to recognize Ag specifically in a complex medium [19].

4 Antibody Immobilization

For developing an immunosensor, the immobilization of antibodies on the surface of a transducer element is of paramount importance. The understanding of the surface chemistry is required for proper binding, optimal orientation, and free movement of antibody molecules to obtain a maximal functional sensor surface [20]. Sensor surfaces consist of an inorganic material (glass, gold, iron oxide, platinum, etc.). The performance of a bio/immunosensor depends on some factors like (1) the ability to immobilize antibodies in their active form (2) its accessibility to the relevant analyte; and (3) low non-specific background. The immobilization step affects the detection limit, sensitivity, and overall performance of the immunosensor [21]. There are several factors which should be taken into account while developing an immunosensor. The attachment of antibodies onto the surface of a transducer may occur in different orientations. If an antibody molecule binds on the sensor surface through the Fc region, then the antigen-binding regions (paratope) will be fully available to the antigen of interest, thus maximizing sensor performance. If an antibody molecule binds on the sensor surface through the antigen-binding regions, it may result in decreased or no sensor activity. The extent of linkage between the Ab and the surface may also need to be carefully controlled. The orientation of Abs on sensor surfaces can be controlled by the interaction between specific reactive groups on the surface and on the Ab. The two main approaches that can be used for immobilization of antibodies are non-covalent and covalent immobilization. However, affinity-based immobilization and engineered antibodies for immobilization are also being used [7].

4.1 Non-covalent Immobilization

Direct physical adsorption of antibodies onto the sensor surface can be performed via simple non-covalent forces, including electrostatic or ionic bonds, hydrophobic interactions, and van der Waals forces. There are some limitations of direct physical adsorption. Though easy to perform, the process is uncontrolled and may cause protein denaturation. When protein denaturation is involved,

some affinity-based assays may lead to ligand leaching from the surface due to extensive washing, thereby decreasing surface bio-activity [20].The orientation of the adsorbed antibody might not be proper as well. Another non-covalent approach used was the entrapment of antibody molecules into a conducting polymer [22], where Abs for human serum albumin were entrapped on a galvanostatically polymerized pyrrole, on to a platinum wire substrate. Some of the common conducting polymers that have been extensively used for immunosensor fabrication are polyacetylene, polythiophene, polyaniline, polyindole, and polypyrrole. But entrapment of antibody molecule may lead to poor accessibility of the antigen-binding sites, leading to poor signal [7].

4.2 Covalent Immobilization

Covalent immobilization of Abs facilitates long-term storage, conformational stability of the ligands, no leaching out of the analyte/ligand and reusability of immunosensors. In covalent bonding, the surface of the sensor is modified so that the reactive groups such as hydroxy, thiol, carboxylic or amino groups are available on the surface for the subsequent Ab immobilization [7]. Coupling of Abs to the sensor surface by targeting amine groups present in the lysine amino acid side chains of Ab is an extensively used form of covalent immobilization, due to the relative ease of access to these groups.

Feyssa and coworkers [23] immobilized anti-C-reactive protein (CRP) antibody in a microfluidic platform via amine covalent linkage. This approach showed signal enhancement in comparison with passive binding. The reproducibility of an amine-coupled biochip was found to be comparable with a human-CRP enzyme-linked immunosorbent assay (ELISA) detection kit. But there are some problems associated with amine coupling chemistry. It usually results in random orientation and less homogeneous binding due to the presence of excessive lysine groups in the Ab. Thiol group on the surface can be targeted for antibody immobilization as it gives more homogeneous immobilization or may allow defined orientation of Abs in comparison with amine coupling. Ab fragments immobilized in defined orientation have been shown to achieve a 20-fold enhanced antigen-binding ability, compared with the randomly immobilized Abs using amine groups [21]. Jarocka and coworkers [24] immobilized Fab Ab fragment on a gold electrode surface via thiol coupling and developed animpedimetric immunosensor for the detection of peptides derived from avian influenza haemagglutinin H5.

Another covalent approach involves the generation of active aldehyde groups (diol groups) which can be linked efficiently on amine-functionalized surfaces, resulting in partially oriented covalent Ab coupling on the sensor surface. Shriver-Lake et al. [25] pointed out some limitations with the use of the carbohydrate-based immobilization procedure, like time delays due to more

steps in the procedure and increased loss of Ab. They observed 50% loss in the Ab prior to immobilization, due to the number of steps in the procedure.

4.3 Affinity-Based Immobilization

In affinity-based immobilization, the surface of the sensor is layered with a baselayer of intermediate binding proteins such as protein A/Gor Fc-specific antibodies, or a nickel surface that binds histidine-tags in the Fc region, or biotin-avidin. These proteins have a high affinity and binding specificity towards the Fc region of a wide range of Abs, thus encouraging Ab immobilization via the Fc region on the sensor surface. This gives the antibody an environment that is not restricted due to proximity to the sensor surface, but instead has more freedom to interact with the analyte. Furthermore, antibodies are properly oriented, so that the antigen-binding sites are freely available for analyte interaction [18]. Hydrazide-containing cross-linkers provide another way of immobilizing Abs via the glycans in the Fc region, thereby exposing the antigen-binding sites to the solution.

de Juan-Franco and coworkers immobilized antibodies using a fusion protein, the Protein A–gold-binding domain (PAG) [26]. The human growth hormone-specific immunosensor fabricated using the PAG immobilization approach showed better sensitivity when compared with conventional methods. Barton and coworkers [27]achieved much higher sensitivities with an avidin–biotin interaction-based immunosensor in comparison with an entrapment-based immunosensor. Ionescu and coworkers [28] immobilized the anti-atrazine antibody fragments via affinity binding onto a polypyrrole film N-substituted by nitrilotriacetic acid (NTA), electrogenerated on a gold electrode. The variable domains of llama heavy-chain antibodies (VHH) was in vivo biotinylated at the lysine position of the Avi-tag, which improved the analyte binding more than 200-fold [21].

4.4 Recombinant Antibodies for Immobilization

With the advent of Recombinant DNA technology, different formats of recombinant antibodies have been generated. Recombinant antibodies have several desirable characteristics including low molecular mass, increased flexibility, high physico-chemical stability, and easy access to the antigen, which makes them a potential substitute for naturally generated Abs. It is now possible to introduce desirable modifications in the antibody molecule by using Recombinant DNA technology [5]. The directed immobilization approaches developed for intact antibody based on the Fc domain or carbohydrate moiety cannot be used with recombinant antibodies or antibody fragments such as scFv (single-chain variable fragment) and dsFv (disulphide-stabilized variable fragment), due to the absence of the glycosylated Fc domain. Various approaches have been developed to engineer scFv or scAb during their generation so as to easily immobilize them on to the solid surface without

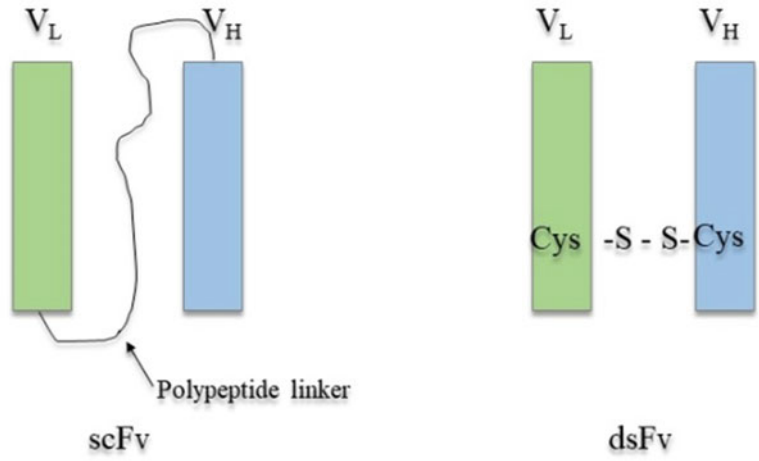


Fig. 6 Structure of scFv and dsFv

denaturation (Fig. 6) [29, 30]. Antibody fragments can be engineered to have positively charged amino acids (e.g. arginine or Arg) in the peptide linker or a 6-histidine amino acid sequence on the C-terminus for immobilization via electrostatic and non-covalent interactions, respectively. A wide variety of sensing transducers have been developed by using rAbs to substitute for intact Abs in immunosensors. The high specificity of recombinant antibodies allows the use of a single recombinant antibody to detect an antigen, thus eliminating the need for a second antigen-specific antibody. Additionally, the small size of recombinant antibodies permits immobilization on to an immunosensor surface at high density, thus resulting in enhanced assay avidity, sensitivity, and stability [7].

5 Biosensor Types

Depending on the method of signal transduction detection, biosensors have been classified as follows:

5.1 Electrochemical Immunosensor

An electrochemical immunosensor measures an electrical signal proportionate to antigen–antibody complex formation. This technique is economical, easy to operate, portable, and simple to construct. Since this is a surface based method, the reaction volume is relatively small and samples are required in minute quantities for detection purposes. With the use of modern and evolving technology, the electrochemical immunosensor is an attractive candidate for wide sensing applications (Fig. 7). One of the main areas where sensors are the most beneficial is in clinical diagnostics [13, 31]. In general, electrochemical immunosensors are classified as amperometric immunosensors, voltammetric immunosensors, potentiometric immunosensors, and impedimetric immunosensors. A more recent event in the field of electrochemical immunosensing is the development of FET-based immunosensors.

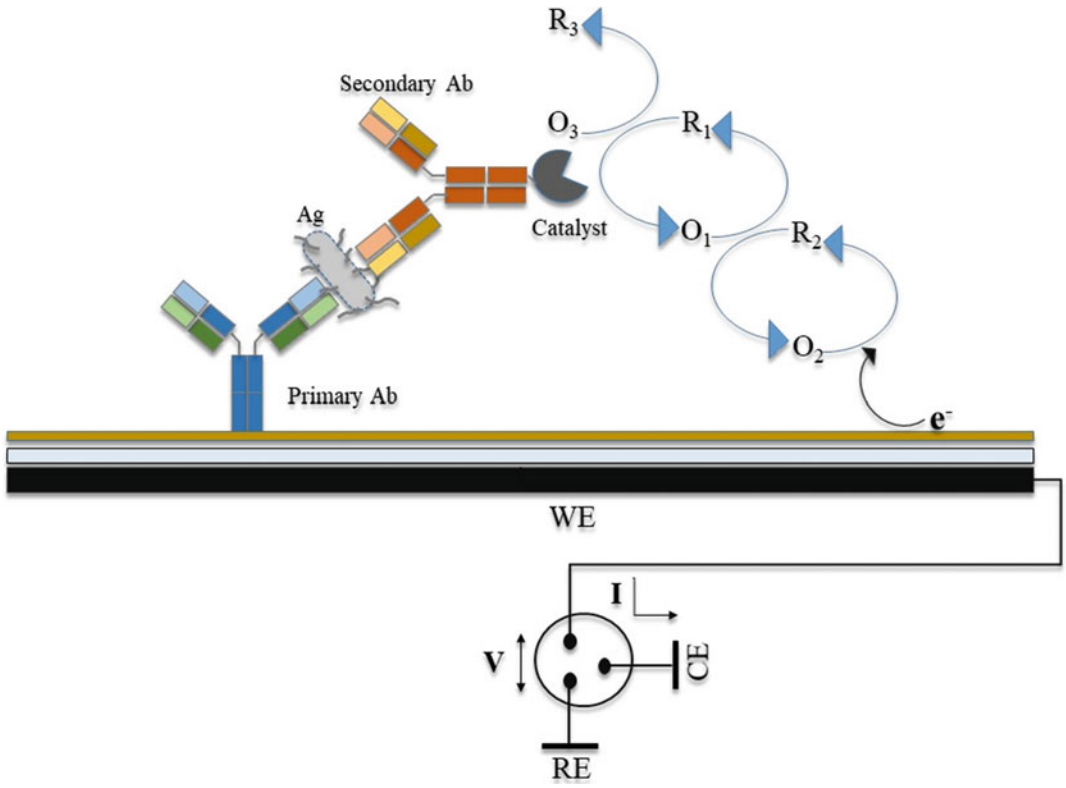


Fig. 7 Schematic of an electrochemical immunosensor (*Ag* Antigen, *Ab* Antibody, *O* Oxidized species, *R* Reduced species, *WE* Working electrode, *RE* Reference electrode, *CE* Counter electrode, *V* Voltage, *I* Current)

An amperometric immunosensor measures the changes in current at a constant potential value. The measurable current output is proportional to the concentration of the analyte of interest. This type of transducer is highly selective in nature as the oxidation or reduction potential characteristic to the target is used to determine its identity. Also, they consume a small percentage of analytes during measurement [32]. The sensitivity of an amperometric immunosensor can be increased using a layer-by-layer construction approach. In one study, gold nanoparticles and methylene blue were layered to form a stable base for hCG sensor. Layering increased the surface area, thereby increasing the capture of analytes, and consequently increased the sensitivity [33]. Platinum nanoparticles have also been used for the layering purpose instead of gold nanoparticles, for the detection of H_2O_2 electroreduction [34].

In voltammetric immunosensors, current and potential are measured over a pre-set potential range. The resulting current is converted into a peak which represents the target of interest, and the height of the peak corresponds to the amount of analyte in a sample. This method is highly sensitive due to its minimal

background noise [14]. In potentiometric immunosensors, changes in ion activity are measured which specifies the mass charge potential. The most common instrument used in potentiometry is the pH electrode. Other ions such as (F, I, CN, Na, K, Ca, NH) or gas (CO, NH) selective electrodes can also be used for potentiometric measurement. But it lacks the sensitivity required to distinguish between two values within a small concentration range, and the occurrence of non-specific binding is also very high [14, 32].

An impedimetric immunosensor measures the electrical impedance of an interface by applying a small sinusoidal voltage at a specific frequency, and the resulting current is recorded. This procedure is performed a range of frequencies. The ratio of current to voltage provides the impedance. Impedance is altered when antigen-antibody complex gets deposited on the surface of the electrode, thus increasing the dielectric layer thickness. The change in capacitance is proportional to the size and concentration of antibody [32, 35].

An FET-based immunosensor consists of a source, drain, and gate electrodes. An external potential (V_{DS}) is applied across the source and drain electrodes, and current (I_D) passes from source to drain through a semiconductor path, which has antibodies immobilized on the surface for antigen binding. The gate electrode is connected to this semiconductor path capacitatively through a dielectric layer. The voltage (V_G) applied to gate creates either an accumulation or depletion of charge carriers in the semiconductor path, controlling I_D. Capture of antigen to this surface changes the surface potential, and influences the magnitude of I_D. For example, a positively charged antigen induces a depletion layer in a p-type semiconductor, and a charge (e^-) accumulation layer in an n-type semiconductor. Accordingly, the conductance either decreases or increases, and is used to estimate the antigen concentration (Fig. 8) [36].

5.2 Optical Immunosensor

Optical immunosensors employ light either coming from a laser, diode or white-hot light bulb for the detection of analytes. As the light passes through or refracts from the Ag-Ab complex, the change in intensity of light can be correlated with Ag-Ab concentration. There is change in phase, polarization, speed or frequency of input light which may correspond to the antigen-antibody complex [37]. The concept involved in optical sensors for the identification of analytes is based on the higher dielectric permittivity acquired by all proteins, cells, and DNA, compared to air and water, causing these biomolecules to reduce the propagation speed of the electromagnetic fields flowing through them (Fig. 9). Because all molecules contain atomic nuclei and electrons in varying orbital states, these molecules are able to interact with the electromagnetic fields that pass through them. By placing these molecules in oscillating electromagnetic fields analogous to the

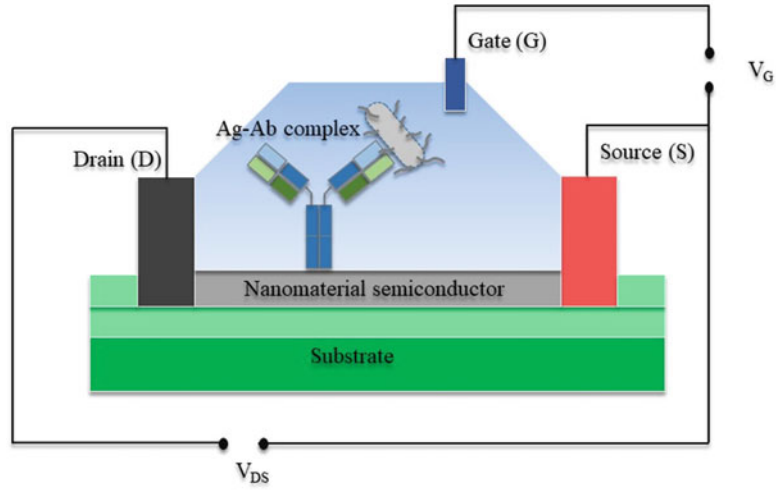


Fig. 8 Schematic of an FET-based immunosensor

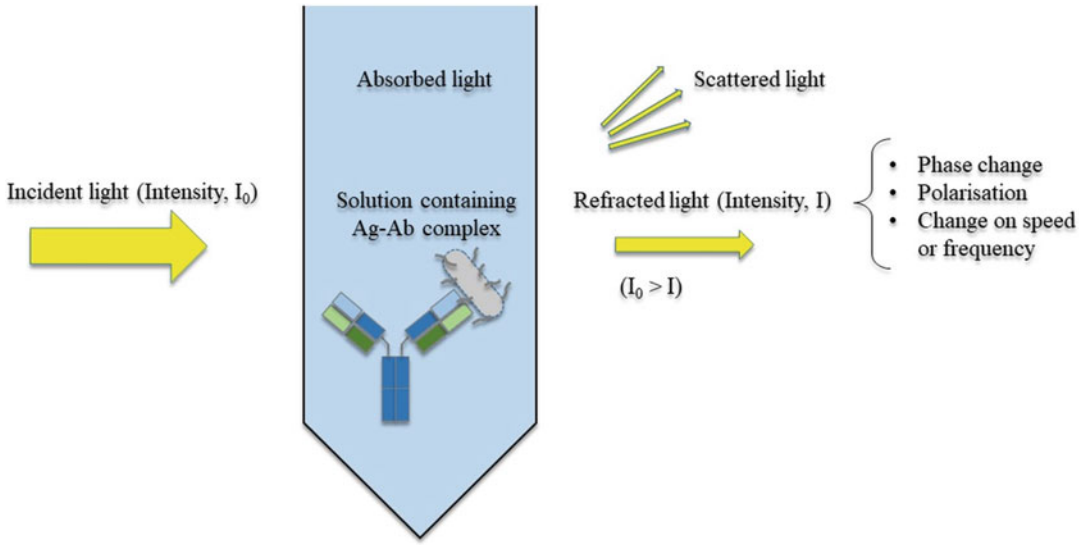


Fig. 9 Schematic of an optical immunosensor

propagation of light, electrons within the molecules vibrate due to the force subjected to them. Free electrons then polarize in the presence of light's magnetic field, generating a polarization current resulting from the movement of electrons, where it moves much slower through a biomolecule than in free space.

The target or bioreceptor molecules can also be attached to chromogenic/fluorescent labels (e.g. dyes) that will cause a change in the fluorescence signal signifying the existence of target analytes. The degree of fluorescence emitted correlates to the magnitude of

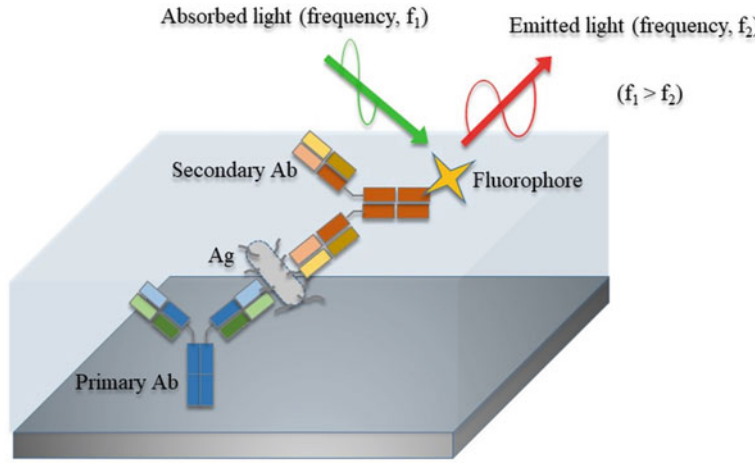


Fig. 10 Schematic of a fluorimetric immunosensor

interaction between the target analytes and bioreceptors (Fig. 10). Popular examples of optical immunosensors include surface plasmon resonance (SPR) based sensors, fiber-optic sensors (FOS), and various fluorescence based sensors, with surface plasmon resonance being the most widely employed sensor [32, 38].

In a SPR immunosensor, antibodies are immobilized on a surface of thin metal film, typically gold, where polarized light is radiated from the back surface through a prism and a target ligand is introduced. The metal film reflects this light and the strength of the reflected light can then be assessed and quantified. When the immobilized antibodies are bound to their target, a shift in the SPR angle can be observed that depends on the concentration of the target (Fig. 11). SPR harnesses refractive index changes caused by the formation of the Ab/Ag complex on a metal surface being related to the concentration of the antigen in the sample being measured [37, 39].Currently, SPR is a leading sensor technology for the observation of biomolecular interactions in real time, which has been commercialized by several companies. Biacore SPR sensor surfaces are the most widely used and are commercially available with various functionalities. The most widely used surface chemistry, called CM5, consists of a SiO_2-base layer, on top of which a thin gold film is deposited. The gold film is further modified by long-chain hydroxyalkyl thiols to introduce organic components for chemical modification [18].

5.3 Piezoelectric Immunosensor

Recently, piezoelectric crystals have been used for the development of piezoelectric immunosensors. When an antigen interacts with antibodies to form immunocomplexes, there is a change in mass, which can be examined by a quartz crystal, the main constituent of a piezoelectric sensor (Fig. 12).The piezoelectric crystal oscillates at

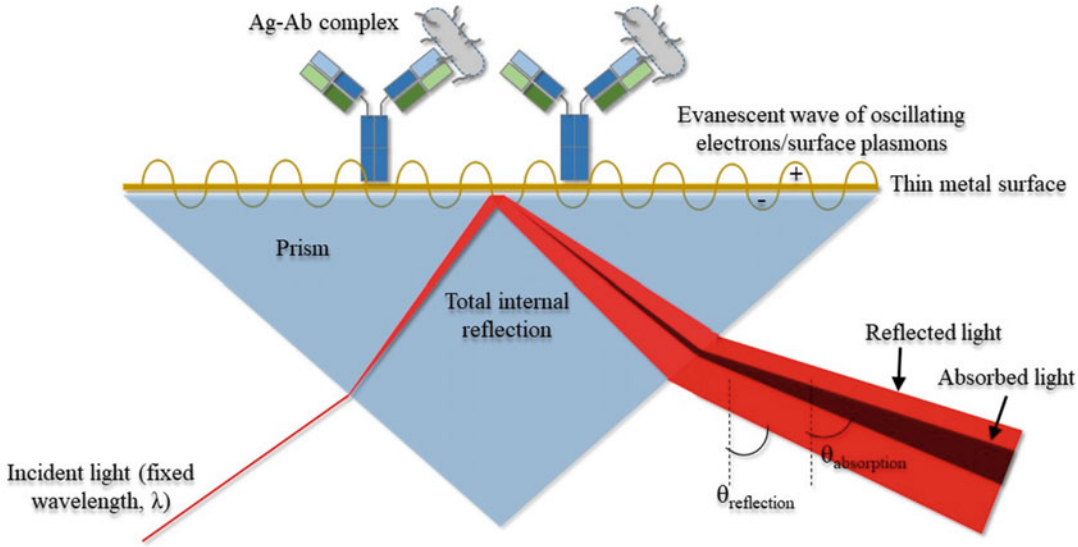


Fig. 11 Schematic of an SPR immunosensor

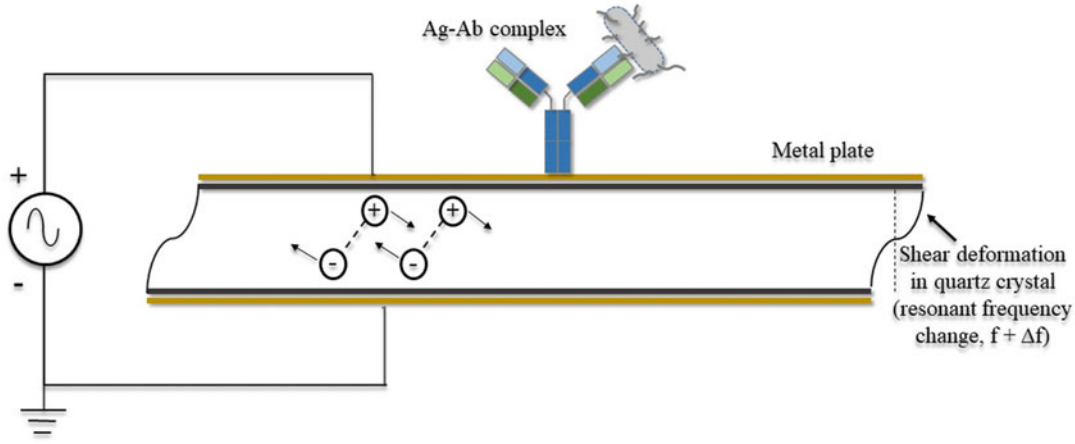


Fig. 12 Schematic of a QCM immunosensor

a specific frequency in conjunction with the use of an electrical signal at a certain frequency. By applying electrical voltage to the quartz crystal via two electrodes, the orientation of the crystal is altered and this causes a distortion in the crystal lattice that causes a mechanical oscillation at a characteristic vibrational frequency, i.e. the crystal's natural resonant frequency [40]. In the event of the formation of immunocomplexes, the surface of the crystal is loaded with an extra mass, which changes the frequency of oscillation of the crystal and the mass change can be determined electrically. Nowadays, piezoelectric crystals are becoming popular as they are simple, highly sensitive, specific, safe, label-free, stable and give

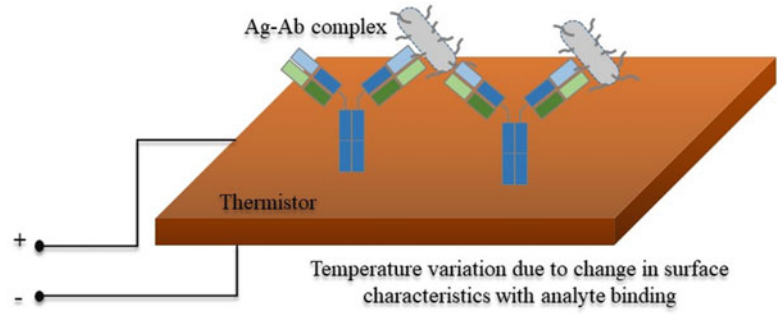


Fig. 13 Schematic of a thermometric immunosensor

fast results. Another advantage of the piezoelectric immunosensor is its ability to detect analytes in real time. They are being used in a number of sectors such as in clinical diagnosis and environmental pollution supervision [41]. In piezoelectric immunosensors, the measurement can be direct or indirect; single step or multi step. In a single step, the ligand binds with the analyte; and in multistep, there is sequential binding of two or more components. In direct measurement, the analyte or antigen binds with an antibody. While in indirect measurement, the analyte interacts with other entities in the solution [14].

5.4 Thermometric Immunosensor

The thermometric immunosensor utilizes absorbance or release of heat in a biological reaction as mode of detection. When the antigen binds with the antibody, there is temperature variation which can be converted into an electrical signal for determining the formation of immunocomplex (Fig. 13). These immunosensors offer the advantages of low operating cost and stability over a relatively longer period of time, and are also not affected by the presence of ions in the solution. However, one major disadvantage of this type of sensors is the lack of specificity. One has to be sure that the related enthalpy changes are not due to the immobilization of any interfering species and are not related with dilution effect [14, 42].

6 Biosensor Fabrication

The fabrication of a biosensor involves four primary parts, each of which has its own set of functions and criteria for design, as discussed below.

6.1 Transducer

Transducers convert a biological event into a proportionate electrical signal. In electrochemical biosensors, a transducer is constructed with one or more materials which possess certain properties such as high electron transfer kinetics, good

conductivity, and biocompatibility. With the advanced tools of nanotechnology available, nanoparticles become an ideal choice, owing to additional properties such as high surface area to volume ratio and ease of functionalization, along with the above mentioned properties. Some commonly used nanomaterials for the preparation of transducers include metal nanoparticles such as gold (AuNP), silver (AgNP), platinum (PtNP) nanoparticles, etc.; carbon based nanomaterials such as carbon nanotubes (CNT), graphene sheets and derivatives, spherical fullerene, etc.; and organic films, dendrimers, and conductive polymers such as polypyrrole, polyaniline, polydopamine, etc. Nanoparticles are fabricated over an electrode surface responsible for conducting the current signal. Common choices of electrodes are indium tin oxide (ITO) electrodes, glassy carbon electrodes (GCE), etc. For transducer fabrication in optical sensors, properties such as absorption of electromagnetic radiation, reflection, scattering, refractive index, fluorescence, and chemiluminescence are targeted. SPR involves the fabrication of a very thin metal surface that has an extended plasmon region/excitable electron band, QCM employs an AT-cut quartz crystal and FET-based biosensors utilize nanomaterials which behave like semiconductors.

6.2 Bioreceptor

As discussed earlier, antibodies and antibody fragments are the most widely used bioreceptors in biosensor fabrication. Antibodies are immobilized on the transducer surface either covalently or non-covalently, through adsorption, electrostatic interactions, affinity binding, and entrapment within polymers. The free sites might cause electrode fouling, and unnecessary background signal, and hence are insulated with common blocking agents such as bovine serum albumin (BSA), self-assembled monolayers (SAM) of ethanolamine, mercaptohexanol, etc.

6.3 Electroactive Labels and Redox Species

In electrochemical biosensors, electroactive labels are often used to generate electrical signals following the antigen-antibody binding event. Some of the common bionanolabels fabricated can be categorized as: electrocatalytic nanoparticles combined with bioreceptors, enzymatic labels with redox substrates, magnetic bead based labels with bioreceptors, heavy metal based labels, etc. One or more redox species are added to the sensing system for signal amplification through coupled, chemical or electrochemical, redox cycling. Common redox species used in sensors are ferrocene ($Fe^{2+/3+}$), potassium ferricyanide/ferrocyanide, hydrazine, etc. Electrochemical detection can also be carried out label-free, as seen in impedimetric biosensors. Electroactive labels and redox species do not form a part of optical, piezoelectric or thermometric biosensors.

6.4 Buffers

The samples used for detection of antigenic species often need to be diluted, lest they cause high background signals or even damage the electrode surface integrity. Buffers are ideal diluents, which have a relatively stable pH similar to that of serum and other biological fluids (~7.4), and can be added without denaturing the biochemical components of the sample. Redox species and electroactive labels are added to this electrolytic buffer. In fluorimetric sensors, fluorescent labels are added to it. Common choices of buffers are phosphate buffered saline (PBS), HEPES buffer, Tris-HCl buffer, etc.

7 Methods for Biosensor Fabrication and Analyte Detection

Given below are two model antibody-based biosensors along with the stepwise preparation of sensor surface and analyte detection.

7.1 Graphene-Based Immunosensor for Rotavirus Detection

Liu et al. developed an electrochemical immunosensor with multilayered reduced graphene oxide (GO) film for transduction, $[Fe(CN)_6]^{3-/4-}$ redox system for signal generation and rotavirus surfaceprotein-specific antibodies for bio-recognition (Fig. 14) [43].

Preparation and detection:

1. GO solution was prepared using modified Hummer's method, and deposited in a free-standing multilayered film by centrifugal vacuum evaporation.
2. The GO film was reduced by subjecting it to thermal annealing at 900 °C in the presence of H_2/Ar flow for 10 h.

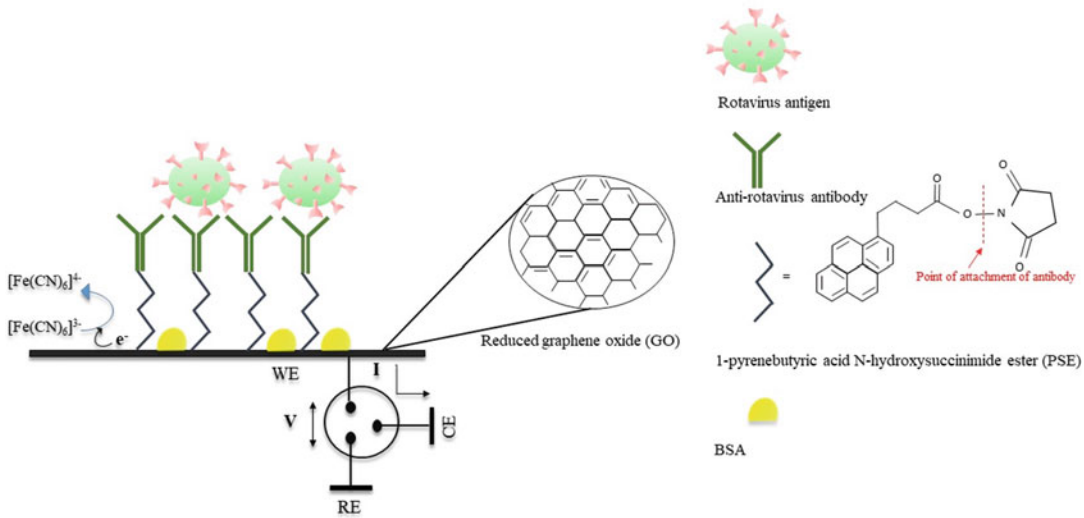


Fig. 14 Schematic for the graphene film-based immunosensor for rotavirus detection

3. The reduced GO film was cut and fixed on a polydimethylsiloxane (PDMS) chamber, with an exposed area of 4 mm^2, used as the working electrode. The reference and counter electrodes were Ag/AgCl and platinum wire electrodes, respectively.
4. 1-pyrenebutyric acid *N*-hydroxysuccinimide ester (PSE) was used as a linker. The pyrene ring of PSE is π-stacked on the reduced GO electrode.
5. The *N*-hydroxysuccinimide group catalyzes the formation of amide bond between PSE and rotavirus-specific antibody.
6. The free sites were blocked with BSA.
7. All fabrication steps were characterized with cyclic voltammetry (CV).
8. The prepared sensor was incubated with a PBS solution containing rotavirus, and the surface was subsequently washed.
9. Specifically bound rotavirus was quantified with CV at a scan rate of 50 mV/s in the presence of $K_3Fe(CN)_6$ in KCl solution.
10. Viral load (measured in plaque forming units or PFUs) was correlated to anodic peak current to obtain a standard curve, and the linear range, limit of detection and sensitivity were determined.

7.2 An Interdigited Gold Micro-Electrode Impedimetric Immunosensor for Label-Free Zika-Virus (ZIKV) Detection

Kaushik et al. developed an interdigited micro-electrode of gold arrays (IDE-Au) functionalized with dithiobis(succinimidyl propionate) (DTSP) for the covalent immobilization of anti-Zika virus envelope protein antibody (Zev-Abs) (Fig. 15) [44].

Preparation and detection:

1. IDE-Au was electrochemically cleaned and incubated with DTSP solution for 2 h for surface immobilization, followed by washing with DI water and drying at 4 °C.

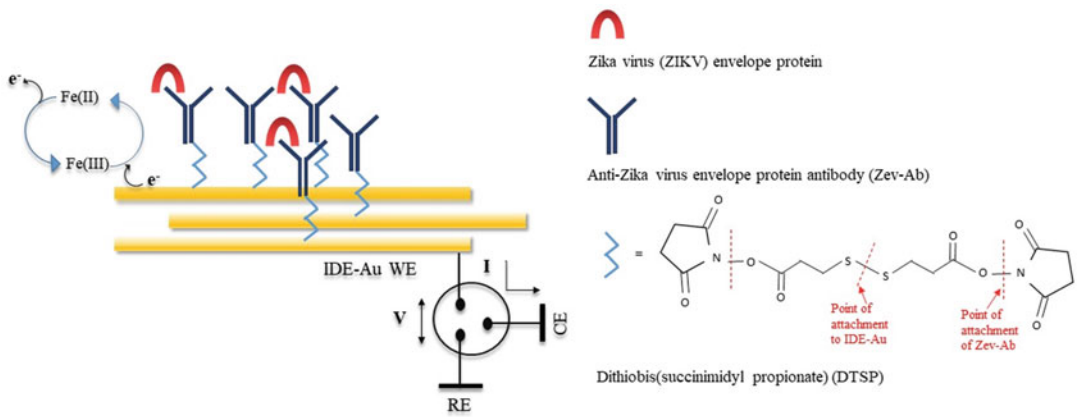


Fig. 15 Schematic of IDE-Au based electrochemical ZIKV immunosensor

2. The functionalized surface was incubated with monoclonal Zev-Abs for 2 h, leading to electrostatic immobilization of Zev-Abs.
3. The free surface was blocked with BSA, followed by washing with PBS, and storage at 4 °C.
4. All fabrication steps were characterized with electrochemical impedance spectroscopy (EIS).
5. The sensor was then incubated with ZIKV in PBS for 30 min, followed by washing with DI water.
6. The specifically bound ZIKV was quantified with EIS in the presence of the redox couple Fe(II)/Fe(III) in PBS.
7. ZIKV concentration was correlated with charge transfer resistance (R_{ct}) obtained from EIS, and the plot was used to estimate the linear range, limit of detection and sensitivity of the sensor.

References

1. Trier NH, Houen G (2020) Antibodies as diagnostic targets and as reagents for diagnostics. Antibodies (Basel) 9(2):15. https://doi.org/10.3390/antib9020015
2. Kohler G, Milstein C (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256:495–497
3. Lu R, Hwang Y, Liu I et al (2020) Development of therapeutic antibodies for the treatment of diseases. J Biomed Sci 27:1
4. Kyowa Hakko Kirin Co. Ltd. Consolidated Financial Summary (IFRS) Fiscal 2018. 2019, February 5
5. Basu K, Green EM, Cheng Y, Craik CS (2019) Why recombinant antibodies - benefits and applications. Curr Opin Biotechnol 60:153–158. https://doi.org/10.1016/j.copbio.2019.01.012
6. Gray AC, Sidhu SS, Chandrasekera PC et al (2016) Animal-friendly affinity reagents: replacing the needless in the haystack. Trends Biotechnol 34(12):960–969. https://doi.org/10.1016/j.tibtech.2016.05.017
7. Sharma S, Byrne H, O'Kennedy RJ (2016) Antibodies and antibody-derived analytical biosensors. Essays Biochem 60(1):9–18. https://doi.org/10.1042/EBC20150002
8. Schroeder HW Jr, Cavacini L (2010) Structure and function of immunoglobulins. J Allergy Clin Immunol 125:S41–S52. https://doi.org/10.1016/j.jaci.2009.09.046
9. Ma H, O'Kennedy R (2015) The structure of natural and recombinant antibodies. In: Houen G (ed) Peptide antibodies. Methods in molecular biology, vol 1348. Humana Press, New York. https://doi.org/10.1007/978-1-4939-2999-3_2
10. Weiner LM, Surana R, Wang S (2010) Monoclonal antibodies: versatile platforms for cancer immunotherapy. Nat Rev Immunol 10:317–327
11. Chiu ML, Goulet DR, Teplyakov A et al (2019) Antibody structure and function: the basis for engineering therapeutics. Antibodies 8(4):55. https://doi.org/10.3390/antib8040055
12. Reverberi R, Reverberi L (2007) Factors affecting the antigen-antibody reaction. Blood Transfus 5(4):227–240. https://doi.org/10.2450/2007.0047-07
13. Mollarasouli F, Kurbanoglu S, Ozkan SA (2019) The role of electrochemical Immunosensors in clinical analysis. Biosensors 9(3):86. https://doi.org/10.3390/bios9030086
14. Lim SA, Ahmed MU (2019) Chapter 1: Introduction to Immunosensors, Royal Society of Chemistry. In: Ahmed MU, Zourob M, Tamiya E (eds) Immunosensors, pp 1–20. https://doi.org/10.1039/9781788016162-00001
15. Clark LC Jr, Lyons C (1962) Electrode systems for continuous monitoring in cardiovascular surgery. Ann N Y Acad Sci 102:29–45. https://doi.org/10.1111/j.1749-6632.1962.tb13623.x

16. Yalow RS, Berson SA (1959) Assay of plasma insulin in human subjects by immunological methods. Nature 184(Suppl 21):1648–1649. https://doi.org/10.1038/1841648b0
17. Turner APF (2000) Biosensors—sense and sensitivity. Science 290(5495):1315–1317
18. Skottrup PD, Nicolaisen M, Justesen AF (2008) Towards on-site pathogen detection using antibody-based sensors. Biosens Bioelectron 24(3):339–348. https://doi.org/10.1016/j.bios.2008.06.045
19. Singh A (2017) Next generation immunoassays for infectious diseases of animals. Res Rev J Immunol 7(2):22–43
20. Cass T, Ligler FS (1998) Immobilized biomolecules in analysis: A practical approach. Oxford University Press, New York
21. Trilling AK, Beekwilder J, Zuilhof H (2013) Antibody orientation on biosensor surfaces: a minireview. Analyst 138:1619–1627
22. John R, Spencer M, Wallace GG et al (1991) Development of a polypyrrole-based human serum albumin sensor. Anal Chim Acta 249: 381–385
23. Feyssa B, Liedert C, Kivimaki L et al (2013) Patterned immobilization of antibodies within roll-to-roll hot embossed polymeric microfluidic channels. PLoS One 8:e68918
24. Jarocka U, Sawicka R, Góra-Sochacka A et al (2014) An immunosensor based on antibody binding fragments attached to gold nanoparticles for the detection of peptides derived from avian influenza hemagglutinin H5. Sensors 14: 15714–15728
25. Shriver-Lake LC, Donner B, Edelstein R et al (1997) Antibody immobilization using heterobifunctional crosslinkers. Biosens Bioelectron 12:1101–1106
26. de Juan-Franco E, Caruz A, Pedrajas JR et al (2013) Site-directed antibody immobilization using a protein A–gold binding domain fusion protein for enhanced SPR immunosensing. Analyst 138:2023–2031
27. Barton AC, Collyer SD, Davis Fet al. (2009) Labeless AC impedimetric antibody-based sensors with pg ml^{-1} sensitivities for point-of-care biomedical applications. Biosens Bioelectron 24:1090–1095
28. Ionescu RE, Gondran C, Bouffier L et al (2010) Label-free impedimetric immunosensor for sensitive detection of atrazine. Electrochim Acta 55:6228–6232
29. Yan H, Shen Z, Mernaugh R, Zeng X (2011) Single chain fragment variable recombinant antibody as a template for fc sensors. Anal Chem 83(2):625–630. https://doi.org/10.1021/ac102087w
30. Zeng X, Shen Z, Mernaugh R (2012) Recombinant antibodies and their use in biosensors. Anal Bioanal Chem 402(10):3027–3038. https://doi.org/10.1007/s00216-011-5569-z
31. Aydin EB, Aydin M, Sezgintürk MK (2019) Advances in electrochemical immunosensors. Adv Clin Chem 92:1–57
32. Holford TR, Davis F, Higson SP (2012) Recent trends in antibody based sensors. Biosens Bioelectron 34(1):12–24. https://doi.org/10.1016/j.bios.2011.10.023
33. Chai R, Yuan R, Chai Y et al (2008) Amperometric immunosensors based on layer-by-layer assembly of gold nanoparticles and methylene blue on thiourea modified glassy carbon electrode for determination of human chorionic gonadotrophin. Talanta 74(5):1330–1336. https://doi.org/10.1016/j.talanta.2007.08.046
34. Fu Y, Li P, Wang T et al (2010) Novel polymeric bionanocomposites with catalytic Pt nanoparticles label immobilized for high performance amperometric immunoassay. Biosens Bioelectron 25(7):1699–1704. https://doi.org/10.1016/j.bios.2009.12.010
35. Bataillard P, Gardies F, Jaffrezic-Renault N et al (1988) Direct detection of immunospecies by capacitance measurements. Anal Chem 60(21): 2374–2379. https://doi.org/10.1021/ac00172a011
36. de Moraes A, Kubota L (2016) Recent trends in field-effect transistors-based Immunosensors. Chemosensors 4(4):20. https://doi.org/10.3390/chemosensors4040020
37. González-Martínez MA, Puchades R, Maquieira A (2007) Optical immunosensors for environmental monitoring: how far have we come? Anal Bioanal Chem 387(1): 205–218. https://doi.org/10.1007/s00216-006-0849-8
38. Velusamy V, Arshak K, Korostynska O et al (2010) An overview of foodborne pathogen detection: in the perspective of biosensors. Biotechnol Adv 28(2):232–254. https://doi.org/10.1016/j.biotechadv.2009.12.004
39. Phillips KS, Cheng Q (2007) Recent advances in surface plasmon resonance based techniques for bioanalysis. Anal Bioanal Chem 387:1831–1840
40. Bunde RL, Jarvi EJ, Rosentreter JJ (1998) Piezoelectric quartz crystal biosensors. Talanta 46(6):1223–1236. https://doi.org/10.1016/s0039-9140(97)00392-5
41. Pohanka M (2018) Overview of piezoelectric biosensors, Immunosensors and DNA sensors

and their applications. Materials 11(3):448. https://doi.org/10.3390/ma11030448

42. Ramanathan K, Danielsson B (2001) Principles and applications of thermal biosensors. Biosens Bioelectron 16(6):417–423. https://doi.org/10.1016/s0956-5663(01)00124-5
43. Liu F, Choi KS, Park TJ, Lee SY, Seo TS (2011) Graphene-based electrochemical biosensor for pathogenic virus detection. BioChip J 5(2):123–128. https://doi.org/10.1007/s13206-011-5204-2
44. Kaushik A, Yndart A, Kumar S, Jayant RD, Vashist A, Brown AN, Li CZ, Nair M (2018) A sensitive electrochemical immunosensor for label-free detection of Zika-virus protein. Sci Rep 8(9700):1–5. https://doi.org/10.1038/s41598-018-28035-3

Chapter 13

Lateral Flow Assay for Diagnosis of Pig Viral Diseases

Aditya Prasad Sahoo and Rajib Deb

Abstract

Several infectious pig viral diseases of economic importance such as foot and mouth disease, Hog cholera, and African swine fever virus (ASFV) pose a serious threat to the swine industry worldwide. Control of these viral diseases is very challenging since there is no specific treatment available and this leads to huge financial loss to pig industries. Prevention of the viral diseases mostly rely on efficient control strategy, zoo-sanitary measures, and culling of infected and exposed animals. Successful implementation of disease control programme essentially require early detection viral infection. Detection of infection can be achieved either by detection of virus which include techniques such as virus isolation, fluorescent antibody test (FAT), detection of viral genome using PCR or RT-PCR or detection of antibody specific to the virus. The major bottleneck of these detection tests in implementing control measure is because of inherent nature of the test like time consuming, require well-equipped laboratories and personnel, delaying the disease diagnosis in remote areas. The lateral flow assay offers a rapid and simple assay which allow early detection of viral infection in resource and equipment limited small laboratories. This chapter describes the method for development of a lateral flow assay using gold nanoparticle probe for detection of viral antigen.

Key words Viral disease, Diagnosis, ELISA, Lateral flow assay, Gold nanoparticles

1 Introduction

Infectious viral diseases of pig such as foot and mouth disease, Hog cholera, African swine fever virus (ASFV), Porcine reproductive and respiratory syndrome (PRRS), and swine influenza are highly contagious and these diseases outbreaks incurs a huge economic loss to pig industry. Although few viral diseases have been eradicated from many developed countries such as USA, Australia, New Zealand, Canada, and few European countries, these diseases are still prevalent in resources limited underdeveloped countries. At present, there is no definitive antiviral treatment for viral disease is available, hence these diseases go unabated which leads to huge financial loss to pig industries. Prevention of the viral diseases mostly rely on efficient control strategy, zoo-sanitary measures, culling of infected and exposed animals. Rapid and early diagnosis of contagious viral

Rajib Deb et al. (eds.), *Protocols for the Diagnosis of Pig Viral Diseases*,
Springer Protocols Handbooks, https://doi.org/10.1007/978-1-0716-2043-4_13,

diseases of pig is crucial in timely implementation of control measures such as restriction of animal movement and quarantine measure to prevent the spread of disease. Detection of virus by virus isolation, direct fluorescent antibody test (FAT), and panel of monoclonal antibodies (mAbs) or viral nucleic acid by polymerase chain reaction (PCR) and reverse-transcription polymerase chain reaction (RT-PCR) in whole blood is the method of choice for detecting infected herds at an early stage of disease outbreak [1]. Serological methods such as enzyme-linked immunosorbent assays (ELISA), fluorescent antibody test (FAT), indirect fluorescent antibody test (IFAT), and complement fixation test (CFT) are valuable for monitoring sero-prevalence in herds [2]. The OIE-recommended tests for virus detection include virus isolation, fluorescent antibody test, and molecular biology techniques such as PCR and RT-PCR. However, these tests cannot be used in field condition for detection of viral diseases in pig farms as these tests are time consuming, expensive, and require dedicated cell culture facilities, well-equipped laboratories, and trained laboratory personnel. In addition, logistics for transportation of clinical samples from actual site of infection to these specialized laboratories is cumbersome and time consuming which delay the disease diagnosis process [3]. Hence, in order to overcome these shortcomings a user friendly, rapid, and economical diagnostic assay is needed for resources limited laboratories for screening clinical samples for disease. This would help immensely in improving disease control programs by assisting field veterinarians to make swift decision in implementing control procedures and effectively at actual site of virus incursion in suspected disease outbreak scenario, especially in disease endemic areas where first evidence of the disease is based only on clinical signs and symptoms.

Lateral flow assay (LFA) is a user friendly easy-to-use rapid diagnostic test which require no special training to perform the test. LFA belongs to category of rapid assays and proved its worth in detection of pathogens, hormones, heavy metals, toxicity, and adulterant detection. Common names used for LFA in different sectors are Lateral flow test, Lateral flow device (LFD), Lateral flow immunoassay (LFIA), Dipstick and Pen-side test. LFA is an immunochromatographic version of ELISA based on specific antigen-antibody interaction but unlike ELISA plate a membrane strip is used to coat reagents required in ELISA which makes this platform simple and rapid to perform. Usually LFA is visually interpreted hence mostly provide qualitative estimate of the analyte, i.e. merely screen the presence or absence of the virus or virus specific antibody in the clinical sample. The LFA can be designed for semi-quantitative estimation of analyte by applying a number of test lines with known concentration of capture antibody and the colour development is measured using a reflectometer or CCD camera. The intensity of colour development is then corelated with the

concentaion of analyte. Since LFA is an immunochromatographic form of ELISA, it can be designed in two formats similar to ELISA namely sandwich LFA and competitive LFA.

Successful development of LFA needs better signal-amplification strategies. Gold nanoparticles (GNP), colored latex beads, carbon nanoparticles, selenium, quantum dots, and enzymes are used as label to increase signal which in turn increases sensitivity of the test. The use of gold nanoparticles as label or probe in LFA is very successful and most widely used. GNP is widely used as a label because of its unique properties like easy to synthesize GNP of different size range, easy to functionalize, and strong light extinction [4]. They can be easily synthesized in sizes ranging from 1 nm to 200 nm using simple one-step aqueous procedures developed by Frens [5], and their surfaces can be modified with nearly any small molecule, polymer, peptide, oligonucleotide, protein, and DNA [6]. Their light extinctions are enormous and can be tuned across the visible and near infrared regions of the spectrum simply by changing particle size and shape. Conjugation of antibodies to the surface of GNP can be achieved by their interaction which can be noncovalent electrostatic interaction, hydrophobic interaction or covalent binding [7].

2 LFA Design and Principle

LFA comprises of five components, namely (1) sample pad, (2) conjugate pad, (3) membrane/solid phase, (4) absorption pad, and (5) test line and control line (Fig. 1).

1. Sample pad made up of cellulose or glass fiber to filter out the unwanted interfering materials present in sample and evenly distribution of the sample.
2. Conjugate pad made up of fiber glass or cross-linked silica to hold bioconjugate detector, i.e. GNP-Antibody complex till test is performed and more importantly able to release the bioconjugate detector once the test is performed.
3. Membrane/Solid phase made up of nitrocellulose membrane of various pore sizes ranging from 0.05 to 12 μm is used as a solid support for applying test line and control line. Pore size is crucial as it is correlated with speed of flow of reagents across the membrane and sensitivity of assay. For example, membrane with bigger pore size results in faster flow rate which leads to poor sensitivity. Like ELISA, blocking agents such as bovine serum albumin (BSA), skimmed milk powder, and casein are used to prevent the nonspecific binding of reagents to test line and control line. The membrane is pasted onto a backing material of polypropylene, polystyrene, or polyethylene.

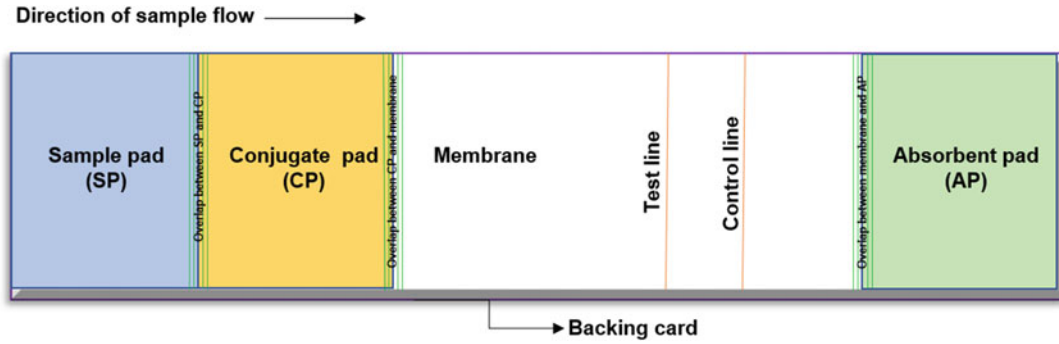


Fig. 1 Schematic representation of a typical LFA test strip

4. Absorbent pad made up of cellulose filter with high wetting capacity to absorb the excess fluid, sample or reagent at the end of LFA test strip.
5. Test line and control line contain primary antibody or capture antibody dried on the running surface membrane. The position of the test line and control line is very important since longer the distance between sample pad and test line better is the interaction between capture antibody and analyte leading to better sensitivity of LFA. Virus in the sample is captured and detected as it interacts with antibody immobilized at test line. The affinity of antibody affects the sensitivity of the test, hence proper antibody combination is crucial foe success of LFA. Polyclonal antibody is used as control line is dried at a distance 0.5–1 cm downstream the test line on the membrane. Control line evaluate the validity of the test since irrespective of presence or absence of virus it should produce a color line else the test is invalid.

The sample passes through the sample pad into the conjugate pad remobilize the already dried GNP-Antibody (secondary antibody) conjugates present on conjugate pad. If virus is present in the sample, it will bind to the GNP-Antibody to form GNP-Antibody-virus complexes which pass through the membrane due to capillary force and reach the test line where the primary antibody against virus is immobilized. This primary antibody binds to the GNP-Antibody-virus complex and produces a color line on test line due to GNP label. Anti IgG antibody immobilized on control line which can bind to secondary antibody with or without analyte bound to it and produce a color line. The control line should always appear as an indicator of a valid test. Excess sample will pass through the nitrocellulose membrane into the absorbent pad which absorb the excess sample.

3 Materials

Materials required for preparation of colloidal gold nanoparticles (GNP) and optimization of pH and concentration of IgG/antibody for bioconjugation with GNP.

1. Hydrogen tetrachloroaurate ($HAuCl_4$).
2. Trisodium citrate ($C_6H_5Na_3O_7{\cdot}2H_2O$).
3. Milli-Q water.
4. Magnetic stirrer with hot plate and magnetic bar.
5. K_2CO_3.
6. NaCl.
7. IgG.
8. 0.45-μm filters.
9. Spectrophotometer.

Materials required for development and assembly of the lateral flow device are as follows.

1. Sample pad.
2. Conjugate pad.
3. Membrane.
4. Backing card.
5. Reagent dispensing system to dispense the test and control line.
6. Centrifuge.
7. Spectrophotometer.
8. Capture antibodies.

4 Methods

4.1 Gold Nanoparticle Synthesis

Gold Nanoparticle can be synthesized in a one-step aqueous preparation in which hydrogentetrachloroaurate ($HAuCl_4$) is brought to boiling and reduced by rapid addition of trisodium citrate.

1. Prepare100 ml of 0.01% of $HAuCl_4$ solution and 2 ml of 1% trisodium citrate solution in Milli-Q water.
2. Heat the solution on a magnetic stirrer with hot plate up to 95 °C (avoid boiling of the solution) with vigorous stirring using a magnetic bar.
3. While heating quickly add 2 ml of trisodium citrate to the stirring solution. The color should change from a pale yellow to a wine-red color in 2–3 min.

4. Allow the solution to boil for another 10 min with vigorous stirring.
5. Finally, cool down the solution to room temperature and filter through 0.8 μm membrane filter.
6. Store the colloidal gold nanoparticle preparation at 4 °C till further use in a dark colored bottle.
7. Measure the size of GNP spectrophotometrically by scanning colloidal gold solution from 200- to 550-nm wave length.

4.1.1 Conjugation of mAb on GNP

Antibodies can nonspecifically adsorbed onto GNP by noncovalent electrostatic and hydrophobic interactions of the antibody and the gold surface. In addition, positively charged amino acids present in antibody form ionic bond with negatively charged citrate capped surface of the GNP [8].

4.2 Optimization of pH of the GNP and mAb Concentration for GNP-mAb Conjugation

4.2.1 Optimization of pH of GNP

1. Take 138 mg K_2CO_3 and add 100 ml of Milli-Q water then filter it with 0.45 μm filter.
2. Take 100 μl GNP in 9 tubes as depicted in the following table.

Tubes	1	2	3	4	5	6	7	8	9
pH of GNP	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0

3. Adjust pH of GNP from 5 to 9 by K_2CO_3.
4. Add 10 μg IgG to 100 μl GNP and mix properly.
5. Incubate for 15 min at room temperature.
6. Add 50 μl of 10% NaCl and incubate for 15 min.
7. Observe the color change.
8. Lowest pH of GNP which maintained wine-red color is the optimum pH of conjugation.

4.2.2 Optimization of mAb Concentration for GNP-mAb Conjugation

Optimization of mAb concentration for GNP-mAb conjugation is required to determine minimum antibody concentration necessary to stabilized GNP to avoid agglomeration. Optimization of working concentration of antigen and GNP conjugated mAb is crucial for successful development of LFA as excessive concentration of any one reagent adversely affect sensitivity of the test.

1. Take 1 ml GNP in 5 tubes.
2. Prepare series of mAb dilutions in 5 mM Borax buffer and add 1 μg, 5 μg, 10 μg, 20 μg, 50 μg IgG/ml GNP.

Tube No.	1	2	3	4	5
IgG conc.	1 μg	5 μg	10 μg	20 μg	50 μg

3. Incubate for 5–15 min at room temperature.
4. Add 50 μl of 10% NaCl to each tube.
5. Incubate for 2 h at room temperature.
6. Observe the change in colour of GNP after NaCl addition. The lowest concentration of mAb which prevent colour change is selected as the the minimum concentration of mAb required to protect GNP from flocculation.
7. Mix optimized concentration of mAb with GNP and stir at room temperature for 1 h to allow complete conjugation between mAb and surface of GNP by noncovalent and ionic interactions.
8. Centrifuge the complex at 12,000 × *g* for 30 min. Decant the supernatant and resuspend the pellet in Milli-Q water.

4.3 Preparation of Lateral Flow Assay

In order to detect virus in clinical sample LFA use monoclonal antibody (MAb) specific for an immunodominant epitope of virus like major capsid protein VP72 of ASFV and the structural protein E2 of CSFV immobilized as test line. In addition, a second MAb specific for another epitope of virus conjugated with GNP as the indicator system applied in conjugate pad. The control band contain recombinant protein A/G or polyclonal antibody IgG.

1. MAb specific for virus is used as the test line capture reagent. Dilute MAb to 1 mg/ml in Tris–HCl 20 mM buffer at pH 7.5 containing 5% sucrose and 0.1% sodium azide as preservative.
2. Polyclonal antibody IgG is used as control line capture reagent. Dilute IgG to 1 mg/ml in the same dilution as the test line capture reagent.
3. Dispense the test and control capture reagents at 1 μl/cm in two parallel lines on nitrocellulose membrane. Keep the distance between two lines at least 5 mm.
4. Dry the test line and control line for 5 min at 45 °C and store the membranes in a desiccator at room temperature.
5. Additionally, membranes can be blocked with PBS containing 1% BSA at room temperature for 5 min to avoid nonspecific binding.
6. Dispense the conjugate mixture (GNP-MAb) at a concentration of 0.2%, in a 25-mM phosphate buffer with 1% BSA onto the conjugate pad and dry for 30 min at 45 °C. Store the conjugate pad in a desiccator at room temperature under dry condition. (The conjugate pad should facilitate the release of label. For effective release of label from the conjugate pad the use of detergents (tween-20) and alcohols (methanol) is recommended in the running buffer).

7. Paste the nitrocellulose membrane, sample pad, conjugate pad, and absorbent pad on the plastic backing with adhesive. Arrange all the components of LFA strip in a manner that they overlap each other for uninterrupted capillary flow.
8. Cut the master card into strips of 4–5 mm width, which are placed individually in a plastic device. Store the LFA strips under dry conditions at 4 °C.
9. Dispense 10 μl of the sample onto the sample pad followed by 120 μl of running buffer (Tris–HCl pH 7.5, NaCl, casein, and NaN_3 as preservative), which allows the mixture to migrate through the conjugate pad and the nitrocellulose membrane by capillarity.

4.4 LFA Test Procedure

The LFA devices should be in dry conditions before test to avoid any negative effect on the result. Sample once applied to the sample pad it migrates through the conjugate pad and the nitrocellulose membrane by capillary force. Results are to be interpreted within the specified time limit after adding the sample. In the presence of virus, the major immunodominant protein of virus is captured by the mAb coated GNP, forming a GNP-mAb-virus immune complex. This immune complex then migrates across the membrane by capillary action and reacts with the immobilized mAb (specific for another epitope of virus) on the test line of membrane, making the test line visible. Irrespective of positive or negative sample control line must appears else the test is invalid.

5 Notes

1. Gold particle diameter can be tuned via citrate:$[AuCl_4]^{1-}$ stoichiometry, i.e. ratio of reducing agent trisodium citrate:tetrachloroauric acid ($HAuCl_4$) regulates gold nanoparticle diameter where citrate act as a reducing as well as capping agent. Gold nanoparticles size ranging from 12 to 147 nm can be achieved by changing the ratio of Na_3Citrate:$HAuCl_4$, i.e. higher the ratio, smaller the size of particles [9]. While preparing gold nanoparticle formation of a deep red color solution indicated the formation of spherical gold nanoparticles and the colors depend strongly on their size and shape [10].
2. The maximum absorbance of surface plasmon band (SPB) of gold nanoparticles is generally around 520 nm. The extinction maximum of SPB shifts from 518 nm to 533 nm when the particle means diameter changes from 10 nm to 48 nm and 20 nm diameter particles has peak absorption at 520 nm [9].

3. Larger size nanoparticle provides better sensitivity but stability of GNP decreases with the increase in size as it tends to aggregate. 40-nm GNP provide best result in terms of sensitivity, better flow through and high signal-to-noise ratio. The GNP-antibody bioconjugate has a high affinity leads to nonspecific binding with interfering molecules present in clinical samples. This problem can be solved by adding blocking agents such as BSA, skimmed milk powder directly to GNP to block nonspecific binding of antibody.
4. Several factors influence the performance of LFA like type of capture reagents, membrane (pore size and flow rate), and complexity of the sample. The capture reagents used in developing LFA influence the specificity and sensitivity of the assay.
5. Sufficient GNP-Antibody-virus should bind to primary antibody at test line to form a colored line at test line as well as sufficient secondary antibody at conjugate pad should be applied to bind with the antibody present at control line.
6. Primary antibody and secondary antibody dilutions must be optimized for successful development of LFA.
7. More complex samples such as muscle tissue and blood contain interfering background which have adverse impact on overall test performance. Hence, complex samples need pre-processing before the actual test to minimize interfering substances [11]. Migration speed of analyte and antibody-gold conjugate on membrane the test strip has the impact on sensitivity of assay. For effective immobilization of capture reagent on the membrane incubation temperature, time, volume of antibody immobilized on the test line must be optimized. Membrane drying temperature after applying test line and control line affects pore structures of membrane as high drying temperature results in smaller pores which in turns decreases the flow rate. Thus, while developing lateral flow assays, the membrane, sample pad, absorbent pad, conjugate pad, and capture antibodies should be very carefully chosen. For more sensitive assays, membranes with slow migration rate are better, whereas for those assays where sensitivity is not an issue but faster result is important, membranes with fast migration rates should be chosen.

References

1. Fernandez-Pinero J, Gallardo C, Elizalde M, Robles A, Gomez C, Bishop R, Heath L, Couacy-Hymann E, Fasina FO, Pelayo V et al (2013) Molecular diagnosis of African swine fever by a new real-time PCR using universal probe library. Transbound Emerg Dis 60(1): 48–58
2. Gallardo C, Nieto R, Soler A, Pelayo V, Fernandez-Pinero J, Markowska-Daniel I, Pridotkas G, Nurmoja I, Granta R, Simon A et al (2015) Assessment of African swine fever diagnostic techniques as a response to the epidemic outbreaks in eastern European union countries: how to improve surveillance and

control programs. J Clin Microbiol 53(8): 2555–2565

3. Oura CA, Edwards L, Batten CA (2013) Virological diagnosis of African swine fever–comparative study of available tests. Virus Res 173(1):150–158
4. Baudhuin P (1989) Colloidal gold: principles, methods, and applications, vol 2. Academic Press, New York, pp 1–17
5. Frens G (1973) Controlled nucleation for regulation of particle-size in monodisperse gold suspensions. Nat Phys Sci 241(105):20–22
6. Mirkin CA (1996) A DNA-based method for rationally assembling nanoparticles into macroscopic materials. Nature 382(6592):607–609
7. Milan RC, Marzan LML (2014) Gold nanoparticle conjugates: recent advances toward clinical applications. Expert Opin Drug Deliv 11(5):741–752
8. Rayavarapu RG, Petersen W, Ungureanu C, Post JN, Leeuwen TG, Manohar S (2007) Synthesis and bioconjugation of gold nanoparticles as potential molecular probes for light-based imaging techniques. Int J Biomed Imaging 2007:29817. https://doi.org/10.1155/2007/29817
9. Daniel MC, Astruc D (2004) Gold nanoparticles: assembly, supramolecular chemistry, quantum-size-related properties, and applications toward biology, catalysis, and nanotechnology. Chem Rev 104(1):293–346
10. Kelly KL, Coronado E, Zhao LL, Schatz GC (2003) The optical properties of metal nanoparticles: the influence of size, shape, and dielectric environment. J Phys Chem 107: 668–677
11. Lee JY, Kim YA, Kim MY, Lee YT, Hammock BD, Lee HS (2012) Importance of membrane selection in the development of immunochromatographic assays for low-molecular weight compounds. Anal Chim Acta 757:69–74

Chapter 14

Droplet Digital PCR-Based Diagnosis for Porcine Viral Diseases

Yoya Vashi and Sachin Kumar

Abstract

Polymerase chain reaction (PCR) is a common and indispensable technique that has been employed since its discovery for the diagnosis of infectious diseases. A biotechnological refinement of the conventional PCR led to the third generation of PCR, called the droplet digital polymerase chain reaction (ddPCR), that can be used to directly quantify and amplify nucleic acids. Presently, ddPCR is widely used in low-abundance nucleic acid detection and is useful in the diagnosis of infectious diseases. The distinctive feature of ddPCR is the separation of the reaction mixture into partitions, followed by a real-time or end-point detection of the amplification. As Poisson distribution describes the distribution of target sequences into partitions, it allows accurate and absolute quantification of the target from the ratio of positive against all partitions at the end of the reaction. ddPCR enables the absolute quantification of nucleic acids without the need to use reference materials with known target concentrations as used commonly in qPCR. A higher resilience to inhibitors in a number of different types of samples is an additional feature of ddPCR. ddPCR provides more sensitive, accurate and reproducible detection of low-abundance pathogens and definitely serves as a better choice than qPCR for clinical applications in the future.

Key words Diagnostics, Droplet digital polymerase chain reaction, Porcine, Viral pathogens

1 Introduction

Almost four decades later, polymerase chain reaction (PCR) still continues to be used as a standard and has become a ubiquitous laboratory tool. During the early 1990s, a concept of "limit dilution PCR" arose when researchers began exploring the possibility of diluting the template to an extent such that, on average, any single PCR reaction contained only a single template molecule. A key advantage of limit dilution PCR is that each DNA molecule is amplified separately, killing interferences between template molecules during PCR and greatly reducing background noise in complex samples. Additionally, when many reactions are performed at this level of dilution, the frequency of positive and negative reactions follows the Poisson distribution and therefore allows for

Rajib Deb et al. (eds.), *Protocols for the Diagnosis of Pig Viral Diseases*,
Springer Protocols Handbooks, https://doi.org/10.1007/978-1-0716-2043-4_14,

calculating the abundance of the target molecule based on the dilution factor. The concept of digital PCR was first described in 1992 [1] using the principles of limiting dilution, PCR, and Poisson statistics. The term "digital PCR" was first used in the 1999 paper by Kinzler and Vogelstein [2] in which they described the quantitation of *ras* mutations in a sample by partitioning the sample in order to perform a series of PCRs in 384 well microplates. Later, the scientific community recognized key advantages of digital PCR over traditional end-point or real-time PCR. The key advantages offered by digital PCR were that it does not rely on a standard curve, had improved accuracy, provides absolute quantification and offered improved detection of low copy-numbers nucleic acids. Other key advantages of digital PCR became evident later, as the method was used more broadly [3]: repeatability of assays over time and across different labs; robustness and tolerance to PCR inhibitors.

Researchers and equipment makers have also made more radical modifications to PCR by borrowing technologies from other fields. One such effort yielded droplet digital PCR (ddPCR), which combines aspects of fluorescence-activated cell sorting with conventional PCR. Though the protocol for ddPCR is somewhat complicated, it has also become highly automated. The ddPCR is a method of dPCR in which a 20 μl sample reaction including assay primers and either Taqman probes or an intercalating dye, is divided into ~20,000 nanoliter-sized oil droplets through a water-oil emulsion technique, thermocycled to end-point in a 96-well PCR plate, and fluorescence amplitude read for all droplets in each sample well in a droplet flow cytometer [4].

ddPCR is a recent technology that has become commercially available since 2011 [5]. As with qPCR, ddPCR technology utilizes Taq polymerase in a standard PCR reaction to amplify a target DNA fragment from a complex sample using pre-validated primer or primer/probe assays. However, there are two distinct differences: (1) the partitioning of the PCR reaction into thousands of individual reaction vessels prior to amplification and (2) the acquisition of data at the reaction end-point (Fig. 1). These factors offer the advantage of direct and independent quantification of DNA without standard curves giving more precise and reproducible data versus qPCR, especially in the presence of sample contaminants that can partially inhibit Taq polymerase and/or primer annealing [7, 8]. In addition, end-point measurement enables nucleic acid quantitation independently of the reaction efficiency, resulting in a positive–negative call for every droplet and greater amenability to multiplexed detection of target molecules [9]. Thereby, ddPCR technology can be used for extremely low-target quantitation from variably contaminated samples where the sample dilution requirements to assure consistent and acceptable reaction efficiency,

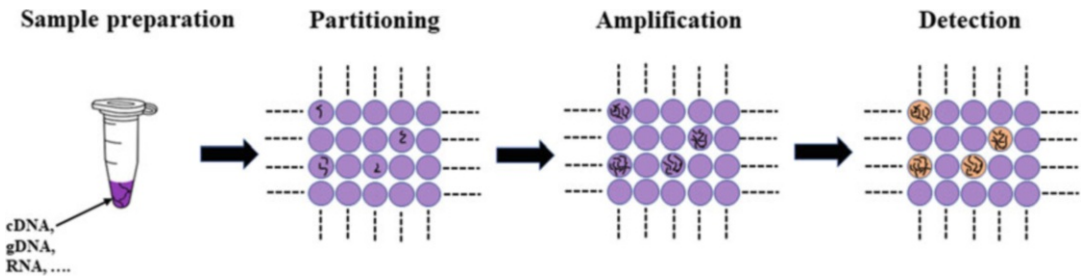


Fig. 1 Principles of digital PCR. The sample is divided into many independent partitions such that each contains either a few or no target sequences. The distribution of target sequences in the partitions can be approximated with a Poisson's distribution. Each partition acts as an individual PCR microreactor and partitions containing amplified target sequences are detected by fluorescence. The ratio of positive partitions (presence of fluorescence) over the total number allows determining the concentration of the target in the sample. (Reproduced from [6])

primer annealing and C_q values for qPCR would likely lead to undetectable target levels [7, 10].

Some of the major applications of ddPCR include absolute quantification, detection of genomic alteration such as gene copy number variation (CNV), detection of rare sequences, gene expression and microRNA analysis, single-cell analysis, etc.

2 ddPCR Experimental Workflow

In this section, we focus on the ddPCR workflow for viral pathogen (DNA or RNA) quantification using the Bio-Rad QX100 (or QX200) system.

2.1 Preparation of Reaction Mixture

The concentrations of primers and probe for each assay with the corresponding 2× ddPCR master mix should be mixed in nuclease-free tubes. Concentrations of primers and probe should be preferably optimized previously (*See* **Note 1**). The preparation of duplex or multiplex assays is also possible. It is to be noted that manganese acetate needs to be added in the case of the one-step master mix. The final reaction volume should be planned to 20 μl. Before adding the sample, the prepared mix should be distributed into nuclease-free tubes, strips, or 96-well plates. Sample (DNA/RNA samples and controls, *see* Tables 1 and 2) should be added into each tube containing master mixes and mixed thoroughly by pipetting, followed by brief centrifugation (*See* **Note 2**). Each tube should contain 20 μl of the reaction mixture.

2.2 Droplet Generation

A DG8™ droplet generation cartridge is placed into the cartridge holder. To each of the 8 wells indicated as "sample" in the droplet generation cartridge, 20 μl of each prepared reaction mixture is

Table 1
Reaction setup for DNA amplification in ddPCR

Component	Volume (μl)	Final concentration
2× ddPCR super mix for probes (*See* **Note 3**)	10	1×
20× target primers/probe (FAM)	1	1× (i.e., 900 nM/150 nM)
20× second target primers/probe (VIC)	1	1× (i.e., 900 nM/150 nM)
Nuclease-free water	Variable	–
DNA sample	Variable	50 fg to 100 ng
Final volume	20	–

Table 2
Reaction setup for RNA amplification in one step RT-ddPCR

Component	Volume (μl)	Final concentration
2× ddPCR super mix for probes	10	1×
Manganese acetate	0.8	1× (i.e., 900 nM/250 nM)
20× target primers/probe (FAM)	1	1× (i.e., 900 nM/150 nM)
20× second target primers/probe (VIC)	1	1× (i.e., 900 nM/150 nM)
Nuclease-free water	Variable	–
RNA sample	Variable	50 fg to 100 ng
Final volume	20	–

transferred (*See* **Note 4**). Precautions should be taken not to form bubbles in the bottom of the well, as they could interfere with the droplet formation. In the wells indicated as "oil," 70 μl of droplet generation oil is added. The oil bottle should not be left open for extended periods of time to avoid evaporation and stability of components. The gasket is then hooked over the cartridge holder using the holes in both sides and the holder is placed with the cartridge in the QX100 droplet generator unit, initiating the droplet generation (*See* **Notes 5** and **6**). Oil and sample are pushed through microfluidic channels and mixed in the cartridge in the process, forming droplets. Droplets are accumulated in the droplet well. The process takes 2–3 min for each cartridge. Once droplets are generated, the gasket is removed and 40 μl of the droplet suspension ("droplets" lane in the cartridge) is transferred from the cartridge to a 96-well PCR plate. The pipetting both for collecting the droplet suspension and for dispensing it in the PCR plate wells should be slow to protect the integrity of the droplets. After all the samples have gone through droplet generation and have been transferred to the 96-well PCR plate, the plate is heat-sealed with a pierceable foil.

Table 3
PCR cycling conditions

	DNA samples			RNA samples		
Step	***T*** **(°C)**	**Time**	**Cycle**	***T*** **(°C)**	**Time**	**Cycle**
RT	–	–	–	60	30 min	Hold
Enzyme activation	95	10 min	Hold	95	5 min	Hold
Denaturation Annealing and extension	94 60	30 s 1 min	40	94 60	30 s 1 min	40
Heat deactivation	98	10 min	Hold	98	10 min	
Hold	4	∞	Hold	4	∞	Hold

2.3 PCR Amplification

The sealed plate is transferred to the thermocycler and the PCR is run as per the conditions shown in Table 3.

2.4 Droplet Reading and Data Analysis

Once the PCR is over, the PCR plate is transferred to the QX100 droplet reader. In the QuantaSoft software (Bio-Rad), the "Setup" button is clicked and the information for each well/sample, including name, type of experiment, type of sample and detectors or channels (FAM and/or VIC), is defined. The reading is started by clicking the "Run" button. The droplet reader acts as a flow cytometer and reads each droplet to determine their signal and amplitude in the selected detectors. Once the run is over, click "Analyze" to start analyzing the results. The critical point is to set a threshold that allows the software to differentiate between negative and positive droplets. The software offers the possibility of defining this automatically or manually. However, other approaches to analyses are available and may, in specific cases, be more suitable (See **Note 7**). The software offers different ways of viewing the results (1D amplitude of one channel, 2D amplitudes of both channels, copy number in each well/channel) that are more or less informative depending on the type of experiment. It ultimately gives a table with parameters resulting from the analysis, such as the concentration of target copies/microliter of reaction, the number of total accepted droplets, the positive ones, and the negative ones.

3 Notes

1. The widely accepted quantitative PCR (qPCR) design guidelines also apply to ddPCR primer design. Some of the important guidelines include the primers to have a GC content of 50–60%, avoiding repeats of Gs or Cs longer than 3 bases and

ensuring that no 3′ complementarity exists. It is to be noted that the QX100™ Droplet Digital PCR system (Bio-Rad) is compatible only with TaqMan hydrolysis probes and QX100™ system is compatible with TaqMan hydrolysis probes and EvaGreen® double-stranded DNA binding dye. The advantage of using hydrolysis probes includes high specificity, a high signal-to-noise ratio, and the ability to perform multiplex reactions. Few considerations during designing probes include choosing the probe sequence between the two primers of the amplicon, higher T_m than that of the primers, probe length of <30 nucleotides, etc.

2. For RNA or DNA isolation, same protocols or kits as those used for qPCR can be used for ddPCR. The higher resilience of ddPCR to inhibitors reported in several recent studies [7, 11–13] allow the use of a broader range of nucleic acid purification methods, including more simple extraction protocols.
3. At present, the Bio-Rad platform requires the use of their proprietary master mixes enabling stable droplets formation, while platforms based on partitioning into chambers allow for testing different commercially available PCR mixes.
4. The highest accuracy when pipetting is required. In addition, when handling the droplets, the pipetting should be done very carefully to preserve their integrity.
5. The QX100 and QX200 systems allow duplexing with fluorescence data acquisition in both the FAM and HEX-VIC spectral regions, respectively. There is an increasing number of studies where multiplexing with ddPCR has been reported [11, 14–16].
6. The process of droplet generation is repeated as many times as required by the sample number. When longer preparation times are expected due to the high number of samples, it is recommended to keep both samples and droplet suspensions at 4 °C using refrigerated blocks. In the case that the droplet reader is unavailable after PCR cycling, the plate can be stored at 4 °C for several hours or even overnight.
7. The variation of signals depends on many factors, e.g., assay characteristics [17], matrix or presence of inhibitors [12], single nucleotide polymorphisms (SNP) in the probe annealing region and others. All these factors can affect the separation between negative and positive droplets in different ways, resulting for example, in the presence of higher signal droplets in negative samples or in the induction of the so-called droplet rain effect. Therefore, in certain cases, automatic analysis can sometimes be misleading, and setting the threshold manually can be necessary. Manual definition of the threshold can also help overcoming other droplet particularities, such as rain

effect. Both in diagnostic and quantitative applications, it is important to evaluate the signal amplitude obtained with negative samples (NTC, isolation controls, matrix controls, closely related pathogens). The threshold can be then manually defined to consider such signals as negative.

4 ddPCR for Porcine Viruses

During the last decades, effective treatments, vaccination, and improved diagnostics have resulted in a reduction in the direct burden of infectious diseases on livestock production [18]. Nevertheless, emerging and re-emerging swine viruses are an emerging threat already ravaging the pig producers worldwide. An early field diagnosis is considered the first line of defense and plays an important role in the treatment of viral infection. However, to date, it can take up to several weeks or months from the time between initial disease outbreak and laboratory confirmation of the viral pathogen. To further prevent the spreading of the disease, reliable, quick, and simple diagnostic testing is crucial so that targeted control strategies can be implemented. Currently, rapid diagnostics systems rely on nucleic acid extraction, purification, and PCR-based amplification and detection. PCR amplification methods are very sensitive but often produce false positives from trace contamination of the specimen/equipment.

The ddPCR is the third generation of PCR technology, which enables absolute quantification of nucleic acid targets without the need to construct a calibration curve as used commonly in qPCR [5, 19]. Therefore, this method has highly precise and sensitive, which is rapidly replacing qPCR as an efficient method for independent nucleic acid quantifications. ddPCR has been used to detect low-abundance nucleic acids in many laboratories and is proving itself to be a powerful tool in detecting the swine viral pathogens. ddPCR has been used to detect major viral pathogens of swine like African swine fever virus [20], porcine circovirus type 2 and 3 [21–23], porcine reproductive and respiratory syndrome virus [24], porcine epidemic diarrhea virus [25], Senecavirus A [26, 27], pseudorabies virus [28], Japanese encephalitis virus [29], and hepatitis E virus [30]. In a few cases, the detection limit for ddPCR was 1 copy/μl, which was about 10 times greater sensitivity than TaqMan real-time PCR [22].

The ddPCR has become an emerging tool for the diagnosis of porcine viral pathogens. The method overrides the variation of amplification with a high level of reliability and reproducibility. Development of ddPCR for more porcine viral pathogens known to affect the swine industry will surely help in the establishment of reliable countermeasures to disease outbreaks and will limit severe animal's health and socio-economic consequences.

5 Conclusion

Nonetheless, researchers worldwide continue to push the capabilities of PCR and find ways to propel it into new territories. The advantages of ddPCR lie in many aspects. Many studies have shown that ddPCR is technically more accurate and sensitive. Also, during disease diagnosis, a faster readout is provided by PCR-based techniques as compared to serological or pathogen culturing tests, and ddPCR overcomes the problems in the accuracy of qPCR, which makes it more suitable as a diagnostic tool. The ddPCR is now a potential diagnostic method for porcine viral pathogens.

References

1. Sykes PJ, Neoh SH, Brisco MJ, Hughes E, Condon J, Morley AA (1992) Quantitation of targets for PCR by use of limiting dilution. BioTechniques 13(3):444–449
2. Vogelstein B, Kinzler KW (1999) Digital PCR. Proc Natl Acad Sci U S A 96(16):9236–9241
3. Kuypers J, Jerome KR (2017) Applications of digital PCR for clinical microbiology. J Clin Microbiol 55(6):1621–1628
4. Wood-Bouwens CM, Ji HP (2018) Single color multiplexed ddPCR copy number measurements and single nucleotide variant genotyping. Methods Mol Biol 1768:323–333
5. Hindson BJ, Ness KD, Masquelier DA, Belgrader P, Heredia NJ, Makarewicz AJ, Bright IJ, Lucero MY, Hiddessen AL, Legler TC, Kitano TK, Hodel MR, Petersen JF, Wyatt PW, Steenblock ER, Shah PH, Bousse LJ, Troup CB, Mellen JC, Wittmann DK, Erndt NG, Cauley TH, Koehler RT, So AP, Dube S, Rose KA, Montesclaros L, Wang S, Stumbo DP, Hodges SP, Romine S, Milanovich FP, White HE, Regan JF, Karlin-Neumann GA, Hindson CM, Saxonov S, Colston BW (2011) High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Anal Chem 83(22):8604–8610
6. Quan PL, Sauzade M, Brouzes E (2018) dPCR: a technology review. Sensors (Basel) 18(4):1271
7. Racki N, Dreo T, Gutierrez-Aguirre I, Blejec A, Ravnikar M (2014a) Reverse transcriptase droplet digital PCR shows high resilience to PCR inhibitors from plant, soil and water samples. Plant Methods 10(1):42
8. Taylor SC, Carbonneau J, Shelton DN, Boivin G (2015) Optimization of droplet digital PCR from RNA and DNA extracts with direct comparison to RT-qPCR: clinical implications for quantification of oseltamivir-resistant subpopulations. J Virol Methods 224:58–66
9. Hughesman CB, Lu XJ, Liu KY, Zhu Y, Poh CF, Haynes C (2016) A robust protocol for using multiplexed droplet digital PCR to quantify somatic copy number alterations in clinical tissue specimens. PLoS One 11(8):e0161274
10. Verhaegen B, De Reu K, De Zutter L, Verstraete K, Heyndrickx M, Van Coillie E (2016) Comparison of droplet digital PCR and qPCR for the quantification of Shiga toxin-producing Escherichia coli in bovine feces. Toxins (Basel) 8(5):157
11. Morisset D, Stebih D, Milavec M, Gruden K, Zel J (2013) Quantitative analysis of food and feed samples with droplet digital PCR. PLoS One 8(5):e62583
12. Racki N, Morisset D, Gutierrez-Aguirre I, Ravnikar M (2014b) One-step RT-droplet digital PCR: a breakthrough in the quantification of waterborne RNA viruses. Anal Bioanal Chem 406(3):661–667
13. Yang R, Paparini A, Monis P, Ryan U (2014) Comparison of next-generation droplet digital PCR (ddPCR) with quantitative PCR (qPCR) for enumeration of cryptosporidium oocysts in faecal samples. Int J Parasitol 44(14): 1105–1113
14. Kelley K, Cosman A, Belgrader P, Chapman B, Sullivan DC (2013) Detection of methicillin-resistant Staphylococcus aureus by a duplex droplet digital PCR assay. J Clin Microbiol 51(7):2033–2039
15. Leibovitch EC, Brunetto GS, Caruso B, Fenton K, Ohayon J, Reich DS, Jacobson S (2014) Coinfection of human herpesviruses 6A (HHV-6A) and HHV-6B as demonstrated by novel digital droplet PCR assay. PLoS One 9(3):e92328

16. Sedlak RH, Kuypers J, Jerome KR (2014) A multiplexed droplet digital PCR assay performs better than qPCR on inhibition prone samples. Diagn Microbiol Infect Dis 80(4):285–286
17. Dreo T, Pirc M, Ramsak Z, Pavsic J, Milavec M, Zel J, Gruden K (2014) Optimising droplet digital PCR analysis approaches for detection and quantification of bacteria: a case study of fire blight and potato brown rot. Anal Bioanal Chem 406(26):6513–6528
18. Perry BD, Grace D, Sones K (2013) Current drivers and future directions of global livestock disease dynamics. Proc Natl Acad Sci U S A 110(52):20871–20877
19. Pinheiro LB, Coleman VA, Hindson CM, Herrmann J, Hindson BJ, Bhat S, Emslie KR (2012) Evaluation of a droplet digital polymerase chain reaction format for DNA copy number quantification. Anal Chem 84(2): 1003–1011
20. Wu X, Xiao L, Lin H, Chen S, Yang M, An W, Wang Y, Yang Z, Yao X, Tang Z (2018) Development and application of a droplet digital polymerase chain reaction (ddPCR) for detection and investigation of African swine fever virus. Can J Vet Res 82(1):70–74
21. Liu Y, Meng H, Shi L, Li L (2019a) Development of a droplet digital polymerase chain reaction for sensitive and simultaneous identification of porcine circovirus type 2 and 3. J Virol Methods 270:34–37
22. Liu Y, Meng H, Shi L, Li L (2019b) Sensitive detection of porcine circovirus 3 by droplet digital PCR. J Vet Diagn Investig 31(4): 604–607
23. Zhao S, Lin H, Chen S, Yang M, Yan Q, Wen C, Hao Z, Yan Y, Sun Y, Hu J, Chen Z, Xi L (2015) Sensitive detection of porcine circovirus-2 by droplet digital polymerase chain reaction. J Vet Diagn Investig 27(6): 784–788
24. Yang Q, Xi J, Chen X, Hu S, Chen N, Qiao S, Wan S, Bao D (2017) The development of a sensitive droplet digital PCR for quantitative detection of porcine reproductive and respiratory syndrome virus. Int J Biol Macromol 104 (Pt a):1223–1228
25. Cao WW, He DS, Chen ZJ, Zuo YZ, Chen X, Chang YL, Zhang ZG, Ye L, Shi L (2020) Development of a droplet digital PCR for detection and quantification of porcine epidemic diarrhea virus. J Vet Diagn Investig 32(4):572–576
26. Pinheiro-de-Oliveira TF, Fonseca-Junior AA, Camargos MF, Laguardia-Nascimento M, Giannattasio-Ferraz S, Cottorello ACP, de Oliveira AM, Goes-Neto A, Barbosa-Stancioli EF (2019) Reverse transcriptase droplet digital PCR to identify the emerging vesicular virus Senecavirus a in biological samples. Transbound Emerg Dis 66(3):1360–1369
27. Zhang Z, Zhang Y, Lin X, Chen Z, Wu S (2019) Development of a novel reverse transcription droplet digital PCR assay for the sensitive detection of Senecavirus a. Transbound Emerg Dis 66(1):517–525
28. Ren M, Lin H, Chen S, Yang M, An W, Wang Y, Xue C, Sun Y, Yan Y, Hu J (2018) Detection of pseudorabies virus by duplex droplet digital PCR assay. J Vet Diagn Investig 30(1):105–112
29. Wu X, Lin H, Chen S, Xiao L, Yang M, An W, Wang Y, Yao X, Yang Z (2017) Development and application of a reverse transcriptase droplet digital PCR (RT-ddPCR) for sensitive and rapid detection of Japanese encephalitis virus. J Virol Methods 248:166–171
30. Mykytczuk O, Harlow J, Bidawid S, Corneau N, Nasheri N (2017) Prevalence and molecular characterization of the hepatitis E virus in retail pork products marketed in Canada. Food Environ Virol 9(2):208–218

Chapter 15

Protocols for Immunofluorescence Techniques

Deepika Bisht, Shikha Saxena, Nitish Singh Kharayat, and Siddharth Gautam

Abstract

Since the initial description of immunofluorescence (IF) assay by Coons, Creech, and Jones in 1941, it has evolved from being an academic technique to a routine laboratory investigative practice. Immunofluorescence technique is a very useful tool to detect protein expression and localize cellular as well as viral antigen (in case of virus infected cell). Immunofluorescence technique detects the target optically using fluorophore conjugated-antibodies. In this chapter, we have described various types of IF techniques, the advantages and limitations associated with each variant. Here we also describe the protocol for performing immunofluorescence technique along with the important points to note and alternative ways for each step involved while performing the IF assay.

Key words Immunofluorescence, Fluorophore, Antibody, Antigen, Fixatives, Permeabilizers

1 Introduction

Protein expression in a biological sample of cellular origin can be evaluated by various techniques such as enzyme linked immunosorbent assay (ELISA) and western blotting, etc. [1, 2]. However, the inability of cellular and subcellular localization of the protein is the most significant limitation of these techniques. Immunohistochemistry (IHC) assays are generally performed to localize the protein; however, Immunofluorescence technique is preferred, when there is a need for co-localization of proteins. Immunofluorescence (IF) technique enables us to visualize and localize the protein in the cells or tissues using antibodies conjugated to a fluorophore [3]. The antigen–antibody interaction and fluorescent signal produced by the conjugated fluorophore on excitation with suitable Ultraviolet (UV) light forms the base of immunofluorescence technique [4]. The fluorophore absorbs UV light in a defined wavelength range and emits the light of higher wavelength. This emitted light is detected by the detector of fluorescent microscope,

Rajib Deb et al. (eds.), *Protocols for the Diagnosis of Pig Viral Diseases*,
Springer Protocols Handbooks, https://doi.org/10.1007/978-1-0716-2043-4_15,

Table 1
Commonly used fluorophores: excitation and emission wavelengths

Fluorophore	Excitation wavelength (nm)	Emission wavelength (nm)
Fluorescein isothiocyanate	492	518
Texas Red	568	590
Rhodamine	555	620
B-Phycoerythrin	546	575
R-Phycoerythrin	490	575

equipped with specific filter for the wavelength of emitted light. Selection of fluorophore to be used in IF imaging is critical to obtain good quality immunofluorescence images (*see* **Note 1**). Some of the most frequently used fluorophores are summarized in Table 1.

Albert H. Coons and his colleagues first described IF, while reporting that antigens in mammalian tissues were detectable optically in ultraviolet (UV) light with a fluorophore (fluorescein isocyanate) conjugated antibody [5, 6]. Various studies have shown that the IF technique is a useful tool in studying bacterial and viral proteins [7–9]. Immunofluorescence technique possesses great value in virology for identification of viral antigens in virus-infected cells [10]. Rapid viral diagnosis using IF staining was first described by Liu in 1956 [11] for the detection of influenza virus. Later, this virus detection system was pioneered by Gardner and McQuillan for other viruses [12]. The detection of viral proteins in the cell indicates active replication, as opposed to latent viral infections. For detecting viral proteins, the fluorophore is conjugated either to the primary/antiviral antibody itself (direct immunofluorescence) or to the anti-antibody/secondary antibody (indirect immunofluorescence), and visualized using ultraviolet light. The type of primary antibody (monoclonal/polyclonal) used depends upon the amount of viral target protein expected in the sample (sensitivity) and cross-reactivity of the antibody with non-target proteins of the host or unrelated pathogen (specificity). The major advantage of immunofluorescence is its ability to localize protein of interest. Another advantage is that it requires lesser time and resources than isolation of virus in cell culture.

Different variants of IF assay, namely, direct immunofluorescence, indirect immunofluorescence, and multicolor immunofluorescence, have their own advantages and disadvantages. Before initiating the immunofluorescence protocol, these advantages/disadvantages should be considered.

1.1 Direct Immunofluorescence

In this protocol, the fluorophore is directly conjugated to the primary antibody for target antigen. Simple protocol and shorter incubation time are few advantages of direct IF. For visualizing multiple proteins, each of the antibody should be tagged with distinct/different fluorophores. The antibodies developed in the same species are compatible and do not pose a problem. Low staining intensity due to the lack of signal amplification phenomena and requirement of tagging fluorophore to each primary antibody are its major limitations.

1.2 Indirect Immunofluorescence

In this protocol, secondary antibodies are conjugated with fluorophore. These secondary antibodies are specific to the unlabelled primary antibodies. As multiple secondary antibodies bind to the target bound primary antibody, indirect IF protocol leads to signal amplification. This phenomenon becomes more desirable while dealing with low abundance targets. Secondary antibodies are able to recognize all primary antibodies derived from a target host species. This improves the flexibility and cost-effectiveness of the protocol. However, while visualizing multiple antigens, the primary antibodies need to be raised in distinct species to prevent cross reactivity. Major limitation of this protocol is high background interfering with fluorescence imaging, when used against proteins in abundance.

The difference of direct and indirect IF is summarized in Fig. 1.

1.3 Multicolor Immunofluorescence

Different fluorophores possess specific excitation and emission wavelength spectra, multiple antigens can be localized on the same sample by conjugating their respective antibodies to different fluorophores with non-overlapping excitation and emission spectra. This form of immunofluorescence is known as Multicolor immunofluorescence [13].

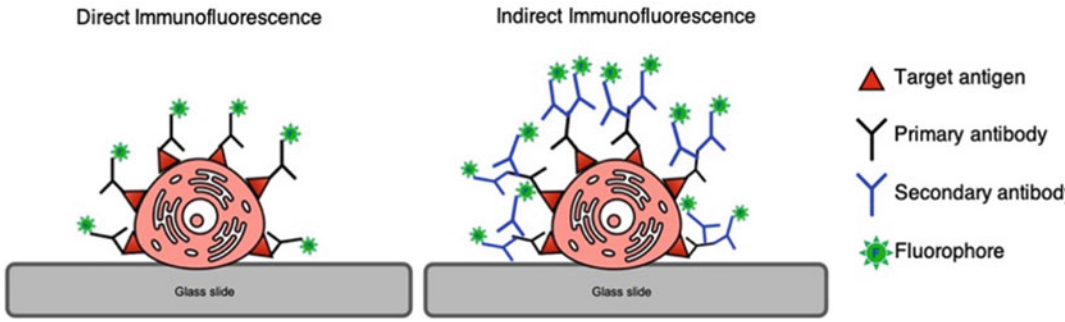


Fig. 1 Direct and Indirect Immunofluorescence. In case of direct immunofluorescence, fluorophore is conjugated to antibody binding to target antigen. Indirect IF protocol involves detection of target antigen, utilizing fluorophore conjugated secondary antibody bound to unlabelled primary antibody specific to target antigen. Signal amplification is seen in case of indirect IF

2 Materials

1. **Phosphate-buffered saline (1×)**: 137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl in double distilled water (pH 7.4).
2. **Fixative solutions (Paraformaldehyde solution)**: 4% Paraformaldehyde (w/v) in PBS (*see* **Note 5**).
3. **Permeabilizer** (0.4% Triton X-100):0.4% (v/v) Triton X-100 in PBS.
4. **Blocking buffer**: 5% (v/v) normal goat serum, 0.05% (v/v) Triton X-100 in PBS.
5. **Antibody dilution buffer**: 1% (v/v) normal serum, 0.05% (v/v) Triton X-100 in PBS.
6. **Primary Antibody**.
7. **Secondary antibody** (FITC conjugated).
8. **Counterstaining stock solution**, DAPI (4′,6-diamidino-2-phenylindole) (5 mg/mL stock): 14.3 mM of DAPI dihydrochloride in DMF. It is diluted to 300 nM in PBS before use.
9. **Mounting medium**: 50% (v/v) glycerol in PBS (*see* **Note 13**).

3 Method

In general, immunofluorescence technique using a single fluorophore conjugated antibody involves the following steps:

1. Sample Preparation.
2. Fixation of cells.
3. Permeabilization of cell membrane.
4. Blocking.
5. Incubation with primary antibody.
6. Incubation with secondary antibody.
7. Mounting.
8. Imaging.
9. Post-imaging analysis.

3.1 Sample Preparation

Immunofluorescence can be performed on adherent cell culture, suspension cell culture as well as on a tissue sample. The main objective of this step is to promote adherence of the specimen to a solid substrate that is optically suitable for imaging at the end of the IF assay.

3.1.1 In Case of Adherent Culture

1. The cells can be cultured on coverslips, kept inside sterile multi-well tissue culture plate or petri-dish (*see* **Note 2**). The sterilization of coverslip can be done by keeping it under UV light in biosafety cabinet for 30 min (*see* **Note 3**).
2. On day 1, the cells are seeded generally at low confluency (cell density approximately 10,000/cm^2) in sufficient volume of cell culture medium (e.g. 500 μL for a well of four well cell culture plate) for immunofluorescence assay.
3. Next day, the cultured cells are washed thrice with 1× phosphate-buffered saline (PBS) to remove cell culture media, unattached dead cells, and debris before fixation.

3.1.2 Suspension Cell Culture

1. Cells grown in suspension can be coated on a slide using the cytospin technique.
2. Approximately 150–200 μL of cells suspension in (1×) PBS are allowed to attach onto the glass slide by spinning at low speed.
3. The slides are pre-treated with L-polylysine to encourage cell attachment. The slide is then air-dried, followed by fixation (*see* **Note 4**).

3.1.3 Tissue Samples

Tissue samples from animals need fixation prior to be cut into smaller pieces (6–8 μm), to get smooth and fine sections. This improves the quality and visibility of immunofluorescence significantly. The fixation is done either by flash freezing the freshly dissected tissue or by embedding formalin fixed tissue in paraffin block.

Flash freezing fixes the tissue by avoiding ice crystal formation. In this method, the freshly dissected tissue is placed on a tissue mold and is covered with a cryo-embedding medium and kept in the dry ice. The storage of the sample is done at −80 °C or in liquid nitrogen. The blocks are cut into thin sections of approximately 4–8 μm and mounted on the glass slide.

For **paraffin embedding**, the tissue is fixed by suspending it overnight in neutral buffered formalin followed by embedding with paraffin. Paraffin embedded tissue is cut into fine sections which are mounted onto the glass slide. Deparafinization is done by multiple xylene washes and rehydration by graded alcohol washes.

In case of paraffin embedding, the antigens get masked, which needs to be unmasked (retrieval of antigen) before proceeding to immunofluorescence assay (*see* **Note 4**).

3.2 Fixation of Cells

Fixation maintains the architecture of the cells as close to native state as possible. It stops the proteolytic enzyme induced cellular autolysis and the process of putrefaction (cellular decay) occurring due to the loss of nutrient supply to the cells. The choice of fixative used may need optimization for each type of antigen-antibody combination as there may be damage of some of the antigenic sites due to certain type of fixative (*see* **Notes 5** and **6**).

3.2.1 Cell Fixation for Membrane-Associated Antigens

Paraformaldehyde (polymerized formaldehyde, PFA) is the most commonly used fixative while staining membrane-associated proteins. Being a cross linking fixative, Paraformaldehyde, causes chemical crosslinking of free amino groups, resulting into proteins interactions that preserve cellular architecture. PFA is fixative of choice while dealing with 3D cultures.

For fixation, freshly prepared 4% Paraformaldehyde in warm 1X PBS solution is used, to cover cells to a depth of 2–3 mm and incubated for 15–20 min at room temperature. The fixative is removed by three washes with 1× PBS for 5 min each (*see* **Note 7**).

3.2.2 Cell Fixation for Intracellular Antigens

Methanol is an example of precipitating fixative. Such fixatives act as strong dehydrating agents and precipitate the cellular proteins. While such fixatives are efficient in maintaining the architecture of cell, they tend to remove small soluble molecules and lipids from the cell.

For fixation of cells with methanol, cells are treated with chilled 100% methanol and incubated at −20 °C for 15–20 min. The methanol is removed by washing three times with 1× PBS for 5 min each.

This fixative is used mostly to stain cytoskeletal proteins or epitopes buried within the internal protein structure as it disturbs hydrophobic bonds of proteins. Its inherent nature to reduce protein solubility limits its use for lipid-associated proteins and proteins localized to the nucleus and mitochondria.

3.3 Permeabilization of Cell Membrane

Typically, antibody molecules are very large and ionic to penetrate the cell membrane and interact with intracellular proteins. Permeabilization allows the antibodies to penetrate the fixed cell by disturbing the cell membrane and subsequently interact with intracellular antigen. Therefore, while performing IF for markers on cell surface, the permeabilization step is not recommended (*see* **Note 8**).

The results of immunofluorescence assay vary, depending upon the type and concentration of permeabilizer used and incubation time given to permeabilizer to act upon the sample. For example, most commonly used permeabilizer, Triton X-100 has been used at 1% in PBS for 1–5 min or at 0.1–0.4% in PBS for 10–20 min. This concludes that for each IF study, the protocol needs to be optimized in such a way to develop good immunofluorescence with minimal distortion of cell morphology. Some of the permeabilizers are enlisted with their commonly used working concentration in Table 2.

3.4 Blocking of Non-specific Sites for Antibody Binding

The non-specific interaction of primary or secondary antibodies with the sample leads to false results in IF assays. These non-specific interactions occur due to trapping of antibody in hydrophobic structure in cell or by cross reactive binding of

Table 2
Commonly used permeabilizers, their working concentrations and preferred uses

Permeabilizer	Working concentration (Needs optimization for each IF assay)	Use as permeabilizer
Triton X 100	0.1–0.4% in PBS for 15–20 min	Non-selective detergent that can extract proteins along with lipids to permeabilize cellular bilayers including the nuclear membrane. Therefore, preferred for staining intracellular and intranuclear antigens
Tween-20	0.1–0.5% in PBS for 15–20 min	Similar to Triton X 100 with milder activity.
Saponin/ Digitonin	0.1–0.5% in PBS for 5–7 min	Reversible nature of action as mainly solubilizes cholesterol from cell bilayer. Maintains the integrity of protein surface antigens **Cannot** permeabilize the nuclear membrane. Common alternative for cells sensitive to triton X-100 Preferred for staining target antigen located at cell membrane

polyclonal antibodies to non-target antigenic sites. The specificity of IF staining is enhanced by blocking these non-specific interactions. Treatment with blocking agent prior to incubation with primary antibodies prevents the non-specific bindings.

The fixed and permeabilized cells are incubated with blocking agent such as serum, bovine serum albumin (BSA), skimmed milk, and gelatine. Generally, 5% serum or 1–5% BSA in 1× PBS with 0.05% Tween-20 or triton X-100 is incubated with the sample for 1 h (*see* **Note 9**).

After blocking, the extra blocking buffer is removed by washing three times with PBS with 0.05% Tween 20 or Triton-X 100 containing washing buffer.

3.5 Incubation with Primary Antibody

After blocking, the sample is ready for incubation with the primary antibodies. The primary antibody is diluted as per prior optimized dilution in antibody dilution buffer and allowed to incubate with the sample. The dilution of primary antibody used depends on abundance of target antigen in the sample, concentration of antibody stock, and affinity of antibody to target antigen (*see* **Note 10**).

The incubation of primary antibody with the sample can be done either in two ways, i.e., an hour at room temperature or overnight at 4 °C. To remove unbound antibodies, washing is done 3–5 times for 5 min each with PBS containing either 0.05% Tween-20 or 0.05% Triton X-100. Care should be taken so that the sample cells do not get dried up between different steps.

In case of direct immunofluorescence assay, incubation of primary antibody (fluorophore conjugated) with the sample is directly

followed by mounting and imaging. In case of indirect immunofluorescence assay, before mounting and imaging, sample is incubated with fluorophore conjugated secondary (anti-species) antibodies.

3.6 Incubation with Secondary Antibody

In indirect IF, fluorophore conjugated secondary antibody is diluted to a pre-optimized dilution using the antibody dilution buffer and incubated with the sample (*see* **Note 11**). Like primary antibody, the secondary antibody incubation is done either for 1 h at room temperature or overnight at 4 °C. To remove excess unbound secondary antibodies, the sample is washed thrice with PBS containing 0.05% Tween 20 or Triton X-100.

3.7 Counterstaining and Mounting

Counterstaining (for cell nuclei or cytoskeleton) is perforemend to visualize the cell with respect to its location and morphology. After incubation with secondary antibody, the sample is equilibrated with PBS and cell nuclei are counterstained with DAPI (4′,6-diamidino-2-phenylindole). The DAPI stock solution is diluted to 300 nM in PBS. This diluted DAPI staining solution is added to the sample, so that the cells are completely covered. Incubation is done for 1–5 min. Sample is then rinsed several times with PBS. Excess buffer is drained from the coverslip followed by mounting (*see* **Note 12**).

For mounting, a few small drops of mounting medium (50% glycerol in PBS), just sufficient to make a thin film over sample, is added prior to gently covering the sample with a coverslip (*see* **Note 13**). The sample is kept on a flat surface for drying and the edges of the coverslips can be sealed with nail polish. After mounting, in order to preserve the fluorescence signal, the sample needs to be stored at −20 °C until imaging.

3.8 Imaging

After IF staining, the sample is visualized by a fluorescence microscope or confocal microscope, depending on the requirement. If only expression of certain protein needs to be confirmed, a regular fluorescent microscope is generally used however for co-localizing proteins inside the cell, a confocal microscope becomes the instrument of choice (Fig. 2). Confocal microscope eliminates outoffocus light and thus captures high optical resolution images in the confocal planes. These images of different confocal planes of any sample can be integrated together to give rise to full three-dimensional picture of any sample [14].

3.9 Post-imaging Analysis and Issues Encountered in IF Staining

Like other biochemical techniques, IF staining results may be affected by many factors. The major issues which are generally encountered are listed in the following.

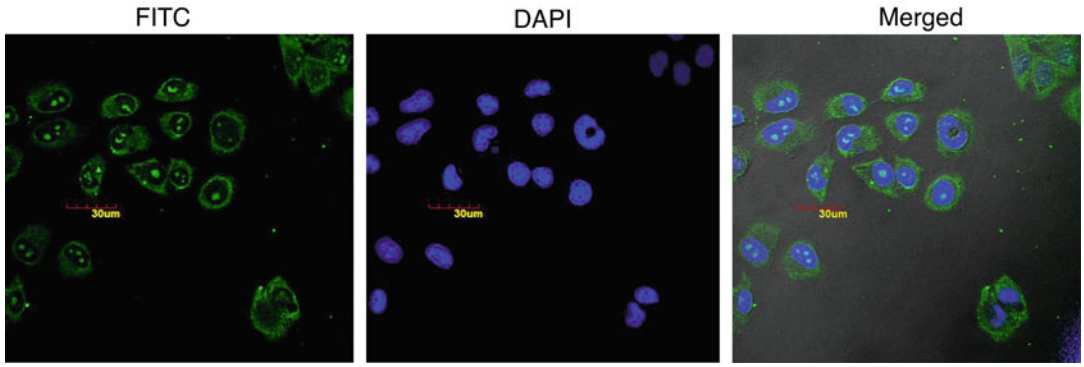


Fig. 2 Confocal Image showing Immunofluorescence assay: Hela cells expressing a recombinant protein were immunostained and confocal imaging was done to localize the recombinant protein inside the cell. Cells grown for 24 h on coverslip kept inside the well of sterile tissue culture plate. Cells fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100 for 10 min, blocked with 5% goat serum for 1 h at room temperature. As primary antibody, cells were incubated for 1 h with polyclonal sera raised in rabbit and finally incubated with Goat anti-rabbit IgG conjugated with FITC. Cells were counterstained with DAPI and examined under confocal microscope

3.9.1 Specificity Validation of Antibodies

In order to analyze specific signals in immunofluorescence staining, we need to validate specific binding of the primary and secondary antibody. For this, it is essential to include appropriate controls in the assay (*see* **Note 14**). In addition, issues regarding antibody specificity may be resolved by optimizing the process of fixation and blocking or by using different specific antibodies having high affinity to the target antigen.

3.9.2 Photobleaching

During the IF imaging, fluorophore conjugated to the bound secondary antibody gets excited and a light of a specific wavelength is emitted which is detected by the detector of the fluorescent microscope. This also results in the generation of reactive oxygen species (ROS). These ROS interact chemically with the fluorophore and cause impediments in its optimal excitation over time. Therefore, after light exposure, the quality of the image is deteriorated overtime, giving a bleached appearance. This phenomenon is known as photobleaching. Photobleaching is prevented by optimizing the exposure time by the excitation light or by using mounting medium containing ROS scavengers (*see* **Note 13**). Another approach is using more robust fluorophores, which are less prone to photo bleaching (e.g., Alexa Fluors, DyLight Fluors, and Seta Fluors).

3.9.3 Autofluorescence

Biological samples contain coenzymes that are important in regulating cellular metabolic activities. Some of them may exhibit autofluorescence such as reduced NADH (Absorption wavelength: 340 nm, emission: 460 nm) and flavin coenzymes like FMN (flavin mononucleotide) and FAD (flavin adenine dinucleotide)

(absorption wavelength: 450 nm, emission: 460 nm). Therefore, sometimes during detection of fluorophores which emit light in the green spectrum, low signal-to-noise ratio is observed. This issue can be resolved by using a different specific antibody having higher-affinity to antigen.

Fixation methods using aldehydes such as glutaraldehyde may also result in high autofluorescence. Quenching the fixation agent to eliminate any free aldehyde groups which could non-specifically bind antibody, reduces the autofluorescence (*see* **Note 15**).

3.9.4 High Background Fluorescence

Due to inefficient blocking or too high concentration of primary antibody used or insufficient washing out of fixative, sometimes there occurs the problem of high background fluorescence. To resolve this issue, we can take following measures:

1. Increase the percentage of blocking agents used in the blocking buffer as well as in antibody dilution buffers. Increase the time for blocking step and/or reducing the time given for antibody incubations.
2. Increasing the dilution of primary and/or secondary antibodies used.
3. Proper washing and quenching of free aldehyde in case of aldehyde fixatives used (*see* **Note 15**).

3.9.5 Fluorophore Overlap

This problem is encountered when multiple target antigens are tagged using different fluorophores having emitted light in similar spectral wavelengths (*see* **Note 11**). For example, if Alexa Fluor 430 (excitation wavelength: 434 nm, emission wavelengths: 539 nm) and Alexa Fluor 514 (excitation wavelength: 518 nm, emission wavelength: 540 nm) are used to stain different antigen in same sample, the detector of fluorescence microscope will not be able to distinguish the light emitted from these two fluorophores due to a significant overlap of their emission wavelengths. Alternatively, if Alexa Fluor 430 is used along with another fluorophore such as Alexa Fluor 594 (excitation wavelength: 590 and emission wavelength: 617 nm), there will be no overlap of the emitted light and the proteins stained with this combination of dyes will be optically differentiated.

4 Notes

There are many tips to follow and alternative methods to perform IF staining in order to achieve better results. Some of them are listed in the following:

1. The thickness of coverslip plays a key role in the quality and intensity of the image. Generally, coverslips having thickness of approximately 170 μm are found compatible with most microscopes.
2. As a crude method for sterilization, coverslip can be dipped in 70% ethanol and kept in the flame using forceps for approximately 15–20 s and directly put inside the well of sterile tissue culture plate. Use of coverslip reduces the volume of primary as well as secondary antibody during incubation, but the fragile nature of coverslip requires extra care in handling during the wash steps.
3. For cells having less adherent strength, the coverslip may be pre-treated with 50 μg/mL L-poly-lysine to aid cell attachment.
4. Unmasking of the antigen is done by heat treatment preferably or by saponin/pepsin treatment. Before the advent of antigen retrieval methods involving heating, techniques to improve immunostaining included treating sections at room temperature with 5 M urea [15] or with detergents [16]. Some of the substances that have been included in solutions for heat induced antigen retrieval are 1% Zinc sulfate, 1% lead thiocyanate [17], 0.1 M Citrate buffer, pH 6 [18], 0.01 M EDTA, pH 8 [19], and Tris buffer, pH 9 [20].
5. Commonly used fixatives other than paraformaldehyde and methanol are as follows:

   (a) **Formalin (10%)**

   A 3.7% Paraformaldehyde solution is equivalent to a 10% formalin solution. Cells can also be fixed with 10% formalin solution for 10–20 min at room temperature followed by three washes with 1× PBS for 5 min each.

   (b) **Acetone**

   Acetone is also a precipitating fixative but it is milder than methanol. For acetone fixation, chilled acetone is used to cover the sample in a thin layer followed by incubation at −20 °C for 5–20 min, depending upon the antigen of interest. Acetone maintains antigenic integrity as compared to methanol. It may be used when methanol fixation is ineffective. Acetone is also used for cytoskeletal proteins. Acetone is very good at cell permeabilization but shares the same limitations as that of methanol fixation.

   In some cases where neither methanol nor acetone alone is effective, 1:1 ratio of methanol and acetone is used (10 min incubation at −20 °C).

   Precipitating fixatives generally denature overexpressed fluorescent proteins (e.g., Green fluorescent

Table 3
Fixatives: Major advantages and disadvantages

Fixatives	Advantages	Disadvantages
Formaldehyde	Universal fixative Preserves cellular morphology Good for staining membrane proteins	Sometimes it may reduce the signals due to excessive cross linking
Methanol	Choice of fixative for aldehyde sensitive epitopes No requirement of additional permeabilization	Highly volatile and flammable Not suitable for overexpressed fluorescent protein Reduces solubility of proteins
Acetone	Choice of fixative for aldehyde sensitive epitopes No requirement of additional permeabilization Milder than methanol	Highly volatile and flammable Not suitable for overexpressed fluorescent protein Reduces solubility of proteins

protein, GFP). Therefore, they are not recommended to fix cells with such proteins.

The advantages and disadvantages of mentioned fixatives are compiled in Table 3.

6. Cell fixation using Paraformaldehyde may sometimes leads to auto-fluorescence-mediated artefacts and therefore it is crucial to have a control sample that skips the step of sample incubation with the primary antibody to differentiate any non-specific background signal.
7. Unlike precipitating fixatives such as methanol and acetone, aldehyde-based fixatives cannot permeabilize the cell membrane effectively and the samples need to be permeabilized before proceeding to staining of intracellular biological molecules.
8. Different permeabilizing agents in increasing order of permeabilizing efficiency are saponins, Tween-20, Triton X-100, and sodium dodecyl sulfate (SDS). Fixatives such as acetone and methanol also perform permeabilization while other fixatives are not very efficient permeabilizers. The permeabilization step can be skipped if the antigen of interest is located on the cell membrane or if acetone is used as fixative. To achieve good signal with minimal cell distortion, it is advised to use the mildest detergent possible that allows antibody penetration into the cell. Milder detergents such as saponin, Tween™ 20, or digitonin at 0.1–0.5% (v/v) for 5–10 min can be used if Triton X-100 or NP-40 do not give good visualization of the target.

9. When using serum as blocking agent, its source should be different animal species than that of primary antibody. In case of indirect IF, the blocking serum should be the same animal species in which secondary antibody was raised. For example, if secondary antibody raised in goat is being used, then blocking is done with 5% normal goat serum. Various commercially available proprietary protein-free compounds or highly purified single proteins may also be used as blocking reagents in IF assays.
10. In multicolor immunofluorescence assay, for staining multiple target proteins, their respective primary antibodies can be combined, provided they are derived from different animal species. This combination is then allowed to incubate with the sample. Alternatively, the primary antibodies can be used sequentially. In order to avoid chances of non-specific binding, the source of the primary antibody should preferably be a species distinct from the species being studied.
11. In immunostaining for multiple target antigens, the secondary antibody corresponding to each primary antibody needs to be conjugated with distinct fluorophore having non-overlapping emission wavelength spectrums.
12. Some mounting media also contains counterstain DAPI to visualize nuclei. DAPI interacts with DNA, emitting light in the blue spectrum. When using such mounting media, counterstaining step is skipped and after secondary antibody incubation, directly mounting step can be performed.
13. The function of 50% glycerol in PBS (mounting medium) is to preserve the sample and to increase the refractive index for obtaining high-quality images using an oil immersion lens. Some commercially available mounting media, such as Fluoromount-G(anti-fade) from Southern Biotech media and Prolong Gold from Molecular Probes also help in minimizing photobleaching caused by free reactive oxygen species (ROS) scavengers.
14. For validating the specificity of antibodies used, appropriate controls should be included to the IF assay.

    Specificity of primary antibody can be tested by the following:

    (a) Blocking primary antibody with its complementary antigen and then performing IF staining using this blocked primary antibody.

    (b) Another way is to produce a sample with targeted deletion or RNAi mediated silencing of the antigen of interest and performing IF staining using this sample as control.

In both cases, specificity of the primary antibody can be validated by the lack of signal on IF staining.

Specificity of secondary antibody can be tested by confirming the lack of signal in a sample incubated only with the secondary antibody and not incubated with the primary antibody.

15. For quenching/attenuation formaldehyde, washing with 0.1 M Tris or Glycine buffer and for quenching glutaraldehyde washing with 0.1% sodium borohydride in PBS prior to antibody incubation is done.

5 Conclusion

In conclusion, immunofluorescence technique is very useful tool to detect protein expression and localize cellular antigen. IF technique detects the target optically using fluorophore-conjugated antibodies. The IF assay has been very useful in rapid identification of viral antigens in the infected cells. Various modifications in the technique have been adopted to develop multiplex immunofluorescence staining systems. The Tyramide signal amplification–avidin–biotin complex (TSA-ABC) method of IF staining is one such example [21]. Such continuous modifications indicate that the IF technique will become more adapted and useful tool for laboratory investigations and identification of pathogenic agents.

References

1. Burnette WN (1981) "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate—polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radio-iodinated protein A. Anal Biochem 112(2):195–203
2. Engvall E, Perlmann P (1972) Enzyme-linked immunosorbent assay, Elisa. 3. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. J Immunol 109(1):129–135
3. Liu JJ, Liu C, He W (2013) Fluorophores and their applications as molecular probes in living cells. Curr Org Chem 17(6):564–579
4. Joshi S, Dihua Y (2017) Immunofluorescence. In: Jalali M, Saldanha F, Jalali M (eds) Basic science methods for clinical researchers, 1st edn. Academic Press, Cambridge, Massachusetts
5. Coons AH, Creech HJ, Jones RN (1941) Immunological properties of an antibody containing a fluorescent group. Proc Soc Exp Biol Med 47(2):200–202
6. Coons AH, Creech HJ, Jones RN, Berliner E (1942) The demonstration of pneumococcal antigen in tissues by the use of fluorescent antibody. J Immunol 45(3):159–170
7. Cherrywbmoody MD (1965) Fluorescent-antibody techniques in diagnostic bacteriology. Bacteriol Rev 29:222–250
8. Hers JF (1963) Fluorescent antibody technique in respiratory viral diseases. Am Rev Respir Dis 88(SUPPL):316–338
9. Kraft SC, Kirsher JB (1964) Immunofluorescent studies of chronic nonspecific ulcerative colitis. Gastroenterology 46:329–332
10. Bourgeois M, Oaks J (2014) Laboratory diagnosis of viral infections. In: Sellon DC, Long MT (eds) Equine infectious diseases, 2nd edn. W.B. Saunders, Philadelphia, Pennsylvania
11. Liu C (1956) Rapid diagnosis of human influenza. Proc Soc Exp Biol Med 92:883–887
12. Gardner PS, McQuillin J (1980) Rapid virus diagnosis. In: Application of immunofluorescence, 2nd edn. Butterworths, London

13. Clark Brelje T, Wessendorf MW, Sorenson RL (1993) Multicolor laser scanning confocal immunofluorescence microscopy. In: Matsumoto B (ed) Methods in cell biology practical application and limitations, vol 38. Academic Press, Cambridge, pp 97–181
14. Kogata N, Howard BA (2013) A whole-mount immunofluorescence protocol for three-dimensional imaging of the embryonic mammary primordium. J Mammary Gland Biol Neoplasia 18(2):227–231
15. Hausen P, Dreyer C (1982) Urea reactivates antigens in paraffin sections for immunofluorescent staining. Stain Technol 57:321–324
16. Feldmann G, Maurice M, Bernuau D, Rogier E, Durand AM (1983) Penetration of enzyme-labelled antibodies into tissues and cells: a review of the difficulties. In: Avrameas S, Druet P, Masseyeff R, Feldmann G (eds) Immunoenzymatic Techniques. Elsevier, Amsterdam, pp 3–15
17. Shi SR, Key ME, Kalra KL (1991) Antigen retrieval in formalin-fixed, paraffin-embedded tissue: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. J Histochem Cytochem 39:741–748
18. Shi SR, Chaiwun B, Cote RJ, Taylor CR (1993) Antigen retrieval technique utilizing citrate buffer or urea solution for immunocytochemical demonstration of androgen receptor in formalin-fixed paraffin sections. J Histochem Cytochem 41:1599–1604
19. Gown AM, Willingham MC (2002) Improved detection of apoptotic cells in archival paraffin sections: immunohistochemistry using antibodies to cleaved caspase 3. J Histochem Cytochem 50:449–454
20. Koopal SA, Coma MI, Tiebosch ATMG, Suurmeijer AJH (1998) Low-temperature heating overnight in tris-HCl buffer pH 9 is a good alternative for antigen retrieval in formalin-fixed paraffin-embedded tissue. Appl Immunohistochem 6:228–233
21. Toda Y, Kono K, Abiru H, Kokuryo K, Endo M, Yaegashi H, Fukumoto M (1999) Application of tyramide signal amplification system to immunohistochemistry: a potent method to localize antigens that are not detectable by ordinary method. Pathol Int 49(5): 479–483

Chapter 16

Polymerase Spiral Reaction (PSR) for the Diagnosis of Porcine Viral Diseases

Vikas Gupta, Nihar Nalini Mohanty, and Vinod Kumar Singh

Abstract

Polymerase spiral reaction is a novel and emerging isothermal nucleic acid-based amplification assay for the detection and diagnosis of microbial pathogens of both veterinary and medical importance. The assay is very cost effective as it does not require any sophisticate equipment or laboratory facilities. The test can be completed within 1 h by employing one set of specifically designed primers unique to target, $MgSO_4$, Betaine, dNTP mix, Bst enzyme, and isothermal buffer in a single tube by incubating in water bath or heat block at constant temperature (isothermal). Amplification of target could be detected by agarose gel electrophoresis, real-time measuring of increase in turbidity in turbidimeter or by naked eye visualization of change in fluorescence of colorimetric dyes such as calcein, hydroxynaphthol blue, SYBR green-I or Eva green. The assay is an amalgamation of isothermal assay and convention polymerase chain reaction and has immense potential to detect the pathogens of animals including swine in a simple, rapid, sensitivity, and specific manner.

Key words Diagnosis, Isothermal, Simple, Sophisticated equipment-free, Naked eye visualization

1 Introduction

Pig production is an important source of global economy and food security. Pigs are susceptible to various types of infectious diseases and among them viral diseases such as African swine fever, Porcine reproductive and respiratory syndrome, Classical swine fever, Pseudorabies, Porcine epidemic diarrhea, Porcine multisystemic wasting syndrome impose constant threat to pig population in the form of heavy morbidity, mortality, restriction in local and international trade leading to massive economical loss and lack of food security. Rapid and accurate diagnosis of infectious diseases play crucial role in the prevention and control of disease. Conventional diagnosis of infectious agents such as agent identification, antigen detection, biochemical identification, and serology are reliable and confirmatory but these approaches are time consuming and require sophisticated laboratory setups. "ASSURED" (Affordable, Sensitive,

Rajib Deb et al. (eds.), *Protocols for the Diagnosis of Pig Viral Diseases*,
Springer Protocols Handbooks, https://doi.org/10.1007/978-1-0716-2043-4_16,

Specific, User-friendly, Rapid and robust, Equipment-free, and Delivered) concept of disease diagnosis is the main focus of research in developing countries for timely diagnosis of diseases [1]. Sequence-based amplification of specific genes has many applications in molecular biology research as well as veterinary and medical diagnostics. At present, for amplifying target sequence, various kinds of polymerase chain reactions (PCR) and isothermal amplification are used. The PCR requires sophisticated instrument for amplifying the target sequence by following chain of three cyclic thermal conditions followed by visualization of products by agarose gel electrophoresis while isothermal amplification assays do not requires any specialized instruments to amplify target sequence at specific temperature. Various types of isothermal reaction such as self-sustained sequence replication reaction (3SR) [2], nucleic acid sequence-based amplification (NASBA) [3], strand displacement amplification (SDA) [4], rolling circle replication (RCR) [5], loop-mediated isothermal amplification (LAMP) [6], helicase-dependent amplification (HDA) [7] and single primer isothermal amplification (SPIA) [8], cross-priming amplification (CPA) [9], transcription-based amplification system (TAS) [10], and polymerase spiral reaction (PSR) [11] have been employed for amplification and detection of genomes of infectious micro-organism in the last three decades. Most of these mentioned assays such as TAS, 3SR, NASBA, SDA, HAD, and SPIA require three or more enzymes and rigorous optimization. Only a few of these isothermal amplification methods (e.g., RCR, LAMP, CPA, and PSR) can efficiently amplify the target sequence by using single enzyme at a constant temperature. However, RCR method can only amplify circular DNA while initial denaturation step is required for the satisfactory result in LAMP assay. Polymerase spiral reaction (PSR) technique was invented by Liu et al. [11] for the detection of antibiotic resistance gene *bla*NDM-1 in *E. coli* pGEX-NDM-BL21 plasmid. This technique is the amalgamation of isothermal amplification techniques, namely RCR and 3SR, and conventional PCR in which only one pair of primers is needed. In contrast to RPA and HDA which need multiple enzymes, the PSR assay requires DNA polymerase enzyme with strand displacement activity (Bst DNA polymerase) showing the similarity with LAMP technique for amplification of target sequence at a constant incubation temperature (60–66 °C). The product can be visualized by DNA binding intercalating dye or with the aid of gel electrophoresis showing specific ladder pattern of amplicons. PSR, when employed for detection and identification of pathogens of both medical and veterinary importance, the assay accurately and efficiently detected both RNA and DNA virus such as Porcine epidemic diarrhea virus [12], Canine provirus-2 [13], Bovine herpes virus-1 [14], Hepatitis C virus [15], Porcine circovirus type-3 [16] from clinical samples. The assay has also been evaluated for diagnosis of pathogens of zoonotic and public health

importance, namely *Brucella* sp. [17], *M. tuberculosis* [11], *E. coli* [11], *Salmonella sp* [18, 19], *Mycoplasma synoviae* [20]; *Candida albicans* [21], *Pseudomonas aeruginosa* [22], and *Vibrio parahaemolyticus* ([23]). Also, [24] developed polymerase cross link spiral reaction (a modification of PSR) for detection of African swine fever virus (ASFV) from blood of infected pigs and wild boars.

2 Materials Required for PSR

1. **Isothermal DNA polymerase having strand displacement activity** e.g. Bst DNA polymerase large fragments (8 U/μl, New England Biolab); Bst 2.0 WarmStar (New England Biolab); GspssD DNA polymerase large fragment (8 U/μl, Optigen west Sussex, UK) or Bsm DNA polymerase large fragment (8 U/μl, Thermo Fisher Scientific) (*see* **Note 1**).
2. **Isothermal reaction buffer** e.g. 10 × thermopol (200 mM Tris–HCl, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$, 1% Tween 20); 10× Bsm buffer (200 mM Tris–HCl, 100 mM KCl, 100 mM $(NH4)_2SO_4$, 20 mM $MgSO_4$, 1% Tween 20,) or 10× Gsp buffer.
3. **Magnesium sulfate (100 mM)**: Generally Magnesium sulphate is required in the range of 2 mM to 10 mM and it is most important factor, affecting the annealing temperature of primers and optimum activity of isothermal DNA polymerase.
4. **dNTPs (10 mM)**: Generally required in the range of 0.4–1.6 mM.
5. **Betaine (*N*,*N*,*N*-trimethylglycine) (5 M)**: It is required for unwinding of duplex form of DNA and generally required in the range of 0.4–1.5 M.
6. **Polymerase spiral reaction primers**: Specifically designed single pair of primer generally required in the range of 20–40 pmole.
7. **Water bath or heat block**: All the isothermal DNA polymerases enzymes have their optimum activity in the range of 60–65 °C.
8. **Visualization device and dyes**:
   (a) **Gel electrophoresis apparatus and accessories**: Amplified product form ladder pattern in gel but generally not recommended to visualize the amplified product due to significant risk of carry over contamination.
   (b) **Real-time fluorimeter (qPCR machine)** or **turibidimeter** for real-time monitoring.

(c) pH indicator dyes: Combination of 0.025 mM phenol red and 0.08 mM cresol red dyes that become yellow from purple-red.

(d) SYTO®-9 for real-time fluorescence.

(e) SYBR® Green 1, Calcein, hydroxynaphthol blue, malachite green or Eva green for endpoint analysis.

3 Methods

3.1 Primer Designing

Generally PSR technique requires one pair of special primer (Table 1) [11, 13–17] and it could be designed by using any PCR primer designing software such as Primer 5, Oligo v7.37, DNAMAN, etc. The software is used to design a pair of specific primer for target sequence. The exogenous sequences (N and Nr) are added to 5′ end of forward and reverse primers. The exogenous sequences must be taken from non-relative source such as from plant to avoid any nonspecific amplification. Both the exogenous sequences should have the same set of nucleotides but in the reverse order and the melting temperature of exogenous sequence should fall 5 °C lower than the PCR primer sequences in order to ensure annealing of complementary forward and reverse sequence to target region occurred earlier than the formation of spiral structure [11]. Further, some time auxiliary/accelerated primers are also used in PSR to enhance the reaction velocity [19, 21, 23].

Step to design PSR primer:

1. **Step-1**: Design a pair of common PCR primer (F and B) for target sequence with product length of around 150–160 bp following the guideline of PCR primer design.
2. **Step-2**: Design a single primer (exogenous sequence N) from non-relative source following the guidelines of PCR primer design but keep in mind the melting temperature of this primer falls 5 °C lower than the target primer. Nr sequence is the reverse of exogenous sequence N.
3. **Step-3**: Add the N and Nr sequences to the F and B respectively at 5′ end to design PSR primers Ft and Bt.
4. **Step-4**: Check the specificity of the PSR primers (Ft and Bt) by primer-blast analysis of NCBI sequence database to avoid the nonspecific reaction.

3.2 Procedure

1. **Setting up of reaction mixture for PSR amplification**: Prepare an initial reaction mixture composition for setting up the amplification run of PSR is as follows (Table 2) (*see* **Notes 2**, **3**, **4** and **5**).

Table 1
Example of primers set for polymerase spiral reaction

Forward primer for target(F)	5-GAACTAGTGGCACACCAACA-3
Reverse primer for target(B)	5-TGGTAAGCCCAATGCTCTATTT-3
Exogenous sequence (N)	5′-acgattcgtacatagaagtatag-3′
Exogenous sequence (Nr) (reverse of N)	5′-gatatgaagatacatgcttagca
PSR-forward primer (Ft)	5′-acgattcgtacatagaagtatagGAACTAGTGGCACACCAACA-3′
PSR-reverse primer (Bt)	5′-gatatgaagatacatgcttagcaTGGTAAGCCCAATGCTCTATTT-3′

Table 2
Initial reaction set up for polymerase spiral reaction

Sl. No.	Reagents	Volume (25 μl)
1	10× isothermal reaction buffer	2.5 μl (1×)
2	100 mM $MgSO_4$	1.5 μl (6 mM)
3	10 mM dNTPmix	3.5 μl (1.4 mM)
4	5 M Betain	4 μl (8 M)
5	40 pmole forward PSR primer	1 μl
6	40 pmole reverse PSR primer	1 μl
7	Bst 2.0 large fragment (8000 U/ml)	1 μl (8 U/μl)
7	DNA template	1 μl
8	Nuclease free water	9.5

2. **Optimization of reaction composition and conditions**: Various concentration combinations of $MgSO_4$, Betaine, Bst enzyme, and different incubation temperature and time could be used to get optimum reaction mixture composition and temperature and time for the rapid and specific amplification (*see* **Note 6**).
3. **Visualization of isothermal amplified product**
   (a) Visualization of isothermal amplified product (LAMP, PSR, and CPA) can be done by agarose gel electrophoresis or by using colorimetric dyes (*see* **Note 7**).
   (b) In **agarose gel electrophoresis** (2.5 to 3%), amplified product after staining with ethidium bromide or other commercial dyes shows specific ladder pattern on excitation with UV light on UV transilluminator at 302 nm.
   (c) **Monitoring of turbidity**: Pyrophosphate released during amplification process form a complex with divalent ion

Table 3
Colorimetric dyes used for detection amplification on visual basis

Type of dye	Concentration	Change in fluorescence
Hydroxynaphthol blue	1 μl of 0.2% solution	Dye changes its color from violet to sky blue
Calcein/MnCl	At a final concentration of 0.5 mM MnCl and 0.05 mM calcein	Orange color of calcein changes to yellow green on excitation either with visible or UV light
pH indicator dye	0.025 mM phenol red and 0.08 mM cresol red dyes at the rate of 1 μl	Become yellow from purple-red
SYBR green, Evagreen	At a final concentration of 1×	Fluoresce strongly on binding with double stranded DNA SYBR green dye is added after end point of reaction Evagreen can be used while setting reaction mix

such calcium, magnesium, and manganese leading to increase in turbidity of the reaction mixture. Increase in turbidity could be recorded in real time in the form of OD at 400 nm at every 6 s by using turbidimeter. Turbidity can also be visualized by naked eyes as pellet on centrifugation.

4. **Visual fluorescence**: Colorimetric dyes such as hydroxynaphthol blue (HNB), calcein, SYBR green, Evagreen, and combination of 0.025 mM phenol red and 0.08 mM cresol red dyes can be employed for visualization of amplified product via naked eyes or with the help of UV light. Various colorimetric dyes, their concentration and change in fluorescence in presence of amplified product are described in Table 3.

3.3 Determination of Specificity of PSR

1. Specificity of PSR could be determined by employing naturally occurring restriction site within the original target sequence and digestion of the amplified sequence with restriction enzyme [11, 13].
2. Excised band from the gel after purification could also be sequenced for the determining authenticity of the amplified product.
3. Restriction enzyme site may be artificially incorporated in variable exogenous sequences (N & Nr) to design PSR primers having restriction cutting site [11, 12]. After the completion of amplification reaction, the product can be digested with corresponding restriction enzyme and digested product can also be sequenced after purification.

4 Notes

1. dUTP and Thermolabile Uracil DNA Glycosylase for carryover contamination prevention (Bst 2.0 is strongly recommended for use with dUTP/UDG systems).
2. Before starting to set the reaction mixture for PSR, keep all the reagents on ice.
3. Do not set the reaction in room used for visualization of PSR product by agarose gel electrophoresis or any other procedure that needs to open the reaction vessel, to avoid possible carry over contamination.
4. For organism having RNA as genomic material, reverse transcriptase enzyme for synthesis of cDNA and then use it as a template for setting the reaction.
5. A layer of mineral oil (~25 μl) could be used to prevent the volatilization of PSR products and subsequently to avoid the possibility of aerosol formation.
6. It is strongly recommended to run a non-template control to ensure amplification specificity and to avoid the possibility of carry over contamination.
7. Gel electrophoresis and other post-amplification detection system which requires opening of reaction tubes are not recommended due to significant risk of carry over contamination leading to false positive results in subsequent reaction.

References

1. Mabey D, Peeling RW et al (2004) Diagnostics for the developing world. Nat Rev Microbiol 2: 231–240
2. Fahy E, Kwoh DY et al (1991) Self-sustained sequence replication (3SR): an isothermal transcription-based amplification system alternative to PCR. PCR Methods Appl 1:25–33
3. Compton J (1991) Nucleic acid sequence-based amplification. Nature 350:91–92
4. Walker GT et al (1992) Strand displacement amplification—an isothermal, *in vitro* DNA amplification technique. Nucleic Acids Res 20:1691–1696
5. Banér J, Nilsson M, Mendel-Hartvig M et al (1998) Signal amplification of padlock probes by rolling circle replication. Nucleic Acids Res 26:5073–5078
6. Notomi T et al (2000) Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 28:E63
7. Vincent M et al (2004) Helicase-dependent isothermal DNA amplification. EMBO Rep 5: 795–800
8. Kurn N et al (2005) Novel isothermal, linear nucleic acid amplification systems for highly multiplexed applications. Clin Chem 51: 1973–1981
9. Fang R et al (2009) Cross-priming amplification for rapid detection of *mycobacterium tuberculosis* in sputum specimens. J Clin Microbiol 47:845–847
10. Kwoh DY et al (1989) Transcription-based amplification system and detection of amplified *human immunodeficiency virus* type 1 with a bead-based sandwich hybridization format. Proc Natl Acad Sci U S A 86:1173–1177
11. Liu W, Dong D, Yang Z et al (2015) Polymerase spiral reaction (PSR): a novel isothermal nucleic acid amplification method. Sci Rep 5: 12723. https://doi.org/10.1038/srep12723

12. Wang Y, Jiao W, Wang Y et al (2019) Simultaneous nucleic acids detection and elimination of carryover contamination with nanoparticles-based biosensor- and Antarctic thermal sensitive UracilDNA-glycosylase-supplemented polymerase spiral reaction. Front Bioeng Biotechnol 7:401. https://doi.org/10.3389/fbioe.2019.0040
13. Gupta V, Chakravarti S, Chander V et al (2017) Polymerase spiral reaction (PSR): a novel, visual isothermal amplification method for detection of canine parvovirus 2 genomic DNA. Arch Virol 162:1995–2001
14. Malla JA, Chakravarti S, Gupta V et al (2018) Novel polymerase spiral reaction (PSR) for rapid visual detection of bovine herpesvirus 1 genomic DNA from aborted bovine fetus and semen. Gene 644:107–112
15. Sun W, Du Y, Li X, Du B (2020) Rapid and Sensitive Detection of Hepatitis C Virus in Clinical Blood Samples Using Reverse Transcriptase Polymerase Spiral Reaction. J Microbiol 30(3):459–468. https://doi.org/10.4014/jmb.1910.10041. PMID: 31893596
16. Ji J, Xu X, Wang X et al (2019) Novel polymerase spiral reaction assay for the visible molecular detection of porcine circovirus type 3. BMC Vet Res 15:322. https://doi.org/10.1186/s12917-019-2072-9
17. Das A, Kumar B, Chakravarti S, Prakash C, Singh RP et al (2018) Rapid visual isothermal nucleic acid-based detection assay of Brucella species by polymerase spiral reaction. J Appl Microbiol 125:646–654
18. Momin KM, Milton AAP, Ghatak S et al (2019) Development of a novel and rapid polymerase spiral reaction (PSR) assay to detect *salmonella* in pork and pork products. Mol Cell Probes 50:101510. https://doi.org/10.1016/j.mcp.2020.101510
19. Xu W, Gao J, Zheng H et al (2019) Establishment and application of polymerase spiral reaction amplification for salmonella detection in food. J Microbiol Biotechnol 29:1543–1552
20. Wu Q, Xu X, Chen Q et al (2019) Rapid and visible detection of mycoplasma synoviae using a novel polymerase spiral reaction assay. Poult Sci 98:5355–5360
21. Jiang X, Dong D, Bian L, Zou D, He X et al (2016) Rapid detection of Candida albicans by polymerase spiral reaction assay in clinical blood samples. Front Microbiol 7:916. https://doi.org/10.3389/fmicb.2016.00916
22. Dong D, Zou D, Liu H, Yang Z, Huang S et al (2015) Rapid detection of *Pseudomonas aeruginosa* targeting the *tox*A gene in intensive care unit patients from Beijing, China. Front Microbiol 6:1100
23. He S, Jang H, Zhao C, Xu K, Wang J et al (2019) Rapid visualized isothermal nucleic acid testing of Vibrio parahaemolyticus by polymerase spiral reaction. Anal Bioanal Chem 412(1):93–101. https://doi.org/10.1007/s00216-019-02209-y
24. Woźniakowski G et al (2017) Polymerase cross-linking spiral reaction (PCLSR) for detection of African swine fever virus (ASFV) in pigs and wild boars. Sci. Rep. 7:42903. https://doi.org/10.1038/srep42903

Chapter 17

Recombinase Polymerase Amplification-Based Diagnostics of Porcine Viral Diseases

Yoya Vashi and Sachin Kumar

Abstract

Recombinase polymerase amplification (RPA) is a highly sensitive isothermal amplification technique, operating at 37–42 °C, with minimal sample preparation and capable of amplifying as low as 1–10 target copies in less than 20 min. The recent advent of RPA is enabling the expansion of this technology for research and diagnostic applications worldwide. By being an affordable, simple, fast, and sensitive method for the identification of pathogens, RPA has been adopted widely as a molecular tool in many diagnostic platforms, and has been used to amplify a wide array of organisms and samples. RPA is undoubtedly a promising isothermal molecular technique for the development of low-cost, rapid, point-of-care diagnostic assay. It is being successfully used for the detection of many viral pathogens.

Key words Diagnostics, Porcine viruses, Point-of-care, Recombinase polymerase amplification

1 Introduction

In the past few decades, swine production has revolutionized from traditional husbandry practices that involved a few pigs or small herds to an intensive concentration of swine raised in multisite production systems. Although this dramatic change has made the production of pork very efficient, it has also changed the ecology of many swine diseases, encouraging the emergence of new infections. There are many swine viruses that have been infrequently associated with the disease; sometimes, one or just a few animals are affected, other times, the disease may spontaneously reach a high incidence in the herd, and then just as quickly, it dissipates. Some of the economically important viral pathogens of swine are shown in Table 1. Early diagnosis of and the immediate establishment of reliable countermeasures to infectious diseases is essential to limit severe biophysical and socio-economic consequences. However, the importance of the many known viral diseases of pigs varies from country to country.

Rajib Deb et al. (eds.), *Protocols for the Diagnosis of Pig Viral Diseases*,
Springer Protocols Handbooks, https://doi.org/10.1007/978-1-0716-2043-4_17,

Table 1
Economic and zoonotic viral pathogens of swine. (Reproduced from [1]

Virus[a]	Economic[b]	Vaccine[c]	Zoonotic[d]
AFSV	+ + + +	No	–
FMDV	+ + + +	Yes	–
CSFV	+ + +	Yes	–
ADV	+ +	Yes	–
PRRSV	+ + + +	Yes	–
IVA-S	+ +	Yes	+ + +
PCV2	+	Yes	–
PEDV	+	Yes	–
SVA	+	No	–
JEV	+	Yes	+ +
HEV	+	No	+ +
Nipah virus	+	No	+ + +
EMCV	+	No	+
Menangle virus	+	No	+
VSV	+	No	+
VESV	+	No	+

[a] *ASFV* African swine fever virus, *FMDV* foot and mouth disease virus, *CSFV* classical swine fever virus, *ADV* Aujeszky's disease virus, *PRRSV* porcine reproductive and respiratory syndrome virus, *IVA-S* influenza virus A- swine, *PCV2* porcine circovirus type 2, *PEDV* porcine epidemic diarrhea virus, *SVA* Senecavirus A, *JEV* Japanese encephalitis virus, *HEV* Hepatitis E virus, *EMCV* encephalomyocarditis virus, *VSV* Vesicular stomatitis virus, *VESV* Vesicular exanthema of swine virus
[b] Economic impact ranging from + (infrequent/mild) to + + ++ (frequent/severe)
[c] Vaccine available to aid in control and prevention: yes or no
[d] Zoonotic potential ranging from + (infrequent/mild) to + + ++ (frequent/severe)

Polymerase chain reaction (PCR) thermal cycling methodology, and more recently, quantitative real-time PCR (qPCR) are often viewed as "gold standards" for molecular diagnosis of viral pathogens. However, these techniques inherently require the use of thermocycler and a reliable power supply, thus restricting their use to laboratories. To address requirements of amplification for the use in low-resource settings or at the point-of-need, isothermal DNA amplification methods have been developed, including nucleic acid sequence-based amplification (NASBA), strand displacement amplification (SDA), rolling circle amplification (RCA), the loop-mediated isothermal amplification (LAMP), helicase-dependent amplification (HDA), as well as the recombinase polymerase amplification (RPA).

One technology, in particular, RPA, despite its comparatively late introduction, is experiencing rapid development and popularity

due to its simplified equipment requirements and fast reaction times [2]. RPA was first introduced in 2006 and was initially demonstrated to be a nucleic acid amplification method for DNA [3]. Later, RNA was shown to be used as a template by the addition of reverse transcriptase in the same reaction tube [4]. RPA is remarkable due to its simplicity, high sensitivity, selectivity, compatibility with multiplexing, extremely rapid amplification, as well as its operation at a low temperature, without the need for the use of multiple primers or an initial denaturation step. Overall, RPA positions itself very favorably for widespread exploitation in kits and assays for use at the point-of-care or point-of-need.

2 RPA Mechanism

The fundamental mechanism of RPA relies on the synthetically engineered adaptation of a key process in DNA metabolism called homologous recombination. The standard RPA reaction reagents comprise of recombinase, recombinase loading factor, and single-stranded binding protein, which subsequently coordinate with ancillary components such as DNA polymerase, crowding agent, energy/fuel components (e.g., adenosine triphosphate, ATP) and salt molecules to perform the RPA reaction mechanism (Table 2) [3]. The RPA process starts when a recombinase protein UvsX from T4-like bacteriophages bind to primers in the presence of ATP and a crowding agent (a high molecular polyethylene glycol), forming a recombinase-primer complex. The complex then interrogates double-stranded DNA seeking a homologous sequence and promotes strand invasion by the primer at the cognate site. In order to prevent the ejection of the inserted primer by branch migration, the displaced DNA strand is stabilized by single-stranded binding proteins. Finally, the recombinase disassembles and a strand displacing DNA polymerase binds to the 3′ end of the primer to elongate it in the presence of dNTPs. The cyclic repetition of this process results in the achievement of exponential amplification (Fig. 1).

3 RPA Operating Parameters

3.1 Primers

The length of RPA primers is relatively long (typically between 32 and 35 nucleotides), unlike PCR primers. However, there are several reports demonstrating that normal PCR primers can be used and efficient amplification can be achieved [6, 7]. It is advised that a large number of small repeats should be avoided, as they could lead to secondary structures and potential primer artifacts. Primer dimers can be avoided by employing self-avoiding molecular recognition (SAMRs) oligonucleotides, where natural bases are replaced by 2-aminopurine-2′ deoxyriboside (A*), 2′-deoxy-2-

Table 2
Summary of RPA reaction components and their functions (Reproduced from [2]

Reaction components	Functions
T4 UvsX protein	Recombinase that possesses pairing and strand-transfer activity that is important in genetic recombination, DNA repair and replication (or *E. coli* RecA; recombinase is a central component in the related processes of recombinational DNA repair and homologous genetic recombination that is the ortholog of the UvsX protein)
T4 UvsY protein	Recombinase loading factor that is classified as a recombination-mediator protein that stimulates the single-stranded DNA-dependent ATPase activity of T4 UvsX and lowers the critical concentration of T4 UvsX required for activity
T4 gp32	Single-stranded binding (SSB) protein is involved in DNA replication, repair and recombination, and binds preferentially to single-stranded DNA. The T4 UvsX, T4 UvsY, and T4 gp32 proteins work co-operatively to initiate the RPA reaction via unwinding, D-loop formation and stabilization of the DNA template
Bacillus subtilis DNA polymerase I (*Bsu*) or *Staphylococcus aureus* polymerase (*Sau*)	DNA polymerase synthesizes new DNA templates homologous to the target nucleic acid by extending nucleotide building blocks from the bound primers, complementary to the original target nucleic acid sequence or "template"
Deoxynucleotide triphosphate (dNTP, N = A, T, C, G)	An equimolar solution of dATP, dCTP, dGTP, and dTTP are building blocks used by the DNA polymerase to synthesize new templates
Forward and reverse primers	Primers are critical to directing the amplification event to the nucleic acid target of interest through homologous binding. After binding, the primers provide the essential 3′-OH for the polymerase to perform strand extension
DNA template	The oligonucleotide that the primers bind to for the synthesis of exact new oligonucleotides
Carbowax20M (a high molecular weight polyethylene glycol (PEG))	The crowding reagent is a good mimic of the real biomacromolecules condition in vivo and facilitates amplification, as the crowding agents can enhance the catalytic activity of the enzymes
Dithiothreitol	Stabilization of the enzymes by baring free sulfhydryl groups
Phosphocreatine Creatine kinase Adenosine triphosphate (ATP)	The three components form the energy-supply system for the activities of the recombinase and the DNA polymerase
Tris(hydroxymethyl)aminomethane (Tris) Potassium acetate	The two components serve to stabilize and solubilize the DNA in the solution
Magnesium acetate	Acts as a cofactor for the performance of the enzymes. The RPA reaction initiates once the magnesium acetate is added

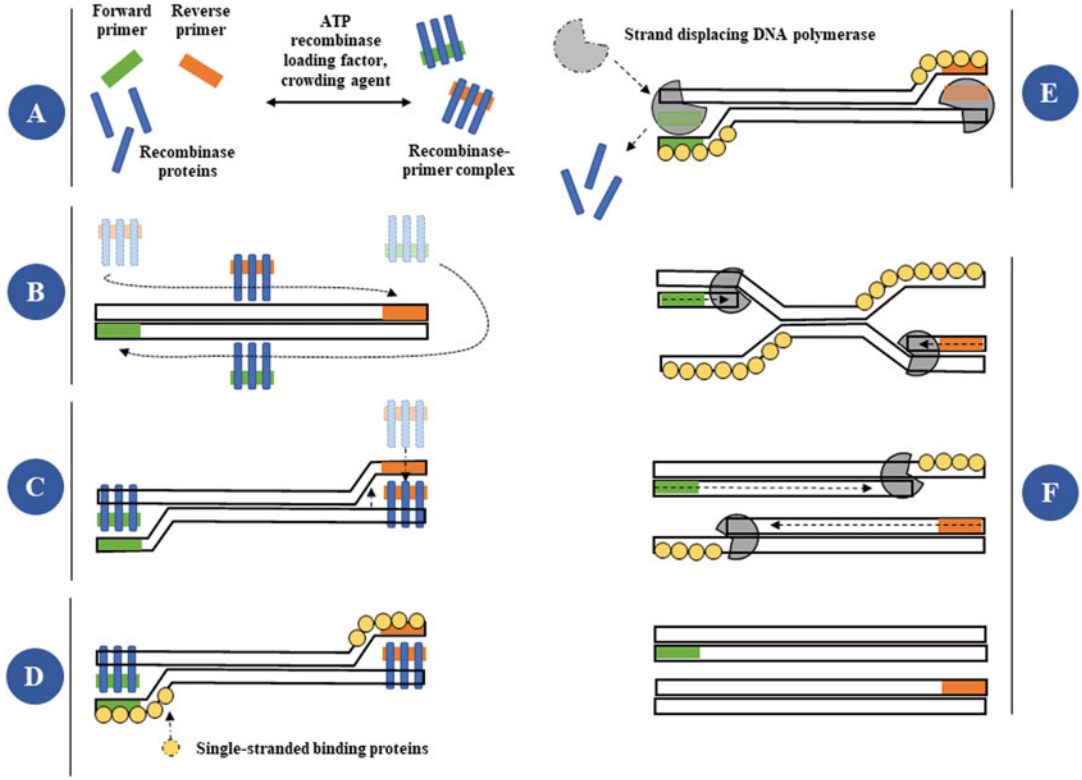


Fig. 1 RPA amplification scheme. Recombinase proteins form complexes with each primer (**a**), which scans DNA for homologous sequences (**b**). The primers are then inserted at the cognate site by the strand-displacement activity of the recombinase (**c**) and single-stranded binding proteins stabilize the displaced DNA chain (**d**). The recombinase then disassembles, leaving the 3′-end of the primers accessible to a strand displacing DNA polymerase (**e**), which elongates the primer (**f**). Exponential amplification is achieved by the cyclic repetition of this process. (Reproduced from [5])

thiothymidine (T*), 2′-deoxyinosine (G*), and N4-thyl-2-′-deoxycytidine (C*) in the primers [8]. Primers with low or high GC content (<30% or >70%) must also be avoided, as they can promote secondary structures or hairpins. RPA can amplify sequences up to 1.5 kb; however, an optimum amplicon size in the range of 100–200 bp yields better results. Additionally, RPA primers and probes have no requirement for melting temperature because primer annealing and elongation are enzyme-mediated and not temperature driven.

3.2 Template

RPA has successfully been used for different kinds of target organisms: bacteria, virus, protozoa, fungi, animals, and plants, with diverse samples types, ranging from cultured microorganisms to body fluids (urine, sputum, respiratory washes, nasal, blood, plasma, saliva, vaginal, and anal swabs), surgical biopsy specimens, organ tissues (skin, lymphatic nodes, liver, lungs, stomach, kidney),

as well as animal and plant products (eggs, shrimps, rice, milk, fruit) [5]. RPA can be used to amplify double-stranded DNA, single-stranded DNA, methylated DNA [9], cDNA generated through reverse transcription of RNA or miRNA [10].

3.3 Temperature

An important element of isothermal amplification is the incubation temperature because it is associated with the complexity of instrumentation. Isothermal amplification usually operates at lower temperatures (e.g., 30–42 °C). RPA reactions can operate at temperatures ranging from 25 to 42 °C without losing reaction efficiency. However, most published reports are optimized for temperatures between 37 and 42 °C. While a suitable working environment for RPA enzymes can be provided by the reaction temperature, agitation can increase the interactions among the RPA components in a homogenous reaction solution.

3.4 Incubation Time

The time required to amplify the DNA to detectable levels inherently depends on the number of starting DNA copies, but 20 min is usually adequate, although amplification times of as low as 3–4 min have been observed [11]. Long incubation times are unlikely to be beneficial in most applications, as for solution phase RPA the recombinase consumes all the available ATP within 25 min [5].

3.5 Inhibitors

RPA has been demonstrated to operate with nucleic acids extracted from various sample matrices such as blood [12], serum [13], fecal [14], nasal [15], vaginal swabs [16], plasma [17], foodstuff [18], plants [19], animal tissues [11], milk [20], stool [21], and urine [22]. RPA method amplified targets even in the presence of certain PCR inhibitors like hemoglobin (50 g/L), heparin (0.5 U), urine (5%), and ethanol (4% v/v) [23, 24]. The robustness of RPA in the presence of traditional inhibitors facilitates amplification from crude extracts, which is not achievable using PCR.

3.6 Specificity and Sensitivity

RPA has been described as highly specific, with 100% specificity for the target sequence in most cases. However, a disadvantage of RPA to discriminate towards closely related species can be observed when these species share high sequence similarity [25–27]. RPA has been reported to be dependent on the number and distribution of mismatches in the sequence of closely related DNA molecules. Studies also showed that mismatches located at the 3′-end of primers effectively prevent or reduce amplification, but mismatches at the 5′-end or center of primers only mildly affect the RPA reaction [28, 29].

The efficiency of RPA is dependent on the target sequence, amplicon size, and type of biological sample tested, as the analytical limit of detection and the turnaround time varied for RNA and DNA detection. RPA is very sensitive and has been shown to detect as little as a few copies per reaction of the analyte, which approaches

the analytical sensitivity of PCR. Furthermore, ultra-sensitive detection down to even a single copy of the analyte can be achieved in RPA [30, 31].

3.7 Multiplexing

Multiplexing with RPA in the same solution is possible but is highly dependent on target sequences, amplicon size, and primer design [32]. Several multiplex assays have been reported despite the requirements for long primers/probes in RPA reactions [3, 13, 16, 20, 32–34]. Multiplex RPA reaction can be performed either in a single tube (homogenous) or in a parallel fashion (sometimes refers to heterogeneous). Primer, probe ratios, and concentrations thus need to be carefully optimized for each multiplexing assay. However, a much higher assay throughput and multiplex capacity can be achieved in a parallel fashion as compared to the single tube multiplex RPA.

4 Detection of Amplicons

RPA enables the amplification of DNA or RNA, and detection of amplification products can be visualized either by agarose gel electrophoresis, lateral flow strips, or real-time fluorescent probes. With lateral flow detection, results can be generated on the dipstick strip within 5 min post-RPA. Three different oligonucleotides (2 primers and 1 probe) and the TwistAmp® nfo kit are typically used for assay designs compatible with lateral flow strip detection (Table 3). The design of a lateral flow probe requires a 5′-fluorophore tag (i.e., FAM) and a tetrahydrofuran (THF) residue for nfo nuclease recognition and cleavage, thus serving as a primer. For lateral flow assay, an opposing amplification primer labeled at the 5′-end with another label (e.g., biotin) is required. The amplicon produced in the presence of the probe and the two primers will include the two labels on one DNA amplicon, ready to be detected in a sandwich assay format by antibodies.

Agarose gel electrophoresis is a widely used technique for visualization of amplification products, but post-amplification, it is necessary to purify the amplicons to avoid smeared bands on the gel due to the presence of the proteins and the crowding agent present in the amplification mix.

Bridge flocculation assay is an equipment-free assay that provides a binary naked eye visual readout, suitable for low-resource settings. The basic principle of bridging flocculation involves the use of long polymers to crosslink multiple particles and thus flocculate out of solution at a specific buffer condition (e.g., pH and salt concentration) [35]. To execute the assay, a bead solution is added to the amplification products and following an ethanol wash, the beads are re-suspended in a low pH buffer and a positive answer is obtained if the beads remain flocculated.

Table 3
Commercial RPA reaction kits by TwistDx™ (Reproduced from [2])

Product name	Category	Nucleic acid detection	Compatible general detection method
TwistAmp® Basic	Lyophilized kit	DNA	Gel electrophoresis
TwistAmp® Basic RT		RNA	
TwistAmp® Liquid Basic	Liquid kit	DNA	
TwistAmp® Liquid Basic RT		RNA	
TwistAmp® exo	Lyophilized kit	DNA	Real-time fluorogenic probe-based
TwistAmp® exo RT		RNA	
TwistAmp® Liquid exo	Liquid kit	DNA	
TwistAmp® Liquid exo RT		RNA	
TwistAmp® fpg	Lyophilized kit	DNA	Real-time and end-point fluorogenic probe-based
TwistAmp® nfo			Lateral flow strip
TwistAmp® exo + ListeriaM	Food safety lyophilized kit	DNA (*Listeria monocytogenes* hly gene)	Real-time fluorogenic probe-based
TwistAmp® exo + Campylobacter		DNA (Campylobacter species including *jejuni* and *coli*)	
TwistGlow® Salmonella		DNA (*Salmonella enterica* INVA gene)	Real-time and end-point fluorogenic probe-based
TwistFlow® Salmonella			Lateral flow strip

Electrochemically active compounds can also be employed as an alternative strategy for the detection of RPA to produce a signal in relation to the amplified nucleic acids. Due to the electrochemically active property of 3,3′,5,5′-tetramethylbenzidine (TMB), amperometric signals from RPA-enzyme-linked immunosorbent (ELISA) assay or RPA-enzymatic assay can be measured for RPA detection. However, although fastidious, hybridization assays, and ELISA tests require several optimizations such as probes and antibody concentrations, hybridization temperature and time, or reaction volume, etc.

DVDs and low reflectivity DVDs are suitable substrates for the immobilization of primers for solid-phase or bridge amplification, facilitating multiplexing through parallelization in individual reactors of the DVD. Once amplification is achieved, a DVD reader can be used to read out the results in reflection or transmission mode [36].

Two kinds of probes, RPA-exo or fpg, can be used for real-time detection of RPA. Nonspecific intercalating fluorophores such as SYBR Green or Eva Green can be employed for real-time detection, but, as in the case of real-time PCR, these dyes cannot discriminate between amplicons and primer-dimer artifacts, thus giving rise to false-positive results. Whereas PCR Taq polymerases are not

compatible with RPA, conventional probes such as TaqMan cannot be used either. DNA amplification is inhibited because the 5′–3′ exonuclease activity of Taq polymerase progressively digests the displaced strand during the strand displacement process. Therefore, strand displacement polymerases that do not have the 5′–3′ exonuclease activity (e.g., *Bsu/Sau* DNA polymerase) are used for TaqMan probe detection.

RPA reactions can also be detected using silicon microring resonator (SMR)-based photonic detection. Amplification of the nucleic acid is performed in an asymmetric manner (pre-immobilized on one of the primers on the SMR, and all the other oligonucleotides and reagents are free in the solution) in the evanescent field of a resonator waveguide [37, 38]. A change in the refractive index proximal to the waveguide surface is induced when the nucleic acids bind to the pre-immobilized primers. Real-time monitoring of the wavelength shift can be monitored on SMR as the nucleic acid amplification progresses.

Surface-enhanced Raman scattering (SERS) has also been exploited for the detection of RPA amplicons. This is the phenomenon of when a laser excites nanoscale roughened metal surfaces (e.g., gold or silver), which resonantly drive surface charges, creating a highly localized (plasmonic) light field. When a molecule is absorbed or lies close to, the enhanced field at the surface, a large enhancement in the Raman signal can be observed [39]. SERS is a highly sensitive spectroscopic detection technique, which shows narrow and distinct spectral peaks of the detection molecules, and is particularly prominent for multiple target molecules detection.

5 RPAs for Porcine Viruses

Infectious diseases of food animals have major impacts on economic returns and public health. Effective surveillance and control of animal diseases are very important, in which rapid and accurate detection of etiological agents play a critical role. Molecular diagnostics is considered a powerful tool for detection and identification of infectious agents and has been used extensively due to its high sensitivity, high specificity, high throughput, and short turnaround time.

RPA has been developed for major swine viral pathogens such as porcine deltacoronavirus [40, 41], African swine fever virus [42, 43], porcine circovirus type 2 [27], porcine parvovirus [44, 45], and porcine reproductive and respiratory syndrome virus [46, 47]. Almost all of the RPAs developed for viral pathogens of swine were highly specific and had no cross-reactions with other porcine associated viruses, including classical swine fever virus (CSFV), porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV), porcine kobuvirus (PKoV), foot and

mouth disease virus (FMDV), and Seneca valley virus (SVV). The analytical sensitivity of RPA ranged from 70 copies to 690 copies per reaction. The positive rate was found to be similar to real-time PCR, but higher than that of conventional PCR. RPA developed for porcine viruses were based on either real-time fluorescent detection or lateral flow dipstick.

6 Conclusion

RPA is a fascinating isothermal amplification technique that has already garnered a huge amount of attention due to its very attractive properties, having a widespread application. The short reaction time (15–30 min) and low incubation temperature make RPA a suitable assay for quick and sensitive detection of viruses. Another advantage of RPA is in designing of primers where annealing temperature is not taken into account as primer forms a complex with the recombinase to target the homologous sequences. RPA is exploited for laboratory-based analysis, portable analysis in laboratory-in-a-suitcase, analysis at the point-of-need/care with biosensors, lateral flow assays and microfluidic devices, and its exploitation in a range of commercial devices for molecular diagnostics. RPA, equipped with field-deployable instruments, offers a sensitive and specific platform for the rapid and reliable point-of-care detection of porcine viruses, especially in the resource-limited settings. Given the tremendous advantages of RPA, as well as some of the current limitations of the technique, it can be expected that there will be exponential growth in the applications of RPA as well as improving and extending its performance. Nevertheless, several diagnostic methods have been developed over the last two decades; seeing the constant evolution of viruses, newer, sensitive, efficient, and rapid diagnostics are still warranted for the effective diagnosis of porcine viruses.

References

1. Lager KM, Buckley AC (2019) Porcine antiviral immunity: how important is it? Front Immunol 10:2258
2. Li J, Macdonald J, von Stetten F (2018) Review: a comprehensive summary of a decade development of the recombinase polymerase amplification. Analyst 144(1):31–67
3. Piepenburg O, Williams CH, Stemple DL, Armes NA (2006) DNA detection using recombination proteins. PLoS Biol 4(7):e204
4. Euler M, Wang Y, Nentwich O, Piepenburg O, Hufert FT, Weidmann M (2012) Recombinase polymerase amplification assay for rapid detection of Rift Valley fever virus. J Clin Virol 54(4):308–312
5. Lobato IM, O'Sullivan CK (2018) Recombinase polymerase amplification: basics, applications and recent advances. Trends Analyt Chem 98:19–35
6. Martorell S, Palanca S, Maquieira A, Tortajada-Genaro LA (2018) Blocked recombinase polymerase amplification for mutation analysis of PIK3CA gene. Anal Biochem 544:49–56
7. Mayboroda O, Gonzalez Benito A, Sabate del Rio J, Svobodova M, Julich S, Tomaso H, O'Sullivan CK, Katakis I (2016) Isothermal solid-phase amplification system for detection

of Yersinia pestis. Anal Bioanal Chem 408(3): 671–676

8. Hoshika S, Chen F, Leal NA, Benner SA (2008) Self-avoiding molecular recognition systems (SAMRS). Nucleic Acids Symp Ser 52:129–130
9. Wee EJ, Ngo TH, Trau M (2015b) Colorimetric detection of both total genomic and loci-specific DNA methylation from limited DNA inputs. Clin Epigenetics 7:65
10. Wee EJH, Trau M (2016) Simple isothermal strategy for multiplexed, rapid, sensitive, and accurate miRNA detection. ACS Sens 1(6): 670–675
11. Xia X, Yu Y, Weidmann M, Pan Y, Yan S, Wang Y (2014) Rapid detection of shrimp white spot syndrome virus by real time, isothermal recombinase polymerase amplification assay. PLoS One 9(8):e104667
12. Aebischer A, Wernike K, Hoffmann B, Beer M (2014) Rapid genome detection of Schmallenberg virus and bovine viral diarrhea virus by use of isothermal amplification methods and high-speed real-time reverse transcriptase PCR. J Clin Microbiol 52(6):1883–1892
13. Teoh BT, Sam SS, Tan KK, Danlami MB, Shu MH, Johari J, Hooi PS, Brooks D, Piepenburg O, Nentwich O, Wilder-Smith A, Franco L, Tenorio A, AbuBakar S (2015) Early detection of dengue virus by use of reverse transcription-recombinase polymerase amplification. J Clin Microbiol 53(3):830–837
14. Amer HM, Abd El Wahed A, Shalaby MA, Almajhdi FN, Hufert FT, Weidmann M (2013) A new approach for diagnosis of bovine coronavirus using a reverse transcription recombinase polymerase amplification assay. J Virol Methods 193(2):337–340
15. Boyle DS, McNerney R, Teng Low H, Leader BT, Perez-Osorio AC, Meyer JC, O'Sullivan DM, Brooks DG, Piepenburg O, Forrest MS (2014) Rapid detection of mycobacterium tuberculosis by recombinase polymerase amplification. PLoS One 9(8):e103091
16. Daher RK, Stewart G, Boissinot M, Bergeron MG (2014) Isothermal recombinase polymerase amplification assay applied to the detection of group B streptococci in vaginal/anal samples. Clin Chem 60(4):660–666
17. Euler M, Wang Y, Heidenreich D, Patel P, Strohmeier O, Hakenberg S, Niedrig M, Hufert FT, Weidmann M (2013) Development of a panel of recombinase polymerase amplification assays for detection of biothreat agents. J Clin Microbiol 51(4):1110–1117
18. Santiago-Felipe S, Tortajada-Genaro LA, Puchades R, Maquieira A (2014b) Recombinase polymerase and enzyme-linked immunosorbent assay as a DNA amplification-detection strategy for food analysis. Anal Chim Acta 811:81–87
19. Zhang S, Ravelonandro M, Russell P, McOwen N, Briard P, Bohannon S, Vrient A (2014) Rapid diagnostic detection of plum pox virus in Prunus plants by isothermal AmplifyRP ((R)) using reverse transcription-recombinase polymerase amplification. J Virol Methods 207:114–120
20. Santiago-Felipe S, Tortajada-Genaro LA, Morais S, Puchades R, Maquieira A (2015) Isothermal DNA amplification strategies for duplex microorganism detection. Food Chem 174:509–515
21. Crannell ZA, Castellanos-Gonzalez A, Irani A, Rohrman B, White AC, Richards-Kortum R (2014a) Nucleic acid test to diagnose cryptosporidiosis: lab assessment in animal and patient specimens. Anal Chem 86(5): 2565–2571
22. Krolov K, Frolova J, Tudoran O, Suhorutsenko J, Lehto T, Sibul H, Mager I, Laanpere M, Tulp I, Langel U (2014) Sensitive and rapid detection of chlamydia trachomatis by recombinase polymerase amplification directly from urine samples. J Mol Diagn 16(1):127–135
23. Kersting S, Rausch V, Bier FF, von Nickisch-Rosenegk M (2014b) Rapid detection of plasmodium falciparum with isothermal recombinase polymerase amplification and lateral flow analysis. Malar J 13:99
24. Rosser A, Rollinson D, Forrest M, Webster BL (2015) Isothermal recombinase polymerase amplification (RPA) of Schistosoma haematobium DNA and oligochromatographic lateral flow detection. Parasit Vectors 8:446
25. Moore MD, Jaykus LA (2017) Development of a recombinase polymerase amplification assay for detection of epidemic human noroviruses. Sci Rep 7:40244
26. Patel P, Abd El Wahed A, Faye O, Pruger P, Kaiser M, Thaloengsok S, Ubol S, Sakuntabhai A, Leparc-Goffart I, Hufert FT, Sall AA, Weidmann M, Niedrig M (2016) A field-deployable reverse transcription recombinase polymerase amplification assay for rapid detection of the chikungunya virus. PLoS Negl Trop Dis 10(9):e0004953
27. Yang Y, Qin X, Sun Y, Cong G, Li Y, Zhang Z (2017) Development of isothermal recombinase polymerase amplification assay for rapid detection of porcine circovirus type 2. Biomed Res Int 2017:8403642

28. Daher RK, Stewart G, Boissinot M, Boudreau DK, Bergeron MG (2015) Influence of sequence mismatches on the specificity of recombinase polymerase amplification technology. Mol Cell Probes 29(2):116–121
29. Lillis L, Lehman DA, Siverson JB, Weis J, Cantera J, Parker M, Piepenburg O, Overbaugh J, Boyle DS (2016) Cross-subtype detection of HIV-1 using reverse transcription and recombinase polymerase amplification. J Virol Methods 230:28–35
30. Lai MY, Ooi CH, Lau YL (2017) Rapid detection of plasmodium knowlesi by isothermal recombinase polymerase amplification assay. Am J Trop Med Hyg 97(5):1597–1599
31. Mondal D, Ghosh P, Khan MA, Hossain F, Bohlken-Fascher S, Matlashewski G, Kroeger A, Olliaro P, Abd El Wahed A (2016) Mobile suitcase laboratory for rapid detection of Leishmania donovani using recombinase polymerase amplification assay. Parasit Vectors 9(1):281
32. Kersting S, Rausch V, Bier FF, von Nickisch-Rosenegk M (2014a) Multiplex isothermal solid-phase recombinase polymerase amplification for the specific and fast DNA-based detection of three bacterial pathogens. Mikrochim Acta 181(13–14):1715–1723
33. Crannell ZA, Rohrman B, Richards-Kortum R (2014b) Quantification of HIV-1 DNA using real-time recombinase polymerase amplification. Anal Chem 86(12):5615–5619
34. Santiago-Felipe S, Tortajada-Genaro LA, Morais S, Puchades R, Maquieira Á (2014a) One-pot isothermal DNA amplification–hybridisation and detection by a disc-based method. Sens. Actuators B Chem 204:273–281
35. Wee EJ, Lau HY, Botella JR, Trau M (2015a) Re-purposing bridging flocculation for on-site, rapid, qualitative DNA detection in resource-poor settings. Chem Commun (Camb) 51(27):5828–5831
36. Tortajada-Genaro LA, Santiago-Felipe S, Amasia M, Russom A, Maquieira Á (2015) Isothermal solid-phase recombinase polymerase amplification on microfluidic digital versatile discs (DVDs). RSC Adv 5(38): 29987–29995
37. Koo B, Jin CE, Park SY, Lee TY, Nam J, Jang YR, Kim SM, Kim JY, Kim SH, Shin Y (2018) A rapid bio-optical sensor for diagnosing Q fever in clinical specimens. J Biophotonics 11(4):e201700167
38. Shin Y, Perera AP, Kim KW, Park MK (2013) Real-time, label-free isothermal solid-phase amplification/detection (ISAD) device for rapid detection of genetic alteration in cancers. Lab Chip 13(11):2106–2114
39. Schlucker S (2014) Surface-enhanced Raman spectroscopy: concepts and chemical applications. Angew Chem Int Ed Engl 53(19): 4756–4795
40. Gao X, Liu X, Zhang Y, Wei Y, Wang Y (2020) Rapid and visual detection of porcine deltacoronavirus by recombinase polymerase amplification combined with a lateral flow dipstick. BMC Vet Res 16(1):130
41. Ma L, Zeng F, Huang B, Zhu Y, Wu M, Xu F, Xiao L, Huang R, Ma J, Cong F, Guo P (2019) Point-of-care diagnostic assay for rapid detection of porcine deltacoronavirus using the recombinase polymerase amplification method. Transbound Emerg Dis 66(3):1324–1331
42. Fan X, Li L, Zhao Y, Liu Y, Liu C, Wang Q, Dong Y, Wang S, Chi T, Song F, Sun C, Wang Y, Ha D, Zhao Y, Bao J, Wu X, Wang Z (2020) Clinical validation of two recombinase-based isothermal amplification assays (RPA/RAA) for the rapid detection of African swine fever virus. Front Microbiol 11:1696
43. Miao F, Zhang J, Li N, Chen T, Wang L, Zhang F, Mi L, Zhang J, Wang S, Wang Y, Zhou X, Zhang Y, Li M, Zhang S, Hu R (2019) Rapid and sensitive recombinase polymerase amplification combined with lateral flow strip for detecting African swine fever virus. Front Microbiol 10:1004
44. Wang JC, Liu LB, Han QA, Wang JF, Yuan WZ (2017a) An exo probe-based recombinase polymerase amplification assay for the rapid detection of porcine parvovirus. J Virol Methods 248:145–147
45. Yang Y, Qin X, Zhang W, Li Y, Zhang Z (2016b) Rapid and specific detection of porcine parvovirus by isothermal recombinase polymerase amplification assays. Mol Cell Probes 30(5):300–305
46. Wang JC, Yuan WZ, Han QA, Wang JF, Liu LB (2017b) Reverse transcription recombinase polymerase amplification assay for the rapid detection of type 2 porcine reproductive and respiratory syndrome virus. J Virol Methods 243:55–60
47. Yang Y, Qin X, Sun Y, Chen T, Zhang Z (2016a) Rapid detection of highly pathogenic porcine reproductive and respiratory syndrome virus by a fluorescent probe-based isothermal recombinase polymerase amplification assay. Virus Genes 52(6):883–886

Chapter 18

Cell Culture System for Porcine Virus Isolation and Propagation

Vishal Rai, Kaushal Kishor Rajak, Kiran, Arfa Fayaz, Monu Karki, Chris Einstein, Mukesh Bhatt, Ashok Kumar, and Ajay Kumar Yadav

Abstract

Cell culture is an integral part of virology as the viruses are the obligate intracellular parasites that require replication inside a living cell to produce copies of themselves. Since the introduction of cell culture, a number of cell culture systems have been developed which are in use to isolate, propagate, and study growth kinetics vis-à-vis host–virus interactions of a number of virus species affecting diverse range of hosts. These systems comprise of primary cell culture and cell lines having finite or infinite life span. Owing to their immortality and other advantages, continuous cell lines are the most frequently used category of cell culture system for animal viruses. During recent years, traditional cell culture system has changed from the use of glass bottles/flasks to the use of plastic tissue culture flasks and diverse range of culture media have been developed to meet the research needs. This chapter elaborates various cell culture systems which have been developed and are most frequently used for isolation and propagation of common DNA and RNA viruses affecting pigs.

Key words Primary cell culture, Cell lines, Secondary cell culture, Porcine viruses

1 Introduction

There are three systems for isolation of viruses, viz. animal host system, embryonated egg system, and cell culture system. Since the advent of cell culture system, isolation and propagation of viruses has become relatively simple compared to the olden days, when the natural or heterologous animal host system was used predominately. As in case of other viruses, isolation and propagation of animal viruses in cell culture has manifold applications. For example, isolation of viruses serves as the gold standard test for diagnosis of many viral infections. In addition, cell culture system has been used extensively for preparation of live attenuated viral vaccines and production of viral antigen in bulk for the development of different diagnostics. Like many other fields of biomedical science, the field

Rajib Deb et al. (eds.), *Protocols for the Diagnosis of Pig Viral Diseases*,
Springer Protocols Handbooks, https://doi.org/10.1007/978-1-0716-2043-4_18,

of cell culture has also undergone tremendous advancement in the past few years enabling the establishment of different types of cell culture system for different viruses, each with its own merits and demerits based on the virus concerned. In such a situation, it becomes increasingly important to choose the correct system for a particular virus. Several swine viruses continue to affect the swine industry worldwide causing huge economic losses. Swine population is susceptible to various DNA and RNA viruses and hence their detection is pertinent for making accurate diagnosis as well as for their epidemiological assessment. Virus isolation in susceptible cell culture system is fundamental and still one of the best methods for virus identification for laboratory diagnosis. The focus of this chapter lies in the different type of cell culture systems that are available and used most frequently for the isolation and propagation of the common DNA and RNA viruses affecting pigs.

2 Types of Cell Culture System

2.1 Primary and Secondary Cell Culture

Primary cell culture is derived straight from the host. Freshly isolated cells derived from animal/human tissue or organ fragments (either enzymatically or mechanically) that grow successfully, constitute primary cell culture. The cells are mainly heterogenous, grow slowly, divide only for a limited time and are much identical with their parental tissue. Primary cell culture is advantageous as the biochemical dynamics of the cells resembles as in vivo, so biological response obtained may be much closer to that in vivo. The cells cannot be transformed and possibility of undergoing mutation is low. Hence the results obtained with primary cell cultures can be more relevant. But primary culture is difficult to establish and have limited life span. Depending on the requirement for attachment for growth, the primary cells can be classified as anchorage-dependent or adherent cells and anchorage-independent or suspension cells.

A culture is considered primary until the cells are passaged or further subcultured. After first subculture, the primary culture is called as a secondary culture or a cell line. Sub-culturing prolongs the lifespan of the cells, but the cells no longer resemble the parent tissue and chances of mutation increases. The maintenance of secondary cell culture or cell line is easier as compared to the primary cell culture.

2.2 Cell Lines

A cell line represents a population of cells obtained after subculture or passaging of a primary culture. It may be of finite or continuous type. Continuous cell lines are those which comprise of immortal cells, i.e., they experience indefinite growth upon subsequent subcultures, whereas finite cell lines are those which experience death of cells upon sub-culturing. Owing to their immortality and other

advantages, continuous cell lines are the most frequently used category of cell culture system for animal viruses. In the subsequent subsections, we will discuss the commonly used primary cell culture and cell lines for common porcine viruses.

3 Cell Culture System for Porcine DNA Viruses

3.1 African Swine Fever Virus (ASFV)

ASFV belongs to the genus *Asfivirus* of the family *Asfarviridae*. The virus is the causative agent of a fatal disease called African swine fever which causes high mortality among infected swine and is a major concern for swine industries worldwide. It is the only known arthropod borne DNA virus which is transmitted by the soft ticks of genus *Ornithodoros*. African swine fever is an OIE notifiable disease spreading to newer regions and the current strain reported from south east Asian countries including India is highly virulent which caused 100% mortality in domestic pigs [1]. Owing to the international importance of the disease the diagnosis becomes crucial and virus isolation is important for detection of the virus.

Primary swine macrophage culture is used for virus isolation. Swine bone marrow or peripheral blood leukocyte cultures are also used. These primary cultures show hemadsorption and CPE occur within few days of inoculation. The virus can be adapted further in different cell lines like Vero cells [2].

MA-104 (Microbiological Associates-104, from African green monkey kidney), a commercially available cell line has been found to be suitable for the isolation of ASFV from infected field samples [1].

3.2 Porcine Circovirus

Porcine circoviruses belong to the genus *Circovirus* of the family *Circoviridae*. PCV1 and PCV2 are found in both domestic and wild pigs [3–6]. PCV1 has been isolated from stillborn pigs but is generally not considered to be pathogenic for swine [2]. PCV2 has worldwide occurrence and causes huge economic losses to swine industry [7–9]. PCV2 is mainly associated with porcine circovirus diseases (PCVDs) [10]. In weanling piglets it causes post-weaning multisystemic wasting syndrome (PMWS). Currently there is no suitable cytopathogenic cell model for studying the pathogenesis of PCV2. Some cell lines reported to be useful are discussed below.

PCV free PK-15 cell line is widely used for virus isolation but it is not much efficient [11] and presence of virus can only be confirmed using immunofluorescence-based assay [3]. Primary porcine kidney cells are also used for virus isolation. Primary porcine hepatocyte culture was found to be more sensitive for PCV-2 as compared to primary kidney cells [12]. IPEC-J2 (Intestinal Porcine Epithelial Cell line-J2) and an immortal porcine lymphoblastoid cell line (lymphoblastoid L35 cell line) have also shown the

replication of PCV2 [13, 14]. An immortalized porcine oral mucosal epithelial cell line (hTERT-POMEC) from neonatal unsuckled piglet produces CPE and can be a better system for PCV isolation [15].

3.3 Herpesviruses of Swine

Suid alphaherpesvirus 1, Aujeszky's disease virus or pseudorabies virus (PRV).

Genus: *Varicellovirus*

Subfamily: *Alphaherpesvirinae*

Family: *Herpesviridae*

The virus has a wide-host range from infecting its natural host pigs to causing fatal disease in cattle, sheep, dogs, rats, etc. [16]. The terms "pseudorabies" and "mad itch" describe disease in cattle. In pigs, the PRV is associated with respiratory signs and CNS involvement in case of neonatal pigs. Since PRV is having a broad host range, it can be grown in cell lines derived from different species, viz. RK-13 (rabbit kidney epithelial cells), PK-15 (adult *Sus scrofa* kidney epithelial cells), MDBK (Madin–Darby bovine kidney), and Vero cells (African green monkey kidney cells). The virus isolation can be done in ML (Mink lung) and ST (swine testicle) cell lines [17]. CPE generally appears within 6–24 h which is evident as cell lysis and syncytia formation.

Suid betaherpesvirus 2, porcine cytomegalovirus (PCMV).

Genus: *Cytomegalovirus*

Subfamily: *Betaherpesvirinae*

Family: *Herpesviridae*

PCMV is present in nearly all the swine population but it rarely causes any disease except in young piglets in which the disease is fatal. Inclusion body rhinitis is an acute or subacute infection of suckling piglets (4-week-old). In cell culture the virus shows no CPE, hence further immunostaining is required. Porcine pulmonary macrophages are used for the isolation of PCMV. Primary pig lung (PL) cells, primary swine testicle (ST), PK-15 cell line and porcine turbinate (PT) can be used for the propagation of PCMV [18].

3.4 Porcine Parvovirus

Genus: *Protoparvovirus*

Subfamily: *Parvovirinae*

Family: *Parvoviridae*

Porcine parvovirus 1 is the main species of porcine parvoviruses which is known to cause reproductive failure in sows. In unvaccinated herds abortion storms can occur. The virus can be isolated in primary pig kidney cell cultures. Primary cell cultures are not

typically used as there is a risk of contamination by adventitious agents. Various continuous cell lines are used, viz. ESK (embryonic swine kidney), PK-15 (pig kidney), SK6 (swine kidney), ST (pig testis), STE (swine testicle epithelial cells), and SPEV (embryonic porcine kidney epithelial cells) [19, 20]. The virus replicates slowly in cell culture and CPE includes granulations, irregular shape, intranuclear inclusions, pyknotic nucleus followed by cell death [19, 21].

3.5 Porcine Adenovirus (PAdV)

Porcine adenovirus belongs to the genus *Mastadenovirus* of the family *Adenoviridae*. PAdV is generally present worldwide and has been isolated from gastrointestinal tract of normal swine [22]. The virus mainly causes subclinical infections but it has been isolated from pigs suffering from diarrhea [23–25], pigs with encephalitis [26], nephritis [27], respiratory disease [28], and reproductive disorders [29].

The virus is easily cultured from the fecal samples or kidney/lung homogenates using primary porcine kidney cells, PK-15 cell line, primary porcine thyroid cells, and primary porcine testicular cells. Cytopathic effects (intranuclear inclusion bodies) can be seen 2–5 days post infection [30, 31]. PAdV is often an adventitious agent in the porcine primary cell cultures derived from kidneys, spleen, or testes [28] creating complications in the virus isolation.

3.6 Swinepox Virus

Genus: *Suipoxvirus*

Subfamily: *Chordopoxvirinae*

Family: *Poxviridae*

The virus is present sporadically worldwide and is associated with poor sanitation. Clinical disease is seen mainly in young pigs with high morbidity but low mortality. Skin lesions are more pronounced on the ventral abdomen, flanks, legs, inguinal areas, and ears and less on the face near the eyes [32]. Virus isolation can be done in primary swine kidney cells, or PK-15 cell line. The characteristic CPEs include cell rounding, vacuolation of nucleus, cell granulation and fusion followed by detachment. The sample should be considered negative only after multiple blind passages have been done in the cell culture.

4 Cell Culture System for Porcine RNA Viruses

4.1 Classical Swine Fever Virus

Classical swine fever virus (CSFV), a member of the genus *Pestivirus* in the *Flaviviridae* family is the causative agent of swine fever or hog cholera disease of pigs. It is considered as one of the most economically devastating disease causing huge mortality. Members of the *Suidae* family such as domestic and wild pigs are considered the only natural reservoir of CSFV [33]. Related pestiviruses

causing disease in other animals include border disease virus (BDV), bovine viral diarrhea virus-1 (BVDV-1), and BVDV-2.

Porcine kidney-15 (PK-15) cells are perhaps the most widely used cells for isolation and propagation of porcine viruses. It has been used extensively for the isolation and propagation of CSFV. PK-15 cells is one amongst the three cell lines recommended for CSFV isolation/diagnosis, with the other two being swine kidney 6 (SK6) and swine testis endothelial (STE) cell lines [34]. It is recommended that circovirus-free PK-15 cells be used for CSFV diagnosis as porcine circovirus 1 (PCV1) is a frequent contaminant of PK-15 cells. In addition, interference in CSFV diagnosis due ruminant pestiviruses is also a possibility since porcine cells are more or less susceptible to these viruses. Hence, it becomes important to demonstrate the absence of ruminant pestiviruses in the cell lines used for CSF diagnosis as well. To minimize the risk of contamination with ruminant pestiviruses, the aforementioned cell lines can be adapted to grow in horse serum instead of the fetal bovine serum or calf serum. Since CSFV are non-cytopathic viruses, they do not cause any visible alteration in the porcine cell lines. Consequently, their detection in the cell culture system relies on indirect techniques such as immunofluorescence or immunoperoxidase staining, RT-PCR, etc. [34].

4.2 Foot and Mouth Disease Virus

Foot and mouth disease is also one of the most economically devastating diseases of pigs. However, this disease is not restricted to swine alone as it affects all cloven-footed animals. The etiological agent is foot and mouth disease virus (FMDV) of *Aphthovirus*, a genus within the *Picornaviridae* family. Seven different serotypes including O, A, C, Asia 1, Southern African Territories (SAT) 1, SAT 2, and SAT 3 have been recognized for this virus till date. The isolation of FMDV in susceptible cell lines has important benefits; first is the accurate diagnosis of the serotype involved in an outbreak and second is the production of vaccines, which in turn are crucial determinants in the control of FMD.

Primary bovine thyroid (BTY) cell culture system has been demonstrated to be the most sensitive for FMDV isolation [35]. But this system has several disadvantages such as difficulty in obtaining thyroid tissue, considerable time and expense required in the preparation, and the relatively short life span of these primary cells. So, the isolation and propagation of FMDV is commonly done using the cell line system in most of the laboratories. Among the cell lines, baby hamster kidney fibroblasts (BHK-21) and pig kidney (IB-RS-2/InstitutoBiologico-Rim Suino-2) cells are the ones which are most frequently used, although they are less susceptible than BTY cell culture system. Nonetheless, IB-RS-2 are considered the most susceptible and thus commonly required for the isolation of pig adapted strains of FMDV as several pig adapted strains do not replicate in BTY cells [36].

Over the years, novel cell lines expressing the cellular receptor for FMDV (bovine αVβ6 integrin) have been engineered. It includes: fetal porcine kidney (LFBK-αVβ6) cells and fetal goat tongue cells (ZZ-R 127). These cell lines have been shown to possess equal sensitivity to BTY cells in isolating FMDV. LFBK-αVβ6 in addition possesses high sensitivity to pig adapted strains of FMDV, highlighting its emergence as a novel cell culture system for diagnosis of FMD in pigs [37].

4.3 Porcine Rotavirus

Porcine rotaviruses (PoRV) are one of the major causes of diarrhea in pigs. They are members of the *Rotavirus* genus within the *Reoviridae* family. Ten groups (A-J) of rotaviruses have been identified till date based on one of the capsid protein (VP6), out of which groups A, B, C, E, and H are associated with disease in pigs [38]. A cell line designated MA-104 (Microbiological Associates-104), obtained from the kidney of African green monkey is the most frequently used cell culture system for the isolation and propagation of porcine rotaviruses.

Permissive cells in addition to MA-104 for propagation of Group A rotaviruses include colon adenocarcinoma, HepG2 liver cells, pancreatic islet cells, and other kidney cell lines [39]. Reports of isolation of group B rotavirus in cell cultures are scarce. A report of isolation of a group B rotavirus (strain SKA-1) in SKL cells (swine kidney cells) combined with pancreatin treatment is available [40]. But, later on it was demonstrated that SKA-1 strain of PoRV actually belonged to group H rotavirus [41]. In addition to MA-104, few strains of group C rotaviruses have been cultured in primary porcine kidney cells, in combination with high pancreatin or trypsin concentrations [42].

Addition of protease such as trypsin, chymotrypsin, or pancreatin in cell culture medium for rotavirus isolation and propagation is important as proteolytic treatment specifically cleaves the spike forming outer capsid protein of rotaviruses (VP4) into polypeptides VP5 and VP8, which in turn enhances the rotavirus infectivity [43]. As some rotavirus strains are difficult to propagate in cell culture, the search for a better cell culture system is going on. In this line, co-cultures of primary porcine intestinal cells (ileocytes and colonocytes) with myofibroblasts have been developed and these primary cells were found to be susceptible to different strains of rotavirus [44]. Whether these cells are more susceptible to rotavirus isolation remains to be seen.

4.4 Porcine Reproductive and Respiratory Syndrome Virus (PRRSV)

PRRSV belongs to the genus *Arterivirus* of the family *Arteriviridae* and is an important pathogen of swine population all over the globe. There are two distinct genotypes of the virus, type 1 porcine reproductive and respiratory syndrome virus (European) and type 2 porcine reproductive and respiratory syndrome virus (North American). The virus affects all age groups and as the name

suggests, PRRSV affects the reproductive and respiratory system of the affected pigs. The virus is the cause of SMEDI syndrome in pregnant sows.

For isolation of PRRSV, primary alveolar macrophage cultures (PAM) from young pig and monkey kidney cell line MA-104 (African green monkey kidney epithelial cell) are frequently used. MARC-145 (a derivative of MA-104) can be used preferentially for isolation of vaccine strains. The cytopathic effects appear as microscopic foci throughout the culture within 72–96 h post inoculation [45]. In contrast to PRRSV 2 which easily adapt to simian cell culture, PRRSV 1 isolates are difficult to adapt to simian cells (like MA-104) but grow readily on porcine macrophages. An efficient MARC-145 cell line expressing porcine CD163 (pCD163) with higher susceptibility and higher isolation rate of PRRSV has been established [46].

4.5 Porcine Coronaviruses

4.5.1 Transmissible Gastroenteritis Virus (TGEV)

TGEV belongs to the genus *Alphacoronavirus* of the family *Coronaviridae*. It is the cause of highly fatal enteritis in swine with 100% mortality in young piglets. The virus can be isolated from fecal samples or gut contents from infected pigs in primary and secondary pig kidney cells (Bohl and [47]), and in ST (swine testicle) cell line [48].

Additional passages should be done after attempting primary isolation from field samples as CPE may not appear upon primary isolation. Infected cells become enlarged, round, and balloon-like [47]. Pig thyroid cells can be used for initial isolation as they show progressive cytopathic effect after TGEV inoculation, but they should be strictly free from adventitious agents like parvoviruses [49]. Supplementing cell culture media with trypsin or pancreatin can result in increased susceptibility and better CPE or plaque detection [50].

4.5.2 Porcine Epidemic Diarrhea Virus (PEDV)

PEDV is also a member of the genus *Alphavirus* under the family *Coronaviridae*. It causes porcine epidemic diarrhea which is clinically similar to transmissible gastroenteritis caused by TGEV. Different strains of PEDV are reported to be circulating worldwide. Neonatal pigs are more susceptible to the infection in which high mortality occurs [51].

Intestinal contents or fecal samples can be used for virus isolation. Virus isolation can be done using vero cells and different porcine cell cultures, like porcine bladder and kidney cells [52]. Trypsin should be added in the cell culture media. The cytopathic effect can be seen around 30 h post inoculation characterized by cell fusion and large syncytia with appearance of floating cells overtime. In absence of CPE, culture should not be considered negative upto 5 days post inoculation [53]. Sometimes blind passages may be required, but for early detection immunofluorescence staining can be done [52, 54].

4.6 Swine Influenza Virus

Swine influenza virus is associated with outbreaks of acute respiratory disease in pigs [55]. The virus belongs to the genus *Influenzavirus A* of the family *Orthomyxoviridae*. Common subtypes of the virus infecting swine are H1N1, H1N2, and H3N2. Pigs are known to play an important role in the reassortment events as intermediate hosts, thus leading to the development of subtypes of pandemic potential [56, 57]. The influenza pandemics of 1918 and 2009 were the results of efficient person-to-person transmission of swine influenza virus [58].

Currently, MDCK (Madin–Darby Canine Kidney) cell line is used most frequently for the isolation, propagation, and titration of swine influenza virus although primary cell cultures from swine organs (kidney, testicle, lung, and trachea) can also be used [59]. Addition of trypsin to basal media is recommended [60]. CPE starts to appear after 24 h of inoculation and is evident as enlarged focal vacuolation of cells followed by cell detachment [61].

4.7 Japanese Encephalitis Virus

Japanese encephalitis is a zoonotic virus transmitted by mosquitoes (*Culex* sp.). Domestic pigs and wild birds are the natural hosts for the virus and humans are dead-end host. Pigs also act as amplifying host for JEV.

The virus isolation can be performed in various cells including primary chicken embryo, Vero cells (African green monkey kidney), and BHK (baby hamster kidney) cells. The CPE is visible after 24 h of inoculation. Mosquito cell line, *Aedes albopictus* C6/36 is also used but as there is no CPE, further culture in other cells or detection of viral RNA/ viral antigen for confirmation is required [62].

4.8 Other Vesicular Disease Viruses

4.8.1 Vesicular Stomatitis Virus (VSV)

The VSV virus belongs to the genus *Vesiculovirus* of the family *Rhabdoviridae*. There are two serotypes of VSV, vesicular stomatitis New Jersey virus (VSNJV) and vesicular stomatitis Indiana virus (VSIV) [63]. Swine disease is associated only with VSNJV while for domestic livestock both the serotypes are pathogenic. Vesicular stomatitis is clinically indistinguishable from other vesicular diseases of swine (FMD, or swine vesicular disease, or vesicular exanthema), therefore laboratory confirmation is imperative for correct diagnosis. Humans in close contact with the infected animals are also susceptible to the virus.

The virus can be easily propagated in cell culture. Cytopathic effects observed in different cell lines such as vero, BHK-21, and pig kidney IB-RS-2 cell line can distinguish VSV from FMD and swine vesicular disease [64].

4.8.2 Swine Vesicular Disease Virus (SVDV)

SVDV belongs to the species *Enterovirus B* of the genus *Enterovirus* under the family *Picornaviridae*. Swine vesicular disease was an OIE-listed disease until 2015, as the disease is clinically similar to FMD. The disease does not cause much production losses and with the advent of newer diagnostic techniques it can easily be differentiated from FMD. Also the clinical signs of the disease are milder than FMD.

Primary or secondary porcine kidney cell cultures are susceptible to the virus [65]. Cell lines such as IB-RS-2, SK6, and PK-15 are also used. Isolation of virus on IB-RS-2 cells is one of the most sensitive methods for diagnosis [66].

4.8.3 Swine Vesicular Exanthema Virus

The virus belongs to the genus *Vesivirus* of the family *Caliciviridae*. The disease caused by the virus is highly infectious but mortality is not common. Clinically the disease is indistinguishable from other vesicular diseases of swine mentioned before. Isolation of the virus can be done in primary porcine cell cultures such as porcine kidney cells and in monkey kidney cells (Vero cell line). The virus replicates rapidly in cell culture resulting in destructive cytopathic effects [67].

References

1. Rai A, Pruitt S, Ramirez-Medina E, Vuono EA, Silva E, Velazquez-Salinas L, Carrillo C, Borca MV, Gladue DP (2020) Identification of a continuously stable and commercially available cell line for the identification of infectious African swine fever virus in clinical samples. Viruses 12 (8):820
2. Maclachlan NJ, Dubovi EJ, Barthold SW, Swayne DF, Winton JR (2017) Fenner's veterinary virology. Academic Press, London
3. Allan GM, Ellis JA (2000) Porcine circoviruses: a review. J Vet Diagn Investig 12(1):3–14
4. Calsamiglia M, Segalés J, Quintana J, Rosell C, Domingo M (2002) Detection of porcine circovirus types 1 and 2 in serum and tissue samples of pigs with and without postweaning multisystemic wasting syndrome. J Clin Microbiol 40(5):1848–1850
5. Segales J, Domingo M (2002) Postweaning-mulstisystemic wasting syndrome (PMWS) in pigs.A review. Vet Q 24(3):109–124
6. Vicente J, Segalés J, Höfle U, Balasch M, Plana-Durán J, Domingo M, Gortázar C (2004) Epidemiological study on porcine circovirus type 2 (PCV2) infection in the European wild boar (Sus scrofa). Vet Res 35 (2):243–253
7. Novosel D, Lipej Z, Cubric-Curik V, Jungic A (2012) Presence of torque tenosus virus in porcine circovirus type 2-associated disease in Croatia. Vet Rec 17:529
8. O'Dea MA, Kabay MJ, Carr J, Wilcox GE, Richards RB (2011) Porcine circovirus-associated disease in weaner pigs in Western Australia. Aust Vet J 89(4):122–130
9. Wilfred E, Mutebi F, Mwiine FN, James OA, Lonzy O (2018) Porcine circovirus type 2–systemic disease on pig farms and associated knowledge of key players in the pig industry in Central Uganda. Int J Vet Sci Med 6 (2):178–185
10. Allan GM, McNeilly F, McNair I, Curran MD, Walker I, Ellis J, Konoby C, Kennedy S, Meehan B (2000) Absence of evidence for porcine circovirustype 2 in cattle and humans, and lack of seroconversion or lesions in experimentally infected sheep. Arch Virol 145(4):853–857
11. Zhu Y, Lau A, Lau J, Jia Q, Karuppannan AK, Kwang J (2007) Enhanced replication of porcine circovirus type 2 (PCV2) in a homogeneous subpopulation of PK15 cell line. Virology 369(2):423–430
12. Hirai T, Nunoya T, Ihara T, Saitoh T, Shibuya K, Nakamura K (2006) Infectivity of porcine circovirus 1 and circovirus 2 in primary

porcine hepatocyte and kidney cell cultures. J Vet Med Sci 68(2):179–182

13. Rodriguez-Carino C, Duffy C, Sanchez-Chardi A, McNeilly F, Allan GM, Segales J (2011) Porcine circovirus type 2 morphogenesis in a clone derived from the l35 lymphoblastoid cell line. J Comp Pathol 144(2–3):91–102
14. Yan M, Zhu L, Yang Q (2014) Infection of porcine circovirus 2 (PCV2) in intestinal porcine epithelial cell line (IPEC-J2) and interaction between PCV2 and IPEC-J2 microfilaments. Virol J 11(1):193
15. Cui H, Liang W, Wang D, Guo K, Zhang Y (2019) Establishment and characterization of an immortalized porcine oral mucosal epithelial cell line as a cytopathogenic model for porcine circovirus 2 infection. Front Cell Infect Microbiol 9:171
16. Pensaert MB, Kluge JP (1989) Pseudorabies virus (Aujeszky's disease). In: Pensaert MB (ed) Virus infections of Porcines. Elsevier, New York, pp 39–64
17. Onyekaba C, Bueon L, King P, Fahrmann J, Goyal SM (1987) Susceptibility of various ceil culture systems to pseudorabies virus. Comp Immunol Microbiol Infect Dis 10 (3–4):163–166
18. Yoon KJ, Edington N (2006) Porcine cytomegalovirus. In: Straw BE, Zimmerman J, D'Allaire S et al (eds) Diseases of swine, 9th edn. Blackwell Publishing Company, Ames, IA, pp 323–329
19. Mengeling WL (1972) Porcine parvovirus: properties and prevalence of a strain isolated in the United States. Am J Vet Res 33: 2239–2248
20. Zimmermann P, Ritzmann M, Selbitz HJ, Heinritzi K, Truyen U (2006) VP1 sequences of German porcine parvovirus isolates define two genetic lineages. J Gen Virol 87 (2):295–301
21. Cartwright SF, Lucas M, Huck RA (1969) A small haemagglutinating porcine DNA virus: I. isolation and properties. J Comp Pathol 79 (3):371–377
22. Horak S, Killoran K, Leedom Larson KR (2016) Vesicular exanthema of swine virus. Swine Health Information Center and Center for Food Security and Public Health
23. Coussement W, Ducatelle R, Charlier G, Hoorens J (1981) Adenovirus enteritis in pigs. Am J Vet Res 42(11):1905–1911
24. Haig DA, Clarke MG, Pereira MS (1964) Isolation of an adenovirus from a pig. J Comp Pathol Ther 74:81–IN11
25. McAdaragh JP, Eustis S, Benfield DA (1980). Adenovirus associated with diarrhea in pigs. Abstract. In Conference of Research Workers in Animal Diseases, Chicago, IL, 10–11 November
26. Kasza L (1966) Isolation of an adenovirus from the brain of a pig. Am J Vet Res 27(118):751
27. Nietfeld JC, Leslie-Steen P (1993) Interstitial nephritis in pigs with adenovirus infection. J Vet Diagn Investig 5(2):269–273
28. Hirahara T, Yashuhara H, Matsui O, Yamanaka M, Tanaka M, Fukuyama S, Izumida A, Yoshiki K, Kodama K, Nakai M et al (1990) Isolation of porcine adenovirus from the respiratory tract of pigs in Japan. Japanese J Vet Sci 52(2):407–409
29. Dee SA (1995) Viral causes of porcine reproductive failure. 1. Compend Contin Educ Pract Vet 17(7):962–972
30. Benfield DAH, Richard A (2012) Porcine adenoviruses. In: Zimmerman JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW (eds) Diseases of Swine, 10th edn. John Wiley & Sons, Inc., New York, pp 392–395
31. Clarke MC, Sharpe HB, Derbyshire JB (1967) Some characteristics of three porcine adenoviruses. Arch Gesamte Virusforsch 21 (1):91–97
32. Delhon G, Tulman ER, Afonso CL, Rock DL (2012) Swinepox virus. In: Zimmerman JJ, Karriker LA, Ramirez A et al (eds) Diseases of swine, 10th edn. Wiley, New York, pp 456–460
33. Everett H, Crooke H, Gurrala R, Dwarka R, Kim J, Botha B, Lubisi A, Pardini A, Gers S, Vosloo W, Drew T (2011) Experimental infection of common warthogs (Phacochoerus africanus) and bushpigs (Potamochoerus larvatus) with classical swine fever virus. I: susceptibility and transmission. Transbound Emerg Dis 58 (2):128–134
34. Ganges L, Crooke HR, Bohórquez JA, Postel A, Sakoda Y, Becher P, Ruggli N (2020) Classical swine fever virus: the past, present and future. Virus Res 289:198151
35. Ferris NP, King DP, Reid SM, Hutchings GH, Shaw AE, Paton DJ, Goris N, Haas B, Hoffmann B, Brocchi E, Bugnetti M (2006) Foot-and-mouth disease virus: a first interlaboratory comparison trial to evaluate virus isolation and RT-PCR detection methods. Vet Microbiol 117(2–4):130–140
36. Dunn CS, Donaldson AI (1997) Natural adaption to pigs of a Taiwanese isolate of foot-and-mouth disease virus. Vet Rec 141(7):174–175
37. Gray AR, Wood BA, Henry E, Azhar M, King DP, Mioulet V (2020) Evaluation of cell lines for the isolation of foot-and-mouth disease virus and other viruses causing vesicular disease. Front Vet Sci 7:426

38. Marthaler D, Rossow K, Culhane M, Goyal S, Collins J, Matthijnssens J, Nelson M, Ciarlet M (2014) Widespread rotavirus H in commercially raised pigs, United States. Emerg Infect Dis 20(7):1203
39. Arnold M, Patton JT, McDonald SM (2009) Culturing, storage, and quantification of rotaviruses. Curr Protoc Microbiol, Chapter 15: Unit 15C.3
40. Sanekata T, Kuwamoto Y, Akamatsu S, Sakon N, Oseto M, Taniguchi K, Nakata S, Estes MK (1996) Isolation of group B porcine rotavirus in cell culture. J Clin Microbiol 34 (3):759–761
41. Zimmerman JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW, Zhang J (2019) Diseases of swine (Eleventh Edition). Wiley-Blackwell, Hoboken, NJ. Print ISBN: 9781119350859, Online ISBN: 9781119350927. https://doi.org/10.1002/9781119350927
42. Saif LJ, Terrett LA, Miller KL, Cross RF (1988) Serial propagation of porcine group C rotavirus (pararotavirus) in a continuous cell line and characterization of the passaged virus. J Clin Microbiol 26(7):1277–1282
43. Arias CF, Romero P, Alvarez V, Lopez S (1996) Trypsin activation pathway of rotavirus infectivity. J Virol 70(9):5832–5839
44. Cui T, Theuns S, Desmarets LM, Xie J, De Gryse GM, Yang B, Van den Broeck W, Nauwynck HJ (2018) Establishment of porcine enterocyte/myofibroblast co-cultures for the growth of porcine Rota-and coronaviruses. Sci Rep 8(1):1–17
45. De Abin MF, Spronk G, Wagner M, Fitzsimmons M, Abrahante JE, Murtaugh MP (2009) Comparative infection efficiency of porcine reproductive and respiratory syndrome virus field isolates on MA104 cells and porcine alveolar macrophages. Can J Vet Res 73(3):200
46. Wu X, Qi J, Cong X, Chen L, Hu Y, Yoo D, Wang G, Tian F, Li F, Sun W, Chen Z (2018) Establishment and characterization of a high and stable porcine CD163-expressing MARC-145 cell line. BioMed Res Int 2018:4315861
47. Bohl EH, Kumagai T (1965) The use of cell cultures for the study of swine transmissible gastroenteritis virus. In: Proceedings of 69th Annual Meeting of United States livestock sanitary association meeting. pp 343–350
48. McClurkin AW, Norman JO (1966) Studies on transmissible gastroenteritis of swine: II. Selected characteristics of a cytopathogenic virus common to five isolates from transmissible gastroenteritis. Can J Comp Med Vet Sci 30 (7):190
49. Dulac GC, Ruckerbauer GM, Boulanger P (1977) Transmissible gastroenteritis: demonstration of the virus from field specimens by means of cell culture and pig inoculation. Can J Comp Med 41(4):357
50. Bohl E (1979) Diagnosis of diarrhea in pigs due to transmissible gastroenteritis virus or rotavirus. In: Bricout F, Scherrer R (eds) Viral enteritis in humans and animals. INSERM, Paris, France, pp 341–343
51. Jung K, Saif LJ, Wang Q (2020) Porcine epidemic diarrhea virus (PEDV): an update on etiology, transmission, pathogenesis, and prevention and control. Virus Res 286:198045
52. Shibata I, Tsuda T, Mori M, Ono M, Sueyoshi M, Uruno K (2000) Isolation of porcine epidemic diarrhea virus in porcine cell cultures and experimental infection of pigs of different ages. Vet Microbiol 72 (3–4):173–182
53. Yang DK, Kim HH, Lee SH, Yoon SS, Park JW, Cho IS (2018) Isolation and characterization of a new porcine epidemic diarrhea virus variant that occurred in Korea in 2014. J Vet Sci 19 (1):71–78
54. Hofmann M, Wyler R (1988) Propagation of the virus of porcine epidemic diarrhea in cell culture. J Clin Microbiol 26(11):2235–2239
55. Terebuh P, Olsen CW, Wright J, Klimov A, Karasin A, Todd K, Zhou H, Hall H, Xu X, Kniffen T, Madsen D (2010) Transmission of influenza a viruses between pigs and people, Iowa, 2002–2004. Influenza Other Respir Viruses 4(6):387–396
56. Subbarao K, Murphy BR, Fauci AS (2006) Development of effective vaccines against pandemic influenza. Immunity 24(1):5–9
57. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y (1992) Evolution and ecology of influenza a viruses. Microbiol Mol Biol Rev 56(1):152–179
58. Kshatriya RM, Khara NV, Ganjiwale J, Lote SD, Patel SN, Paliwal RP (2018) Lessons learnt from the Indian H1N1 (swine flu) epidemic: predictors of outcome based on epidemiological and clinical profile. J Family Med Prim Care 7(6):1506
59. Zhang J, Gauger PC (2020) Isolation of swine influenza a virus in cell cultures and Embryonated chicken eggs. In: Animal Influenza Virus. Humana, New York, NY, pp 281–294
60. Herman M, Haugerud S, Malik YS, Goyal SM (2005) Improved in vitro cultivation of swine influenza virus. Int J Appl Res Vet Med 3 (2):124–128
61. Zuhairi FR, Maharani TM, TAN M (2012) The role of trypsin in the internalisation

process of influenza H1N1 virus into Vero and MDCK cell. ITB J Sci 44:297–293

62. Williams DT, MacKenzie JS, Bingham J (2019) Flaviviruses. In: Zimmerman JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW, Zhang J (eds) Diseases of Swine, 11th edn. Jon Wiley & Sons, Inc., New York, pp 530–544
63. Cartwright B, Brown F (1972) Serological relationships between different strains of vesicular stomatis virus. J Gen Virol 16 (3):391–398. https://doi.org/10.1099/0022-1317-16-3-391. PMID: 4342823
64. Lambert T, Leedom Larson KR (2016) Vesicular stomatitis virus. Swine Health Information Center and Center for Food Security and Public Health
65. Nardelli L, Lodetti E, Galandi GL, Burrows R, Goodridge D, Brown F, Cartwright B (1968) A foot and mouth disease syndrome in pigs caused by an enterovirus. Nature 219 (5160):1275–1276
66. De Castro MP (1964) Behaviour of the foot-and-mouth disease virus in cell cultures: susceptibility of the IB-RS-2 cell line. Arq Inst Biol 31:63–78
67. Horak S, Leedom Larson KR (2016) Porcine adenovirus. Swine Health Information Center and Center for Food Security and Public Health

Chapter 19

An Overview of Mouse Monoclonal Antibody Production

Kaushal Kishor Rajak, Kiran, Arfa Fayaz, Vishal Rai, Monu Karki, Chris Einstein, Mukesh Bhatt, Ashok Kumar, Ajay Kumar Yadav, and R. P. Singh

Abstract

Antibodies are an important tool in the field of diagnostics and therapeutics owing to their affinity to bind with specific antigen. These can be of two types: polyclonal antibodies that are produced by a mixture of various B lymphocyte clones, and monoclonal antibodies (mAbs) which are secreted by a single clone of B lymphocytes. mAbs have a higher affinity to the target protein and are highly selective in nature which makes them the best choice for specific purposes. Since the development of hybridoma technology in 1975, a number of mAbs have been developed and are in use for therapeutic and diagnostic purposes. Besides the recent advances in high throughput mAb generation technologies, hybridoma is the most preferred method due to its nature to maintain the inherent antibody structure and functional information. This chapter focuses on the basics of hybridoma technology including various steps involved in production of mAbs and various critical points which must be considered while generation of monoclonal antibodies.

Key words Hybridoma, Monoclonal antibodies, Therapeutics, Diagnostics, mAb production

1 Monoclonal Antibody (mAb)

Antibodies are the serum immunoglobulins (Igs) that have ability to bind with a particular antigen. Antibodies are therefore of enormous utility in applications such as experimental biology, medicine, biomedical research, diagnostic testing, and therapy. Polyclonal antisera are still used in diagnostic serology and in antigen detection. However, polyclonal antibodies are often unsuitable for such assays because they contain immunoglobulins that vary in specificity, affinity, class, and subclass. The sensitivity of immunoassays such as immunofluorescence antibody test (IFAT) or enzyme-linked immunosorbent assays (ELISA) is limited largely by antibody affinity to antigen [1, 2]. To this end, high affinity monoclonal antibodies appear to be an attractive option and the antibodies with the

Rajib Deb et al. (eds.), *Protocols for the Diagnosis of Pig Viral Diseases*,
Springer Protocols Handbooks, https://doi.org/10.1007/978-1-0716-2043-4_19,

highest affinity for an antigen may be obtained conveniently by screening among pertinent monoclonal antibodies (mAbs).

[As an aside, mini-antibodies are artificial immunoglobulins lacking CH1 domain. Both the chains of mini-antibody are expressed separately in prokaryotic and eukaryotic expression system linked to single chain consisting of different specificities. The decreased size of mini-antibodies helps easy penetration of tissue hence improved neutralization of the antigen.]

In 1975, Kohler and Milstein bio-engineered the exquisite cells imitating the role of a single unit with capabilities to function as natural plasma cell and immortalized myeloma cell [3]. The technique allows producing monoclonal antibody (mAb) indefinitely with programmed specificity. However, it can only produce antibodies for the specific epitope. Therefore, to produce different antibodies targeted against different epitopes, there is a need of increase in the number of mAb producing lymphocytes to the desired extent. Consequently, on exposure to certain antigen there is sensitization of lymphocyte which has the capacity to recognize the particular antigen, and replicate to create more similar cells to produce monoclonal antibodies [4].

Myeloma cells are clones of plasma cells, a white type of blood cell in the bone marrow and important part of immune system. B cells mature and grow into plasma cells which are produced in large number when body is under the attack of any microbe to produce an army of antibodies to fight against the infection and disease [5]. Sometimes, body loses control on the process of development of a normal antibody-producing cell and it becomes cancerous which leads to the formation of a tumor, loses the ability to identify a particular antigen and causes abnormalities in the immune response. These cells are called myeloma cells (National Research Council 1999). Although, plasma cells are highly specialized cells as they are under the control of biological laws and lack the capacity to survive indefinitely in vitro. However, this control mechanism reverses in the myeloma cells and they learn to divide infinitely [6].

2 How Hybridomas Are Produced?

The production of hybridoma is a tedious work. It starts with the preparation of the antigen and ends with characterization of the monoclonal antibody. Below, there is a brief description of the steps involved in the process (Fig. 1).

2.1 Antigen/ Immunization Materials

Selection of immunizing material is very critical for production of mAbs. For production of monoclonal antibodies, various workers have opted different material as an immunizing agent like ultra-purified virus antigen and crude virus antigen concentrated with poly ethylene glycol (PEG) [7, 8]. Even some people have used

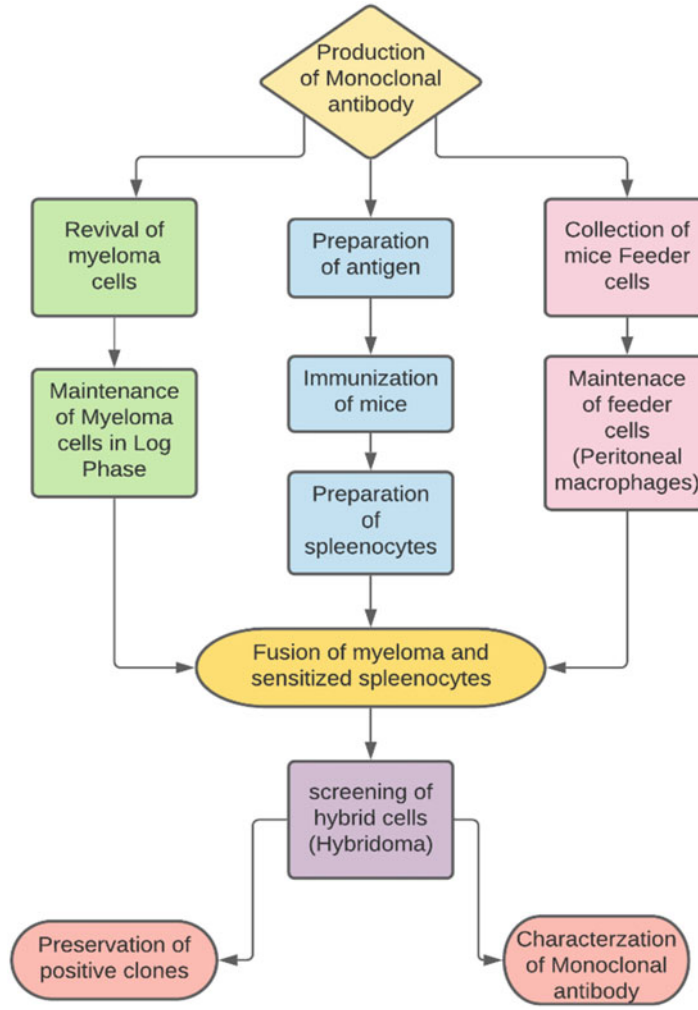


Fig. 1 Outline of production of monoclonal antibody

virus infected cell culture lysate for immunization after subjecting it to sonication and centrifugation [9, 10]. The immunization protocol does not show significant variations among various workers. The immunizing material was inoculated on two, three, or more occasions. The efficiency of the fusion varied and primarily depended on immunizing material and the protocol of immunization.

2.2 Production of Antigen

The most commonly used method for antigen production is by infecting the cell monolayer with virus. The culture is maintained and checked routinely for cytopathic effect (CPE) specific to virus, viz. structural changes in the morphology of the host cell, granulation, rounding of the cell, syncytia formation, etc. When the flask attains 90% of the CPE, the culture is harvested. The antigen

produced in bulk is precipitated using polyethylene glycol (PEG) with sodium chloride overnight at 37 °C. The solution is then subjected to ultracentrifugation. The antigen is emulsified with Freund's adjuvant or other adjuvants used for immunization.

Another method is the production of recombinant antigen in which the gene of interest is cloned in the bacterium by inserting a positive plasmid into *E. coli*. The expression of recombinant protein is followed by screening of transformant for plasmid construct with gene of interest. The expression of recombinant protein is then induced by IPTG (isopropyl-β-D-thiogalactoside). These bacteria are then harvested and further lysed by sonication. For purification of the sonicated product, it is centrifuged and washed with PBS (Phosphate buffered saline) followed by affinity chromatography. Sometimes, microorganisms, intact cells, and whole membranes are also chosen for immunization [11].

Mostly, whole virus antigen is preferred for the production of monoclonal antibodies as they are similar to the shape and structure which gives us monoclonal antibody with specificity to diverse epitope. However, this process is cumbersome as it becomes tedious while screening and expansion for all the positive clones obtained after fusion. Hence, some researchers prefer recombinant virus particle as they provide antibodies against specific epitope. However, it has its own drawback in the sense that it is only preferred where the antigen is used as a positive control and required in bulk.

2.3 Mice for mAb Production

Generally, Balb/c mice of 4–6 weeks of age of either sex weighing 16–20 g are used for production of monoclonal antibodies. Although swiss albino mice can also be used, this may result in low efficacy of mAb production as compared to Balb/c mice. In specific cases, immunocompromised (e.g., severe combined immunodeficient [SCID]) mice or other animal species such as the rat and hamster may also be used [6]. The rationale behind choosing the BALB/c mice is their capability to induce humoral immune response and production of plasmacytoma upon injection with mineral oil which are important for monoclonal antibody production. Further, as myeloma cell lines are of BALB/c mice origin and the ascites fluid from hybridomas is grown in mice which should share BALB/c histocompatibility, BALB/c mice appear to be the best choice for immunization to prevent the tumor rejection [12].

2.4 Immunization of Mice

Firstly, mice are primed with the antigen and then boosters are given every 2–3 weeks to produce the desired antibody titer. After several weeks of immunization, mice are assessed for serum antibody titer by collecting the blood samples. Serum antibody titer is determined by various techniques including serum neutralization test (SNT), ELISA, flow cytometry, etc. If the antibody titer is high enough, then mice are boosted 2–3 days before the fusion (Fig. 2).

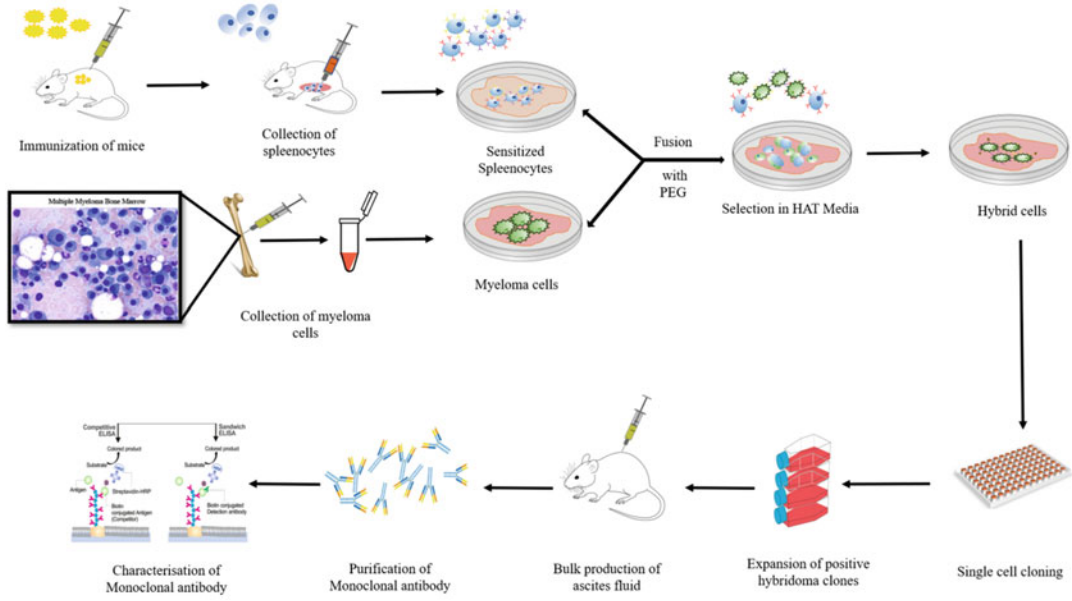


Fig. 2 Schematic representation of principle steps involved in monoclonal antibody production

2.5 Revival of Myeloma Cells and Feeder Cells Preparation

Myeloma cells are revived from liquid nitrogen at the required time and maintained for fusion. Generally, myeloma cells are grown with 8-azaguanine or 6-thioguanine to ensure their sensitivity for HAT (Hypoxanthine Aminopterin Thymidine) media. Myeloma cells used for fusion could be HGPRT (hypoxanthine-guanine phosphoribosyltransferase) negative or TK (thymidine kinase) negative that means they cannot use the salvage pathway for nucleotide synthesis [13] as per Table 1.

Additionally, aminopterin in medium blocks the pathway that allows for nucleotide synthesis via de novo pathway. Hence, unfused myeloma cell dies because they are unable to synthesize nucleotide. Although unfused B cells can synthesize nucleotide via salvage pathway, they die because of the limited life span. Only the fused cell survives in the HAT medium. The feeder cells are basically the peritoneal macrophages that are collected from the mice and seeded approximately 48–72 h before the fusion.

2.6 Fusion

The immunized mice are sedated using volatile ether/chloroform and spleen is taken out from the peritoneal cavity followed by collection of sensitized splenocytes under aseptic conditions. Both the myeloma cells and spleen lymphocytes are counted on Neubauer's chamber followed by dilution in the ratio of 1:10, respectively for fusion. Fusion is completed with both myeloma and spleen cells in polyethylene glycol (PEG) strictly at 37 °C which helps in cell membrane fusion [14]. Then, fused cells are incubated in the HAT medium. The fused cells are screened for reactivity with

Table 1
Sensitivity of different cells to HAT media

Genotype	Cell type	HAT medium	Result
TK^- or $HGPRT^-$	Immortal cell	Dies	Unable to synthesize nucleotide
TK^+TK^-or $HGPRT^+HGPRT^-$	Fused hybrid	Survives	Indefinite life span with ability to synthesize nucleotide
TK^+or $HGPRT^+$	Mortal cell	Dies	Mortal

Note: *TK* Thymidine Kinase, *HGPRT* Hypoxanthine-guanine phosphoribosyl transferase

the antigen and selected for expansion in a 25 cm^2 flask. Positive clones are amplified and preserved in liquid nitrogen.

2.7 Single-Cell Cloning

The positive clones are subjected to single-cell cloning and sub-cloning using limiting dilution method in 96-well plate [15]. Single-cell cloning is done twice to confirm the monoclonality of hybridomas clones. These clones are expanded and preserved in liquid nitrogen. An adequate number of monoclonal antibodies are produced via ascitic fluid and subsequently used for characterization.

2.8 Bulk Production

The mAbs are produced in bulk by mainly two methods, viz. cell culture and ascites fluid method (National Research Council 1999). Though cell culture method, wherein culture supernatant is used as mAb, is easy and cheap compared to the other (ascites fluid), ascites fluid method gives highly concentrated biological material which are mostly used for development of pen-side diagnosis/chromatographic strip tests. Mice ascites are useful in an experiment where an adequate amount of mAb is required for improved efficacy or specificity. Mice are injected intraperitoneally with an immunological adjuvant like pristane (0.5–1 ml per mouse) prior to the implantation. Clones are suspended in serum free media (SFM) or PBS and injected into the peritoneal cavity of mice. Dosage can be increased for subsequent formation of ascites. Mice are examined daily for the growth of tumor in the belly. Generally, mice become morbid after 1–4 weeks. Ascitic fluid high in concentration of mAb is harvested with humane techniques. Halder and some other scientists quoted that ascitic monoclonal antibody contains other factors like cytokines, which could render the use of ascitic fluids "scientifically wrong" [16]. However, where purity is not a concern this method is widely used.

2.9 Purification

The monoclonal antibody produced from mouse ascitic fluid is subsequently purified. Some ascitic fluid contains blood clots that should be removed by centrifugation. The whitish lipid layer and pristane over the ascitic fluid are also removed after centrifugation. The aliquots are pooled and immunoglobulins are precipitated using different commercially available kits following the manufacturer's instruction. This method provides antibodies with high purity. Further antibody purification can be done via an ion exchange column or affinity chromatography. These purified antibodies are tested for antibody/protein titer by either ELISA or protein estimation kits (Lawry or Bradford method).

3 Characterization of mAb

After successful selection, monoclonal antibodies are harvested. The mAbs are characterized for structural characterization (like amino acid sequence, peptide map, sulfhydryl groups, disulfide bridges, and carbohydrate structure) and physiochemical characterization (like weight, size, isoform patterns, etc.) of antibody. The main parameters for characterization of monoclonal antibodies chosen by various workers are class and subclass determination, protein specificity of mAbs using RIPA and western blot assays, virus neutralization activity of mAbs and reactivity of mAbs in ELISA test with various isolates of the same virus and other viruses of same genus. The class and subclass of the monoclonal antibodies are determined using commercially available kits. These studies are helpful when the monoclonal antibodies are subjected to purification for the purpose of labeling with enzymes or biotin. Different classes of antibodies have been shown to have different binding and elution profiles in protein-A chromatography [17]. Radio immune-precipitation assay followed by SDS-PAGE and autoradiography has been extensively used for determining protein/antigenic specificity of monoclonal antibodies. However, various simple and easy methods using non-radioactive labels have also been developed for identifying the specificity of monoclonal antibodies at an early stage [18, 19]. Immunofluorescence assay has been used to assess the reactivity of monoclonal antibodies with different viruses and to determine the virus specificity of mAbs [9, 20].

4 Application of mAbs in Diagnosis

Ever since the development of Hybridoma Technology in 1975 by Kohler and Milstein, the vision for antibodies as tools for research in animal disease diagnosis, antigenic characterization of pathogens, vaccine production and in the study of genetic regulation of immune responses has been revolutionized. A very distinctive

advantage in production of hybridomas is generation of specific antibody using a mixture of antigen. The monoclonal antibodies directed against single epitopes are highly specific in nature and in addition, these can be produced in huge quantities without the use of animals.

4.1 Detection of Antigen and Antibody

For a sensitive and rapid diagnosis of diseases, monoclonal antibodies have been used in different test formats and offer an added advantage of specifically binding with the target. Monoclonal antibodies have been deployed in different tests like ELISA and lateral flow assays for detection of viral antigen in tissues, secretions (conjunctival and nasal swabs), lymphocytes, and blood by various workers in different tests (Potgeiter et al., 1989; [21–23]). Another approach of using mAb-based ELISA for antigen detection is *cell-ELISA* which is a very simple, sensitive, rapid, inexpensive assay that has been used for detection and quantification of virus infected cells using monoclonal antibody directed against one of the viral proteins. The cell-ELISA has been used for quantification of molecules that are being expressed on the surface of cells [24]. The technique was developed based on two basic immunological methods, i.e., (a) Immunohistochemistry: which involves identification/localization of antigen in tissue sections and (b) ELISA: which helps in quantification of soluble antibodies/antigen by making one of the immune-reactant immobilized on a solid phase. One of the most important uses of cell-ELISA has been in hybridoma technology for rapid screening of virus-specific hybridoma clones after fusion.

Several tests have been developed for the detection of antibodies against particular pathogen. The mAb-based detection of antibody employing assays like ELISA or chromatographic strip test is coming in big way for detection of seroconversion due to vaccination or infection.

4.2 Antigenic Profiling of Viruses

Various studies in the past have shown by the use of monoclonal antibodies that viruses exhibit antigenic differences between the strains [25–28]. mAbs play crucial role in differentiating viruses of same family, genus, or even the vaccine and wild type strains of same virus species. So, antigenic profiling of viruses using mAbs is very useful to know their antigenic behavior which help in designing the diagnostics and prophylaxis for better control of the diseases.

5 Conclusion

Monoclonal antibodies have proven to be very useful tool especially in diagnostic assays. mAbs enable researchers to easily identify and characterize various pathogens and have therefore revolutionized the diagnostic science in all fields. The use of monoclonal antibodies in diagnostic platforms like lateral flow assay/chromatographic

strip test has changed the field of diagnosis. Since mAbs are directed against a single epitope, multiple antigenic determinants can be identified from a complex mixture by using the approach of multiplex LFA tests. One of the blooming topics nowadays is DIVA-based diagnostic strategy. Using mAbs of distinct epitope can help in devising an epitope-based marker vaccine. Monoclonal antibodies also find their utility in developing anti-idiotypic vaccines using an anti-idiotypic mAb which mimics an antigen and can prove to be more immunogenic as compared to the original one.

References

1. Avrameas S (1983) Enzyme immunoassays and related techniques: development and limitations. In: Bachmann PA (ed) New developments in diagnostic virology. Springer-Verlag, New York, NY, pp 93–99
2. Yolken RH (1983) Use of monoclonal antibodies for viral diagnosis. Curr Top Microbial Immunol 104:177–l95
3. Köhler G, Milstein C (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256:495–497. https://doi.org/10.1038/256495a0
4. Pandey S (2010) Hybridoma technology for production of monoclonal antibodies. Int J Pharm Sci Rev Res 1(2):88–94
5. Alberts B, Johnson A, Lewis J et al (2002) B cells and antibodies. In: Molecular biology of the cell, 4th edn. Garland Science, New York
6. Leenaars M, Hendriksen CF (2005) Critical steps in the production of polyclonal and monoclonal antibodies: evaluation and recommendations. ILAR J 46(3):269–279
7. Potgieter LN, Ajidagba PA (1989a) Quantitation of canine distemper virus and antibodies by enzyme-linked immunosorbent assays using protein a and monoclonal antibody capture. J Vet Diagn Investig 1:110–115
8. Singh RP, Bandyopadhyay SK, Sreenivasa BP, Dhar P (2004) Production and characterization of monoclonal antibodies to pests des Petitis Ruminanats (PPR) virus. Vet Res Commun 28(7):623–639
9. Liu Y, Hao L, Li X, Wang L, Zhang J, Deng J, Tian K (2017) Development and characterization of canine distemper virus monoclonal antibodies. Monoclon Antib Immunodiagn Immunother 36:119–123
10. Masuda M, Sato H, Kamata H, Katsuo T, Takenaka A, Miura R, Yoneda M, Tsukiyama-Kohara K, Mizumoto K, Kai C (2006) Characterization of monoclonal antibodies directed against the canine distemper virus nucleocapsid protein. Comp Immunol Microbiol Infect Dis 29:157–165
11. Parray HA, Shukla S, Samal S, Shrivastava T, Ahmed S, Sharma C, Kumar R (2020) Hybridoma technology a versatile method for isolation of monoclonal antibodies, its applicability across species, limitations, advancement and future perspectives. Int Immunopharmacol 85:106639
12. Lerner EA (1981) How to make a hybridoma. Yale J Biol Med 54(5):387–402
13. Hundekar YR, Chimkode RM, Salagare MV (2017) Hybridoma technology: a new scientific tool for AIDS recovery. Int J Pharm Pharm Sci 9(4):1–16
14. Greenfield EA (2018) Screening for good batches of fetal bovine serum for myeloma and Hybridoma growth. Cold Spring Harb Protoc 1(3). https://doi.org/10.1101/pdb.prot103150
15. Fuller SA, Takahashi M, Hurrell JGR (2001) Cloning of Hybridoma cell lines by limiting dilution. Curr Protoc Mol Biol; Chapter 11: Unit11.8. https://doi.org/10.1002/0471142727.mb1108s01
16. Halder H, Embelton M, Fischer R, de Geus B, Hendriksen C, de Leeuw W, Marx U, Balls M (1998) Comments on appendix C of the National Institutes of Health response to the petition of the American anti-vivisection society to prohibit the use of animals in the production of monoclonal antibodies. Altern Lab Anim 26(4):549–554
17. Seppala IJT, Sarvas H, Peterfy H, Makala O (1981) The four subclasses of IgG can be isolated from mouse serum by using protein A-Sepharose. Scand J Immunol 14:335–342
18. Cole SR, Ashman LK, Ey PL (1987) Biotinylation: an alternative to radioiodination for the identification of cell surface antigens in immunoprecipitates. Mol Immunol 24:699

19. Schuh R, Kremmer E, Ego E, Wasiliu M, Thierfeldcr S (1992) Determination of monoclonal antibody specificity by immunoadsorption and Western blotting. J Immunol Methods 152:9–67
20. Bi Z, Xia X, Wang Y, Mei Y (2015) Development and characterization of neutralizing monoclonal antibodies against canine distemper virus hemagglutinin protein. Microbiol Immunol 59:202–208
21. An DJ, Kim TY, Song DS, Kang BK, Park BK (2008) An immunochromatography assay for rapid antemortem diagnosis of dogs suspected to have canine distemper. J Virol Methods 147: 244–249
22. Li Y, Cheng S, Xu H, Wang J, Cheng Y, Yang S, Luo B (2012) Development of a combined canine distemper virus specific RT-PCR protocol for the differentiation of infected and vaccinated animals (DIVA) and genetic characterization of the hemagglutinin gene of seven Chinese strains demonstrated in dogs. J Virol Methods 179:281–287
23. Zhang Y, Xu G, Zhang L, Zhao J, Ji P, Li Y, Liu B, Zhang J, Zhao Q, Sun Y, Zhou E (2020) Development of a double monoclonal antibody–based sandwich enzyme-linked immunosorbent assay for detecting canine distemper virus. Appl Microbiol Biotechnol 104:10725–10735
24. Ogino T, Wang X, Ferrone S (2003) Modified flow cytometry and cell-ELISA methodology to detect HLA class I antigen processing machinery components in cytoplasm and endoplasmic reticulum. J Immunol Methods 278: 33–44
25. Giraudon P, Wild TF (1981) Differentiation of measles virus strains and a strain of canine distemper virus by monoclonal antibodies. J Gen Virol 57:179–183
26. Russell PH, Alexander DJ (1983) Antigenic variation of Newcastle disease virus strains detected by monoclonal antibodies. Arch Virol 75(4):243–253
27. Sheshberadaran H, Chen SN, Norrby E (1983) Monoclonal antibodies against five structural components of measles virus. I. Characterisation of antigenic determinants on nine strains of measles virus. Virology 128:341–353
28. Ter Meulen V, Loffler S, Carter MJ, Stephenson JR (1981) Antigenic characterization of measles and SSPE virus Haemagglutinin by monoclonal antibodies. J Gen Virol 57:357–364

Chapter 20

Nucleic Acid Hybridization Techniques for Viral Disease Diagnosis: A Detailed Perspective

B. V. Sunil Kumar, Himalaya Bhardwaj, Ankita Gurao, Naveen Kumar, and Yashpal Singh Malik

Abstract

Despite the newer techniques that offer rapid diagnosis of viral diseases by demonstration of viral antigen or viral nucleic acid in the specimens, virus isolation in cell culture remains the gold standard against which newer methods must be compared. But virus isolation requires stringent attention by the clinician and is time-consuming. Hybridization techniques offer direct detection of viral nucleic acid in the clinical specimens and in tissue sections. However, hybridization techniques are still not widely used in the clinical laboratory. Recent developments in molecular cloning and the development of non-radioactive probes have made hybridization techniques more accessible. In this chapter, we present an overview of the various nucleic acid hybridization techniques used for diagnosis of viral diseases. Emphasis has been made on optimization of different factors for better results. Southern blotting, northern blotting, and spot hybridization techniques have been detailed with respect to their utility in disease diagnosis. Since a hybridization experiment depends on various effectors, modification of any one effector usually impacts several others. Because of this complex interplay of cause and effect, thorough optimization of the experiment is required to ensure accurate results.

Key words Nucleic acid hybridization, Viral diagnosis, Sensitivity, Specificity, Southern blotting, Northern blotting, Spot hybridization, Probes, Labeling

1 Introduction

The significance of viral infections has been overwhelmingly recognized in recent years, primarily as a cause of mortality and morbidity among immunocompromised subjects. The awareness on the part of people and the medical fraternity of the importance of various viral infections such as herpes infection or acquired immunodeficiency syndrome (AIDS) or recent coronavirus disease (COVID-19) has contributed to the spread of viral diagnostic laboratories in community hospitals and universities [1, 2]. The number of commercial companies dedicated to the manufacture of diagnostic kits has also increased.

Rajib Deb et al. (eds.), *Protocols for the Diagnosis of Pig Viral Diseases*,
Springer Protocols Handbooks, https://doi.org/10.1007/978-1-0716-2043-4_20,

To achieve an effective therapy, it must be tailored to the specific disease and should begun at an earlier stage of the disease [1, 3]. In order to do this, a prompt and accurate diagnosis of infection must be made. With the availability of newer antiviral agents and other therapeutic methods, the physicians also need precise laboratory diagnosis of their patients' illnesses which has led to the burgeoning of viral diagnostic facilities. In addition, medical professionals themselves should be well acquainted with the methods for diagnosing viral disease for better interpretation [1].

Many of the newer viral diagnostic assays are also compared to the traditional virus isolation technique which remains the "gold standard" test for most of the newer methods, but the conventional virus isolation technique is labor-intensive, time-consuming and requires maintenance of a dedicated cell culture facility. The identification of viral antigens and nucleic acids is increasingly challenging the traditional paradigm [4]. Many rapid "same day" diagnostic methods have been used for viral diagnosis, viz. viral cytopathology, electron microscopy, immunofluorescence (IF), enzyme immunoassay (EIA), polymerase chain reaction (PCR), serology, and nucleic acid hybridization.

Nucleic acid hybridization is used to identify particular nucleic acid sequences using specific denatured oligonucleotide probes that are typically chemically synthesized and fluorescently or radio labeled [5]. Recent developments in molecular cloning and the development of non-radioactive probes have made hybridization techniques more accessible. Availability of extensive cloned viral DNA has made the nucleic acid hybridization technique more feasible for the detection of such viruses in clinical specimens [6]. Various hybridization tests are available commercially for the identification of herpes simplex virus, cytomegalo virus (CMV), and human papillomavirus (HPV). Probes for hepatitis B virus, human immunodeficiency virus (HIV), Epstein–Barr virus (EBV), adenovirus, and type A rotavirus are also currently available [6]. In this chapter, our discussion will be limited to nucleic acid hybridization (NAH) and its role in viral diagnosis. In order to achieve optimal results in NAH, all the steps from the sample collection to the analysis of results need to be optimized.

2 Sample Collection

Sample collection still remains a critical step for NAH, as for any other laboratory diagnostic technique. Ultimate care should be given to any minute detail such as timing, collection, handling, etc., otherwise the results may be misinterpreted. In order to get successful virus isolation, samples should be collected within a few days after the symptoms begin [6]. Depending on the type of

infection, different samples, such as swabs of mucosal surfaces, skin scrapings, tissues, or some other biological fluid, may be collected for diagnosis. However, sterility should be taken care of for any sample, and the sample should be transported quickly to the concerned laboratory at ice-cold temperature (preferably in a virus transport medium with antibiotics), as any delay in transport could lead to degradation of the viral nucleic acid or the virus itself. The type of sample to be collected should be correlated with the clinical presentation, and in the case of unknown disease, it is often advised to collect samples from multiple sites. If rhinovirus, parainfluenza, influenza, adenovirus, enterovirus, adenovirus, rotavirus, norovirus, etc., are suspected, samples of throat swabs, stools, urine, or serum should be collected, while samples of vesicular fluid or serum are preferred for suspected infections with varicella-zoster virus, and herpes simplex virus (HSV-1 and HSV-2) [6].

3 Sample Preparation

The collected sample needs to be processed prior to the hybridization step. There are different sample processing methods that depend on the type of hybridization experiment and the sample type itself.

3.1 Stool Samples

3.1.1 RNA Isolation [7]

The stool samples should be diluted in 10% phosphate-buffered saline (PBS) and centrifuged for 10 min at a maximum speed; the supernatant should be stored at −20 °C for further use. The supernatant has to be mixed with equal volume of phenol-chloroform (3:2 v/v) mixture and mixed well for 1 min. The final mixture should be centrifuged at 1200 × *g* for 10 min, and the resulting clear upper aqueous layer should be removed and treated with 0.5 mL of isopropanol. After incubation at room temperature for 20 min, the tube should be centrifuged again at 14,000 × *g* for 10 min at 4 °C. The supernatant should be discarded and the pellet containing RNA washed with 0.5 mL of 75% (v/v) ethanol by centrifuging at 5000 × *g* for 5 min at 4 °C. After removal of the supernatant, the pellet is finally allowed to air-dry briefly and suspended in an appropriate volume of nuclease-free water. To denature the RNA, the samples should be incubated at 65 °C for 10 min, and snap chilled on ice for 2 min.

3.1.2 DNA Isolation

Approximately 1–1.5 g of fecal sample is needed for fecal DNA extraction, which is vortexed and washed using 5 mL ethanol and centrifuged at 4000 × *g* for 2 min. The pellet should be washed with 5 mL Tris EDTA (TE) buffer, then 3 μL of TNE buffer (10 mmol/L Tris-Cl, 0.5% SDS, and 1 mmol/L $CaCl_2$) and 50 μL of Proteinase K (20 mg/mL) are added to the pellet. The whole mixture must be incubated at 55 °C for 1–2 h. Then, the

lysate is centrifuged at 4000 × *g* for 1 min and the supernatant to be collected. The supernatant is mixed with 3 g of potato starch and vortexed for 1 min to suspend the particles properly. Further, the mixture should be incubated at room temperature for 1 min and centrifuged at 8000 × *g* for 3 min. The supernatant (600 μL) should be mixed with 150 μL NaCl solution (3.5 mol/L) and 250 μL Cetyl trimethyl ammonium bromide (CTAB) solution (10% CTAB +0.7 M NaCl). The step is followed by incubation at 70 °C for 10 min. The mixture is extracted twice using equal amount of phenol: chloroform: isoamyl alcohol (25:24:1 v/v) and equal volume of binding buffer (4 M guanidine hydrochloride, 1 M potassium acetate, pH 5.5). Further, any spin column-based kit for DNA extraction can be used to process the mixture.

3.2 Nasal Swabs

3.2.1 RNA Isolation [8]

After the collection of nasal swabs in RNA*later*, the collection tube should be briefly vortexed and mixed. Later, after 15 min of centrifugation at the maximum speed, the RNA*later* is decanted and collected in a microcentrifuge tube. The pellet must then be resuspended in 500 μL of TRIzol and incubated for 1 h at room temperature. Further RNA isolation steps should be carried out following the TRIzol manufacturer's instructions. All the centrifugation steps should be performed at 19,000 × *g* at 4 °C. RNA is precipitated with one volume of chilled isopropanol, 10% of 3 M sodium acetate, and 1 μL of glycogen at −20 °C. Extracted RNA is resuspended in a sufficient volume of nuclease-free water.

3.2.2 DNA Isolation

DNA can be isolated from the processed samples using any standard kit or follow the protocol mentioned in Subheading 3.1.2.

4 Probes

4.1 Preparation and General Considerations

A probe is essentially a primer which is chemically bonded to a label at its end. The DNA probes are short, single-stranded, labeled DNA sequences used to detect the presence or absence of the nucleic acid of interest in a sample. The probe corresponding to a specific gene or part of genome which is highly conserved in the virus, can be labeled in either ways, radioactive or non-radioactive. Similarly, synthesis of a probe can be achieved using in vitro synthesis or polymerase chain reaction (PCR) amplification. For preparing the probe, molecular cloning is an easy yet powerful tool. Viruses with smaller genome size can be cloned into plasmid vector and those with a larger genome size using the cosmid or yeast artificial chromosome (YAC).

Once the viral DNA fragments are cloned, they can be used for preparing genomic libraries that serve as a potential repository for choosing the probes. It is possible to obtain DNA probes from RNA viruses by synthesizing cDNA from the viral RNA [9, 10]. In

general, the gene probes are longer than 500 bases and can be synthesized by cloning or direct amplification from genes of interest using PCR. The oligonucleotide probes, on the other hand, are usually 15–30 bases and offer more precise hybridization with the target. For both the aforesaid probes, some general requirements, including GC content of 40–60%, absence of self-complementarity within the probe, and <70% homologies to non-target sequence should be met [11]. The labeling protocols are described in depth below.

4.2 Probe Labeling

Several isotopes have been utilized for labeling the probes, which include ^{3}H, ^{32}P, ^{35}S, and ^{125}I, for which detection can be done using autoradiography or Gieger–Muller counter. In the recent times, however, the use of radioactive labeled probes has drastically reduced considering their disposal and safety.

4.2.1 Radioactive Labeled Probes

Classically, for synthetic oligonucleotides, end labeling is most widely used, although if 5′ overhangs are present, double-stranded DNA can also be labeled. Using the Maxam–Gilbert chemical cleavage method, end-labeled oligonucleotides are used for hybridization probes, primer extension analysis, or DNA sequencing. Bacteriophage T4 polynucleotide kinase could be used for labeling the 5′ ends of DNA (or RNA) with γ[^{32}P]ATP. Polynucleotide kinase transfers the γ-phosphate group from ATP to the free 5′ hydroxyl group of DNA (or RNA). Dephosphorylation of the 5′ phosphate group is required prior to labeling using calf intestinal alkaline phosphatase. The dephosphorylated substrate is then re-phosphorylated by transfer of γ-phosphate from γ[^{32}P]ATP. Terminal transferase (terminal deoxynucleotidyl transferase) could be employed for labeling 3′ ends of DNA, with which transfers dNTPs to free hydroxyl groups at the 3′ ends of DNA. End-labeled probes generated with polynucleotide kinase have relatively low specific activity because only one mole of ^{32}P is incorporated per mole of template. Since terminal transferase can add many nucleotides to each molecule of template, this enzyme is frequently preferred when higher specific activity is required, e.g., in situ hybridization [12]. Nowadays, many brands use Klenow fragment for incorporation of radiolabels into the probes.

4.2.2 Non-radioactive Labeled Probes

The non-radioactive labeling of probes is preferred over the radioactive counterpart because they are safer and highly stable. In recent times, many non-radioactive labeling methods have been developed including biotin-avidin/streptavidin, enzyme labeling, e.g., horseradish peroxidase (HRP), fluorescent labeling of probes, digoxigenin (DIG) system, etc. The following protocol discusses 3′ end labeling with DIG-ddUTP or Biotin-ddUTP.

Digoxigenin is a steroid of plant origin; this molecule has been widely used as a hapten and can be conjugated with nucleic acids using conjugation reaction. The protocol for oligonucleotide

3′-end labeling with DIG-ddUTP or Biotin-ddUTP is as follows [13]. Dissolve the oligonucleotide to be labeled in nuclease-free water and prepare 1 mM solution of DIG-ddUTP [available commercially as Digoxigenin-3-O-methylcarbonyl-ε-aminocaproyl-{5-(3-aminopropargyl)-2′,3′-dideoxyuridine-5′-triphosphate}, Triethylammonium salt]. Next, add 4 μL of 5× concentrated reaction buffer [1 M potassium cacodylate, 0.125 M Tris-HCL, 1.25 mg/mL Bovine Serum Albumin (pH 6.6)]. Add 4 μL of $CoCl_2$, 100 pmol of oligonucleotide, and 1 μL of DIG-ddUTP. Further add 1 μL of terminal transferase (50 U) and nuclease-free water to a final volume of 20 μL. Mix well, centrifuge, and incubate at 37 °C followed by snap chilling. To stop the reaction, add 2 μL of EDTA-glycogen mixture (200 μL of 0.2 M EDTA (pH 8.0) mixed in 1 μL of 20 mg/mL glycogen). Finally, precipitate the labeled oligonucleotide.

5 Optimizing Conditions for Hybridization

The hybridization step is very important for any nucleic acid detection technique, whether DNA or RNA. However, as it needs a lot of optimization and standardization, there is no common protocol for hybridization. Optimization involves playing with the choices of buffers required in the method: temperature conditions and duration of the experiment. Another group of effectors that need to be optimized in a series of experiments for better results are the type of membranes, probes, and the target constitute [14]. Suboptimal conditions dramatically decrease the efficacy of all assays by introducing considerable bias [15].

5.1 Temperature

One of the key determinants of a hybridization experiment is the hybridization temperature, which relies on the melting temperature (Tm) of the probe, and the nature of the target. Although the Tm may be determined using different formulas depending on the type of the probe and the target-probe hybrid, it is often advisable to obtain it empirically as different variables (type of labels, probes, or buffer composition) influence the melting temperature and consequently the end point of the experiment. A smaller base modification, or non-radioactive tags, for example, can change the characteristics of the probe and the Tm [16]. In addition, at higher temperatures, probes with horse radish peroxidase (HRPO) tag are less stable than those with alkaline phosphatase. Therefore, lower hybridization temperatures or addition of thermal stabilizers such as trehalose to the buffers can be considered for improved label stability when using HRPO tagged probes [14]. The type of probes also determines its melting temperature, with RNA probes having a higher Tm as compared to their DNA counterparts. However, due to the instability of RNA at higher temperatures, the buffers may

alternatively be supplemented with denaturing agents like such as formamide or urea to allow hybridization at a lower temperature [14].

Enzyme-coupled probes should be hybridized at the lowest possible temperature to warrant enzyme stability. However, lower hybridization temperature compromises with binding specificity and can lead to background noise, while too higher hybridization temperature compromises with the strength of end signal [15]. Between particular signal strength and background levels, a trade-off often lies. Therefore, for each experiment, the optimum hybridization temperature should be standardized and the acceptable limit of background noise should also be determined accordingly [15].

5.2 Probe Concentration

The probe concentration is application dependent, but it is usually kept higher than the target concentration. The probe concentration should be selected in such a way that minimum background noise and optimum specific signal strength are present. In general, in the absence of any rate enhancers, a probe concentration is 5–10 ng/mL of the hybridization buffer, whereas rate accelerating buffers require a reduction in the concentration of the probe to 0.1–5 ng/mL of hybridization buffer [14, 17].

5.3 Hybridization Time

For majority of the probe-target complexes, overnight hybridization should work well. There are some determinants, however, which determine the optimum duration of hybridization. For instance, shorter single-stranded probes need less time compared to longer double-stranded ones for hybridization [17]. Higher probe concentration buffers (more than 10 ng/mL) and rate enhancers also facilitate shorter hybridization times [14].

5.4 Buffer Components

Denaturing buffers such as formamide buffers are preferred for thermolabile probes, membranes, or labels over salt/detergent buffers. Formamide (30% to 80%), urea (3 M to 6 M), ethylene glycol, sodium perchlorate (2 M to 4 M), or tertiary alkylamine chloride salts could be used as denaturants [18]. The stability of probe-target hybrids is influenced by the cations of the salts that counteract the repulsive forces between the probe phosphodiester bonds and target and thus stabilize the hybrid formed with the target. By including sodium chloride upto a final concentration of 1.2 mM, stability may be improved and 80 to 90 mM citrate buffer or 50 mM sodium phosphate buffer are other alternatives for improving the stability [14, 19, 20]. Detergents such as 1% to 7% sodium dodecyl sulfate (SDS), 0.05% to 0.1% Tween-20, 0.1% N-lauroylsarcosine, or Nonidet P-40, blocking reagents such as bovine serum albumin (BSA), skimmed milk powder, and genomic DNA (calf thymus, herring, or salmon sperm), or poly A could be included in the buffer to prevent non-specific binding on to the membrane [21].

Hybridization rate enhancers/accelerators such as dextran sulfate, ficoll, and polyethylene glycol if included in the hybridization buffers, significantly shorten the hybridization time. These enhancers work by reducing the free water molecules (volume excluders) and effectively increasing the probe concentration per mL of the buffer. Their concentration can be determined empirically, based on the acceptable background noise limit [14].

6 Nucleic Acid Hybridization Protocol

6.1 Southern Blotting Using Radioactive Labeled Probe

The following buffers have been standardized for Southern blotting by [22]:

A. *20× saline sodium citrate (SSC) solution* 175.3 g NaCl 88.2 g sodium citrate Bring the final volume to 1 L with double distilled water.	C. *Neutralizing solution* 500 mL of 1 M Tris-HCl (pH 7.4) solution 300 mL of 5 M NaCl solution Bring to 1 L with double distilled water.
B. *Denaturing solution* 300 mL of 5 M NaCl solution 100 mL of 5 M NaOH solution Bring to 1 L with double distilled water.	D. *Nylon wash solution (pH 7.2)* 40.6 g Na_2HPO_4 18.65 g EDTA 500 g SDS Bring the final volume to 3.58 L with double distilled water.

6.1.1 Protocol

Digest the extracted DNA with one or multiple restriction enzymes. Separate the digested DNA on the agarose gel (percentage of agarose according to the band size of interest) at 30–50 V and remove the gel from the electrophoresis tank and incubate for 30 min in the denaturing solution on a platform shaker at approximately 25 rpm. Rinse the gel twice in double distilled H_2O. Again, incubate the gel for 30 min in neutralizing solution on a platform shaker at approximately 25 rpm. Re-incubate the gel in fresh neutralizing solution for 30 min. Rinse the gel twice with double distilled H_2O. Before assembling the Southern blot setup, prepare the SSC buffer. Essentially, the assembly has the neutralized gel sandwiched in between a nylon membrane and Whatman filter paper, while the other Whatman filter paper is positioned above the nylon membrane. Multiple paper towels are stacked and a glass plate is placed over it topped with 200–500 g of weight (Fig. 1). The entire setup is kept in a tray filled with 20×SSC buffer. Incubate the stacked gel overnight to allow capillary transfer of the nucleic acid from the gel to the nylon membrane. Next day, disassemble the setup and remove the membrane followed by rinsing with 2×SSC and air-dry. The DNA embedded membrane is

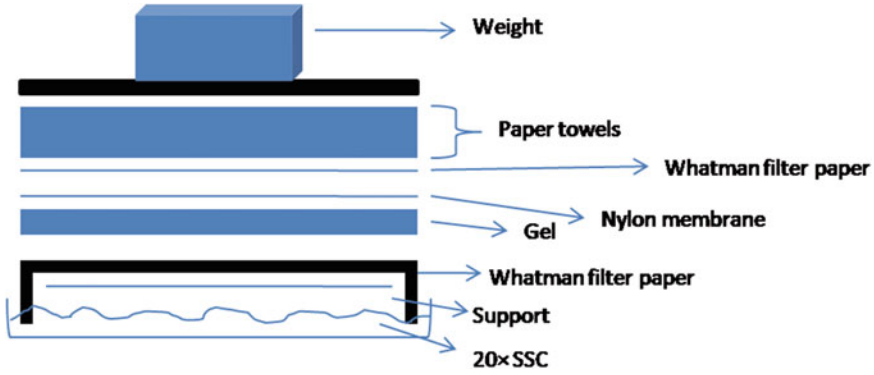


Fig. 1 Schematic representation of Southern blotting setup

exposed to UV light to aid cross-linking. Pre-hybridize the membrane in 20 mL 0.75× nylon wash solution (sodium phosphate dibasic, ethylenediaminetetraacetic acid, sodium dodecyl sulfate) for 1 h at 65 °C. Add the ^{32}P labeled probe to the hybridization mixture and incubate overnight at 65 °C. Wash membrane consequently in 0.5× nylon and 0.1× nylon wash solution for 20 min at 65 °C every time. Expose the membrane to the Phospho imager screen for at least 2 h and record it.

6.2 Northern Blotting Using Radioactive Labeled Probes

The following buffers may be required for Northern blotting.

A. *10× MOPS (500 mL):* 41.9 g MOPS 6.8 g NaAc 10 mL 0.5 M EDTA Store away from light at 4 °C	B. *Loading buffer (1000 µL):* 100 µL 10× MOPS 500 µL Formamide 185 µL Formaldehyde 40 mg Ficoll 400 Bromophenol blue 215 µL H_2O Store at −20 °C
C. *Pre-hybridization buffer (100 mL):* 25 mL 20× SSC 50 mL Formamide 5 mL 100× Denhardt's 1 g SDS 1 mL 10 mg/mL DNA	D. *Hybridization buffer:* Pre-hybridization buffer with 5% dextran sulfate and without non-homologous DNA
E *100× Denhardt's solution (500 mL):* 10 g Ficoll 400 10 g polyvinylpyrrolidone MW 360,000 10 g BSA fraction V Distilled water upto 500 mL Store at −20 °C.	F. *20× SSC:* 175.3 g NaCl 88.2 g Sodium Citrate

(continued)

G. *Strip solution (500 mL):*
2.5 mL 1 M Tris
200 µL 0.5 M EDTA
5 mL 5% NaPP
1 mL 50× Denhardt's

6.2.1 Protocol

The RNA (~30 µg) is loaded into the wells of 1.3% agarose-formaldehyde gel and is run until the RNA is resolved properly. Examine the gel under UV after staining with ethidium bromide and rinse gels with 20× SSC buffer (0.03 M sodium acetate, pH 7.0 and 0.3 M NaCl). Soak gel 3 times 5 min in distilled water (to remove Formaldehyde) and cut nylon membrane to the exact gel size. Presoak the membrane. Set up the capillary transfer stacking as described in Southern blotting protocol. After overnight blotting, place the membrane on wet Whatman paper and UV-crosslink damp membrane. Further bake the membrane at 80 °C for 1–2 h. Pre-hybridize membrane for 1–4 h @ 42 °C with 5–10 mL pre-hybridization buffer. Heat radioactive labeled probe for 3 min @ 95 °C, cool on ice. Discard the pre-hybridization buffer, add the hybridization buffer and probe, incubate at 42 °C. Wash the membrane once for 15 min and twice with SSC at room temperature, followed twice with SSC, 0.1% SDS at 65 °C until background is clear. Optionally wash again with 0.1×SSC, 0.1× SDS at 65 °C. Expose the wet membrane at −80 °C under saran wrap. For stripping, wash membrane for 30 min to 3 h in strip solution at 75–85 °C until no radioactivity can be detected on the membrane. The membrane can now be air-dried and stored at RT and the hybridization protocol is followed for re-hybridization.

6.3 Spot Hybridization

For spot hybridization, the following reagents/materials are needed [23]. Proteinase K, Phosphate-buffered saline (PBS), Sodium chloride (NaCl), sodium citrate, nitrocellulose sheet, formamide, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), DNA probe, sodium dodecyl sulfate (SDS).

6.3.1 Protocol

Pick infected cells and store them at −70 °C, if the hybridization needs to be done at a later time. Take out the cells, thaw and treat with proteinase K at a concentration of 0.1 mg/mL in PBS. Incubate at 37 °C for 1 h, dilute the specimens in stock solution of 20× SSC (l× SSC = 0.15 M NaCl, 0.015 M sodium citrate) to a final concentration of 6× SSC. Divide each sample into two equal parts and put them separately onto nitrocellulose filters. Then, incubate the nitrocellulose sheet at 80 °C for 2 h and pre-hybridize at 42 °C for 2 h in 50% formamide 50 mM HEPES (pH 7.4). Label the DNA probe, allow denaturing and addition of a hybridization solution. Incubate the filters at 42 °C overnight for hybridization with the labeled probe. After hybridization, wash the filters three

times in 2× SSC with 0.5% SDS (w/v) at 42 °C. Visualize by autoradiography overnight for the results.

7 Advantages and Limitations of Nucleic Acid Hybridization Methods [23, 24]

7.1 Advantages

1. Hybridization methods do not require cultivation of the organisms or any technology-based gene amplification.
2. These techniques have high sensitivity, specificity and rapid turnover with a high efficiency of hybridization and detection.
3. These methods can be used to study chromosomal aberrations in non-dividing cells, which are useful for the visualization of chromosomal aberrations directly in cytological preparations and tissue sections.
4. They proved to be invaluable in both diagnostics and research.
5. DNA is very stable, so there are less chances of degradation in tissues compared with protein.
6. Southern blot approach improves the detection resolution to a level that may be useful for examining specific genomic loci.
7. Northern blot reagents are not too expensive, which allows the running of many gels at low cost.
8. Northern blot helps in determining transcript size and spliced transcripts.
9. Spot hybridization makes the maximum use of tissue that is difficult to obtain (e.g., embryos and clinical biopsies) and hundreds of different hybridizations can be performed on the same tissue.

7.2 Limitations

1. Difficulty in identifying targets with low copies of DNA and RNA.
2. The chemicals used in most northern blots can pose a risk to the researcher, as formaldehyde, radioactive material, ethidium bromide, DEPC, and UV light are all dangerous under such exposure.
3. RNA molecules are often degraded in tissues, and even a slight degradation of RNA can compromise data quality and thus the ability to quantify gene expression.
4. Northern blot technique is a labor-intensive technique.
5. Prior knowledge on the nucleic acid sequence of the target organism is required. These methods are not capable of detecting unknown viruses.

8 Applications of Nucleic Acid Hybridization in Viral Disease Diagnosis

The conventional cell culture-based techniques for virus identification need live, infective virus particles to be identified, whereas the nucleic acid hybridization-based methods can detect both live and non-infectious viruses [9, 10]. In addition, if the transportation or delayed sampling causes loss of infectivity of the viruses, such specimens yield negative results on cell culture but may be appropriate for nucleic acid hybridization techniques. However, nucleic acid hybridization can only detect viruses with known sequences. Unknown viruses cannot be detected by this method.

8.1 Applications in Rapid Viral Disease Diagnosis

DNA hybridization technique can be used to detect Herpes Simplex virus (HSV) and its major two subtypes, HSV-1 and HSV-2 on the nitrocellulose membrane [25]. Due to close similarity between these two subtypes, utmost care should be taken while designing the probes. Similarly, in the case of Epstein–Barr Virus (EBV) and rotaviruses infections, in which the infection is either non-productive or virions are released in low quantity, DOT blot hybridization is of particular importance [26–28]. Earlier, rotaviruses were detected by electron microscopy, analysis of RNA on polyacrylamide gel electrophoresis, RIA, or ELISA. Radioisotope-labeled probes like ^{32}P for spot and Southern blots, and ^{3}H, ^{35}S, or ^{125}I for in situ hybridization have also been used. Biotin-labeled non-radioactive probes have been used to identify viruses such as human papilloma virus (HPV), human immunodeficiency virus (HIV), and cytomegalovirus (CMV) [27, 29, 30]. With cytomegalovirus, the viral DNA is detected not only in the cytomegalic inclusion cells, but also in the nucleus and cytoplasm of histologically unremarkable hepatocytes, pneumocytes, endothelial cells, and other cells [31]. This approach allows the identification of viral agents at a much earlier stage than would have been possible with routine histologic sections.

8.2 Applications in Histopathology

Hybridization-based methods often play an importance in their use in histopathology, mostly when a small proportion of cells in a population are infected. Therefore, correlated study between hybridization and histology were very helpful in detection of viruses. In situ hybridization was used to examine the replication of HBV in liver sections from patients with chronic hepatitis [32]. ^{3}H radioactive probe was used to detect HBV in the liver section. However, non-radioactive, biotin-labeled DNA/RNA probes may also be used to detect viral genomes both in tissue culture (parvovirus, HSV, adenovirus, and retrovirus) and in paraffin sections of tissue (HSV, and adenovirus) [33]. Biotin-labeled probes decrease hybridization and detection time to less than 24 h, although the sensitivity is lower compared to radiolabeled probes [33].

8.3 Applications in Viral Disease Epidemiology

Hybridization techniques may be used to study viral epidemiology when serological assays distinguishing different strains of virus (such as CMV) are unavailable. Restriction endonuclease digestion of CMV from random clinical isolates has shown that no two viruses generate the same pattern of DNA fragments, when more than one enzyme is used. Thus, Huano et al. [34] showed that endogenous virus carried by mothers during pregnancy was the most frequent cause of recurrent infection, and transmission to their babies. Re-infection with another strain has rarely occurred. Spector [35] demonstrated that CMV could be spread from one child who had viruria to another, in a hospital setting. This finding has clear consequences for the management of patients in renal and bone marrow transplant units and in pediatric wards.

HSV isolates have also been typed and classified using restriction endonuclease digestion, as do some adenoviruses, which are not clearly identified by serological methods [36]. Genome mapping has provided valuable information about the source of herpetic infection in nosocomial outbreaks.

9 Conclusion

Conventional methods for diagnosing viral disease are either based on virus isolation and identification in cell culture, or on the detection of viral-specific antibody, or on the recognition of viral inclusion bodies by light microscopy. These methods, while tedious, remain the "gold standard" for the viral diagnostic laboratory. Hybridization assays offer good sensitivity as diagnostic assays, but labeling techniques should be refined to make them cost-effective. While this technique can be applied to identify dead or non-infective viruses, that are not detectable by cell culture-based methods, this technique cannot be used to detect those viruses whose nucleic acid sequence has yet to be identified. In order to be widely applied, hybridization assays must compete with traditional and the newer diagnostic laboratory approaches, such as enzyme immunoassays, in terms of sensitivity, cost, and ease of use.

References

1. Landry ML, Mayo DR, Hsiung GD (1989) Rapid and accurate viral diagnosis. Pharm Ther 40(2):287–328
2. Malik YS, Verma AK, Kumar N, Touil N, Karthik K, Tiwari R, Bora DP, Dhama K, Ghosh S, Hemida MG, Abdel-Moneim AS, Bányai K, Vlasova AN, Kobayashi N, Singh RK (2019) Advances in diagnostic approaches for viral etiologies of diarrhea: from the lab to the field. Front Microbial 10:1957–1963
3. Richman DD, Clevland PH, Redfield DC, Oxman MN, Wahl GM (1984) Rapid viral diagnosis. J Infect Dis 149:298–310
4. Landry ML (1990) Nucleic acid hybridization in viral diagnosis. Clin Biochem 23:267–277
5. Bhagavan NV, Ha CV (2011) Nucleic acid hybridization. In: Bhagavan NV, Ha CV (eds) Essentials of medical biochemistry with clinical cases, 1st edn. Academic. ISBN: 9780120954612

6. Al-Hajjar S (2012) Laboratory diagnosis of viral disease. In: Elzouki AY, Harfi HA, Nazer HM, Stapleton FB, Oh W, Whitley RJ (eds) Textbook of clinical pediatrics. Springer, Berlin. https://doi.org/10.1007/978-3-642-02202-9_75
7. Herring AJ, Inglis NF, Ojeh CK, Snodgrass DR, Menzies JD (1982) Rapid diagnosis of rotavirus infection by direct detection of viral nucleic acid in silver-stained polyacrylamide gels. J Clin Microbiol 16:473–477
8. Espinosa-de Aquino W, Olvera-Ramirez A, Arellano-Carbajal F, Lanz-Mendoza H, Villagran-Herrera E, Acevedo-Whitehouse K (2017) Protein and RNA extraction from mucosal swabs: a minimally invasive source of ecological datta for studies of natural populations. Methods Ecol Evol 8(3):370–378
9. Kulski JK, Norval M (1985) Nucleic acid probes in diagnosis of viral diseases of man. Brief review. Arch Virol 83:3–15
10. Kulski JK, Norval M (1985) Nucleic acid probes in diagnosis of viral diseases of man. Arch Virol 83:3–15
11. de Muro MA (2005) Probe design, production, and applications. In: Walker JM, Rapley R (eds) Medical biomethods handbook. Springer protocols handbooks. Humana Press. https://doi.org/10.1385/1-59259-870-6:013
12. Igarashi P (1999) Radioactive labeling of DNA and RNA probes. In: Hildebrandt F, Igarashi P (eds) Techniques in molecular medicine. Springer lab manual. Springer, Berlin. https://doi.org/10.1007/978-3-642-59811-1_9
13. Eisel D, Grünewald-Janho S, Krushen B (eds) (2002) DIG application manual for nonradioactive in situ hybridization, 3rd edn. Roche Diagnostics, Penzberg
14. Herzer S, Englert DF (2001) Nucleic acid hybridization. In: Molecular biology problem solver: a laboratory guide. A.S. Wiley-Liss, Gerstein, pp 401–453
15. Sykacek P, Kreil DP, Meadows LA, Auburn RP, Fischer B, Russell S, Micklem G (2011) The impact of quantitative optimization of hybridization conditions on gene expression analysis. BMC Bioinformatics 12:73. https://doi.org/10.1186/1471-2105-12-73
16. Pearlman DA, Kollman PA (1990) The calculated free energy effects of 5-methyl cytosine on the B to Z transition in DNA. Biopolymers 29:1193–1209
17. Anderson MLM (1999) Nucleic acid hybridization. Springer, New York
18. Howley PM, Israel MA, Law MF, Martin MA (1979) A rapid method for detecting and mapping homology between heterologous DNAs. Evaluation of polyoma virus genomes. J Biol Chem 254:4876–4883
19. Nakano S, Fujimoto M, Hara H, Sugimoto N (1999) Nucleic acid duplex stability: influence of base composition on cation effects. Nucleic Acids Res 27:2957–2965
20. Spink CH, Chaires JB (1999) Effects of hydration, ion release, and excluded volume on the melting of triplex and duplex DNA. Biochemist 38:496–508
21. Church GM, Gilbert W (1984) Genomic sequencing. Proc Natl Acad Sci U S A 81: 1991–1995
22. Bardelli M, Zarate-Perez F, Agundez L, Jolinon N, Linden RM, Escalante CR, Henckaerts E (2017) Analysis of replicative intermediates of adeno-associated virus through hirt extraction and southern blotting. Bio Protoc 7(9):e2271
23. Hyypiä T, Stålhandske P, Vainionpää R, Pettersson U (1984) Detection of enteroviruses by spot hybridization. J Clin Microbiol 19(3): 436–438
24. Furrer D, Sanschagrin F, Jacob S, Diorio C (2015) Advantages and disadvantages of technologies for HER2 testing in breast cancer specimens. Am J Clin Pathol 144(5):686–703
25. Singh A, Preiksaitis J, Ferenczy A, Romanowski B (2005) The laboratory diagnosis of herpes simplex virus infections. Can J Infect Dis Med Microbiol 16(2):92–98
26. Fleckenstein B, Muller I, Collins J (1982) Cloning of the complete human cytomegalovirus genome in cosmids. Gene 18:39–46
27. Flores J, Boeggeman E, Purcell RH, Serene M, Perez I, White L, Wyatt RG, Chanock RM, Kapikian AZ (1983) A dot hybridization assay for detection of rotavirus. Lancet 1:555–559
28. Sixbey JW, Nedrud JG, Raab-Traub N, Hanes RA, Pagano JS (1984) Epstein-Barr virus replication in oropharyngeal epithelial cells. N Engl J Med 310:1225–1230
29. Galloway DA, Fenoglio C, Shevchuk M, McDougall JK (1979) Detection of herpes simplex RNA in human sensory ganglia. Virology 95:265–268
30. Myerson D, Hackman RC, Meyers JD (1984) Diagnosis of cytomegaloviral pneumonia by in situ hybridization. J Infect Dis 150:272–277
31. Durst M, Gissmainn L, Ikenberg H, Zur Hausen H (1983) A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. Proc Natl Acad Sci U S A 80:3812–3815

32. Burrell CJ, Gowans EJ, Jilbert AR, Lake JR, Marmion BP (1982) Hepatitis B virus DNA detection by in situ cytohybridization: implications for viral replication strategy and pathogenesis of chronic hepatitis. Hepatology 2: 85S–91S
33. Brigati DJ, Myerson D, Leaky JJ, Spaliiolz B, Travis SZ, Fong CK, Hsiung GD, Ward DC (1983) Detection of viral genomes in cultured cells and paraffin-embedded tissue sections using biotin-labeled hybridization probes. Virology 126:32–50
34. Huano ES, Alford CA, Reynolds DW, Stango S, Pass RF (1980) Molecular epidemiology of cytomegalovirus infections in women and their infants. New Engl J Med 303: 958–962
35. Spector SA (1983) Transmission of cytomegalovirus among infants in hospital documented by restriction-endonuclease-digestion analyses. Lancet 1:378–381
36. Waddell G, DeJong JC, Wolontis S (1981) Molecular epidemiology of adenoviruses: alternating appearance of two different genome types of adenovirus 7 during epidemic outbreaks in Europe from 1958 to 1980. Infect Immun 34:368–372

Chapter 21

Ligase Detection Reaction-Fluorescent Microsphere Assay

A. Raja

Abstract

Disease outbreaks in swine production cause severe economic loss. Conventional diagnostic methods are laborious and less sensitive or time consuming. Recent molecular methods of diagnosis of diseases especially genome detection play a major role in the diagnosis and control of diseases. Although several molecular diagnostic techniques are available, still not all the techniques are employable to detect different pathogens simultaneously. Most of the recent outbreaks are involved with more than one pathogen. Hence, a simple and rapid technique which can detect different targets/pathogens simultaneously is in need of time. LDR-FMA is such an assay where multiplexing is possible. Although LDR-FMA is mainly used to detect single nucleotide polymorphism, this technique still could be adapted for simultaneous detection of different pathogens.

Key words Ligase detection reaction, Fluorescent microscopy, MOL-PCR

1 Introduction

Swine disease outbreaks cause serious economical loss worldwide. Porcine circovirus (PCV), porcine reproductive and respiratory syndrome virus (PRRSV), classical swine fever virus (CSFV), porcine parvovirus (PPV), pseudorabies virus (PRV), and Japanese encephalitis virus (JEV) are six swine viruses involved in reproductive and/or respiratory infections which are severe and frequently reported in swine production. Efficient detection methods are needed for early diagnosis of these infections. Several methods and techniques have been proposed for detection of swine viruses, such as virus isolation, enzyme linked immunosorbent assays (ELISAs), and immunofluorescence assay (IFA). But conventional methods for viral diagnosis are laborious, less sensitive, or time consuming. For example, virus isolation is the "gold standard" for detection but requires 7–10 days and is often insensitive.

DNA ligase catalyzes the annealing of two DNA strands on a DNA template by the formation of a phosphodiester bond at a nick junction. DNA ligase is involved in DNA replication, repair, and

Rajib Deb et al. (eds.), *Protocols for the Diagnosis of Pig Viral Diseases*,
Springer Protocols Handbooks, https://doi.org/10.1007/978-1-0716-2043-4_21,

recombination. DNA ligases are classified into two families based on adenylation cofactor dependence. ATP-dependent ligases are found in bacterial and eukaryotic viruses, Archaea, yeast, mammals, and eubacteria, whereas NAD^+-dependent ligases are found almost exclusively in eubacteria with the exception of the sequenced entomopoxvirus genomes *Melanoplus sanguinipes* and *Amsacta moorei*. Some simple eubacteria genomes encode both NAD^+- and ATP-dependent ligases, whereas many eukaryotic organisms encode multiple ATP-dependent ligases to fulfill diverse biological functions.

Biotechnological application of DNA ligase started with the advent of recombinant DNA techniques. The development of the polymerase chain reaction (PCR) in the mid-1980s enabled genetic materials to be amplified in vitro [1, 2]. A few years later, a mutation detection technique based on the use of T4 DNA ligase was invented, which provided a conceptual foundation for the development of many current ligase-based techniques [3]. Similar to PCR, DNA ligase-mediated strand-joining reactions can be repeated on product denaturation and primer annealing to achieve signal or target amplification [4–6] by using thermostable DNA ligase [7].

The LDR was first developed to detect low amounts of pathogens [8]. Since even a single base mismatch at the ligation junction prevents successful ligation, the technique is highly specific [9, 10].

The ligase detection reaction-fluorescent microsphere assay (LDR-FMA) was designed for multiplex detection of various targets and for SNP genotyping [11]. This technology has been applied for the multiplex detection of 15 genetically distinct plant pathogens including Citrus tristeza virus (CTV), *Xanthomonas* genus, and *Xylella fastidiosa*. LDR-FMA has also been used in the detection of pathogens along with antibiotic (doxycycline and ciprofloxacin) resistance in Yersinia pestis, F. tularensis, and Bacillus anthracis [11, 12]. LDR-FMA has also been useful in identification and differentiation of the six main human-associated lineages of Mycobacterium tuberculosis complex (MTBC) with sensitivity approaching 99.2% [13]. In 2008, Bruse et al. made few improvements to LDR-FMA assays by using fewer beads and universal biotinylated oligonucleotides to reduce costs significantly but also maintaining accuracy [14].

2 Principles of LDR

LDR (Ligase Detection Reaction) is a ligation dependent methodology that, unlike LCR (Ligase Chain Reaction), involves only one pair of probes complementary to one strand of target DNA. Cycling in LDR results in linear amplification of the ligation product. This method can be used to confirm the presence of a particular SNP in a target sequence that has been amplified by another

method (such as PCR). As with LCR, the method uses a high fidelity thermostable ligase that can discriminate against the ligation of mismatched probes, such as Taq DNA Ligase.

3 Principles of LDR-Fluorescent Microsphere Assay (LDR-FMA)

Deshpande et al. [15] described the LDR-FMA as multiplex oligonucleotide ligation-PCR (MOL-PCR) where fluorescent microspheres were used to detect different targets. The assay comprises of three steps: detection, signal amplification and hybridization and readout.

3.1 Detection

Detection is achieved by using 40–70 nucleotide-long, single-stranded DNA probes called MOLigo pairs. When the target for a particular MOLigo pair is present in a reaction, the MOLigo pairs will anneal adjacent to each other on the target. If annealing occurs, DNA ligase recognizes this structure and covalently links MOLigo1 and MOLigo2. Ligation only occurs if the terminal bases of MOLigo1 and MOLigo2 at the junction site are complementary to the target sequence, and this property confers SNP discrimination. This step creates single-stranded DNA molecules of approximately 100–120 nucleotides long that serve as PCR templates for signal amplification and labeling. The ligation reaction can be multiplexed to allow the simultaneous screening for many signatures in the same reaction tube.

3.2 Signal Amplification

The ligation products undergo PCR amplification with a universal primer pair. If the ligation reaction is multiplex, use of a universal primer pair allows the amplification of multiple ligated products in the same reaction tube. In the absence of a target, ligation will not occur, the forward and reverse primer sites will not be linked, and the unligated MOLigo pairs will not produce geometrically amplified and labeled fragments that contain tags.

3.3 Hybridization and Readout

The reverse PCR primer is tagged with a fluorescent dye (e.g., Alexa Fluor 532) at the 5′ end, which allows the detection of the amplified ligation products. The PCR products are hybridized to a microsphere array using tag sequences complementary to anti-tags covalently linked to the microspheres. The microspheres are then analyzed by a flow cytometer, which identifies each bead type and measures fluorescence intensity emitted by the bead-PCR product complex. A microsphere in the array will only show fluorescence if it is hybridized to a labeled PCR product amplified from ligated MOLigo pairs, thus confirming the presence of the target DNA in the sample.

4 Materials

1. Primers and Probes (Sigma).
2. Taq DNA Polymerase (New England Biolab).
3. 96-well PCR plates (Qiagen).
4. MES buffer ((Fisher Bioreagents).
5. 96-well round bottomed plate (Costar).
6. Luminex flow cytometer.

5 Methods

Method for carrying out the LDR-FMA is adopted from Deshpande et al. [15] and it is as follows:

1. Ligation and PCR is conducted in a single tube as follows:
   (a) Final volume of each reaction is 10 μl, consisting of 4 units/μl of thermostable DNA ligase, 5 nM of each MOLigo pair (5′ end of MOLigo 1 is phosphorylated to ligate with the MOLigo 2), 4 mM Mg^{2+}, 1× Taq buffer, 200 μM dNTPs, 0.04 units of Taq DNA polymerase, 500 μM NAD, 0.125 μM forward PCR primer, 0.5 μM Alexa Fluor 532 (Life Technologies) labeled reverse PCR primer and nuclease free water to 9 μl volume, combined with 1 μl of template DNA.
   (b) Ligase-mediated PCR is carried out in a thermal cycler with the following cycling conditions. Initial denaturation at 94 °C for 1.5 min followed by 30 cycles of 50 °C for 30 s, and 94 °C for 25 s to complete the ligation process.
   (c) Amplification of ligated product is carried by 40 cycles of 94 °C for 20 s, 58 °C for 20 s, and 72 °C for 20 s. At the end of the reaction, the tubes are stored at 4 °C until further process.
   (d) A separate reaction is set for each template/sample. All reactions are carried out in a 96-well plate. This corresponds to one plate of a singleplex assay per probe. For simultaneous detection of different targets/SNPs, in the same tube, different labeled probes are added and the remaining components are same.
2. Tagging fluorescent microspheres with the LCR products.
   (a) Anti-tag-conjugated Luminex xTAG® microspheres of each set specific to each MOLigo pair is used.

(b) Mixture is prepared in such a way that it contained 1000 microspheres/μl of each set, 800 mM NaCl, and 50 mM MES buffer.

(c) 5 μl of this mix is then added to the LCR product (10 μl) well and gently mixed.

(d) Then the mix is heated to 94 °C for 1 min, followed by a slow ramp down in temperature to 25 °C with a hold of 1 min at 5 °C decrements in temperature in a thermal cycler. Samples are stored at 4 °C until further process.

(e) Two control tubes are also set up for calculation purpose—a bead-only control and a no template control. The bead-only control is to report background fluorescence obtained from the microspheres alone and the no template control to report cross-reactivity of MOLigo pairs in the absence of any template.

3. For analysis on the Luminex flow cytometer, the reaction volume is increased to 50 μl by adding 35 μl of buffer that contains 10 mM Tris–Cl (pH 8.0), 0.1 mM EDTA, 90 mM NaCl, and 0.02% Tween 20. Then, the reaction is transferred to a 96-well round bottomed plate and analyzed on a Luminex flow cytometer.

4. Analysis of the result.

(a) Median fluorescence intensity (MFI) is used to calculate signal-to-noise. The MFI for this control is also the "noise" used for calculating the signal-to-noise ratio.

(b) MEI values for all samples and controls are imported into an Excel file.

(c) The signal-to-noise ratios are calculated by first subtracting the MFI of the bead-only control from all MFIs, and then dividing the MFI obtained for a sample by the MFI for the no template control.

(d) A signal-to-noise ratio of 4 and a MFI of 50 are criteria used to determine "positive samples."

References

1. Mullis K et al (1986) Specific enzymatic amplification of DNA in vitro:the polymerase chain reaction. Cold Spring Harb Symp Quant Biol 51:263–273
2. Saiki RK et al (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487–491
3. Landegren U et al (1988) A ligase-mediated gene detection technique. Science 241:1077–1080
4. Barany F (1991) Genetic disease detection and DNA amplification using cloned thermostable ligase. Proc Natl Acad Sci U S A 88:189–193
5. Barany F (1991) The ligase chain reaction in a PCR world. PCR Methods Appl 1:5–16
6. Wu DY, Wallace RB (1989) The ligation amplification reaction (LAR)—amplification of specific DNA sequences using sequential rounds of template-dependent ligation. Genomics 4: 560–569

7. Barany F, Gelfand DH (1991) Cloning, overexpression and nucleotide sequence of a thermostable DNA ligase-encoding gene. Gene 109:1–11
8. Muller R, Ditzen A, Hille K, Stichling M, Ehricht R, Illmer T, Ehninger G, Rohayem J (2009) Detection of herpesvirus and adenovirus co-infections with diagnostic DNA-microarrays. J Virol Methods 155:161–166
9. Niederhauser C, Kaempf L, Heinzer I (2000) Use of the ligase detection reaction-polymerase chain reaction to identify point mutations in extended-spectrum beta-lactamases. Eur J Clin Microbiol Infect Dis 19:477–480
10. Wang XL, Xie SG, Zhang L, Yang WX, Wang X, Jin HZ (2008) Comparison of ligase detection reaction and real-time PCR for detection of abundant YMDD mutants in patients with chronic hepatitis B. World J Gastroenterol 14:120–124
11. Mark JA, Green LD, Deshpande A et al (2007) System integration and development for biological warfare agent surveillance. Proceedings of SPIE, optics and photonics in global homeland security III (65401D)
12. Song J, Li PE, Gans J et al (2010) Simultaneous pathogen detection and antibiotic resistance characterization using SNP-based multiplexed oligonucleotide ligation-PCR (MOL-PCR). Adv Exp Med Bio 680455–680464
13. Stucki D, Malla B, Hostettler S, Huna T et al (2012) Two new rapid SNP-typing methods for classifying Mycobacterium tuberculosis complex into the main phylogenetic lineages. PLoS One 7:e41253
14. Bruse S, Moreau M, Azaro M et al (2008) Improvements to bead-based oligonucleotide ligation SNP genotyping assays. BioTechniques 5:559–571
15. Deshpande A, Gans J, Graves SW, Green L, Taylor L, Kunde YA, Leonard PM, Li P-E, Mark J, Momchilo V, Scott White P, Song J, Kim HB (2010) A rapid multiplex assay for nucleic acid-based diagnostics. J Microbiologic Methods 80:155–163

Chapter 22

ELISA as a Diagnostic Weapon

Ramya Kalaivanan and Sankar Palanisamy

Abstract

The soundness of animal health is gauged by their ability to withstand infection and remain resistant to reinfection. The robustness of animals can be directly attributed to their sturdy immune system. The animals are often inflicted with potential infectious agents hindering their growth and productivity and occasionally succumb to the disease. Hence, diagnosis of diseases at the right stage is a critical step to adapt a suitable and effective control measures to contain their spread. Scientists rely on different immunological assays with varying degrees of sensitivity and specificity to determine the immune status of the animal. These assays are inevitable in detecting either the etiology or the product of an infection manifested as humoral or cell-mediated immunity. ELISA is a form of immunoassay using an enzyme-linked conjugate and enzyme substrate producing colored products depending on the concentration of the analyte present in the test system. It is one of the simple, versatile, reliable technique available in different formats which primarily detects antibody or antigen with highest level of sensitivity and specificity. This chapter describes the different types of ELISA procedures available for the diagnosis of foot and mouth disease infection in swine species.

Key words Enzyme-linked immunosorbent assay (ELISA), Diagnosis, Foot and mouth disease (FMD), Conjugate, Optical density, Non-structural proteins

1 Introduction

Assays using enzyme-linked conjugate and a substrate to demonstrate antigen–antibody interactions qualitatively/quantitatively are generally termed as Enzyme immunoassays (EIA)/ Enzyme-linked immunosorbent assay (ELISA). Enzyme-linked immunosorbent assay (ELISA) is a kind of immunological assay used to determine or quantitate the amount of analyte/antigen present in the biological fluids through the color change obtained by using an enzyme-linked conjugate and enzyme substrate. The technique relies on the significant attributes of an immune response, specificity of the antibody to its antigen. ELISA is classified as a primary binding assay which directly measures the binding of antigen to antibody with a sensitivity of detecting 0.0005 μg/

Rajib Deb et al. (eds.), *Protocols for the Diagnosis of Pig Viral Diseases*,
Springer Protocols Handbooks, https://doi.org/10.1007/978-1-0716-2043-4_22,

mL. ELISA is a highly versatile, sensitive, and quantitative technique requiring little equipment and for which critical reagents are readily available emphasizing its implication as a diagnostic weapon.

1.1 ELISA as a Diagnostic Weapon for FMD

FMD is an economically significant, acute, contagious disease of cloven-hoofed animals posing severe threat across the globe despite its eradication from few countries. Despite of active vaccination strategies undertaken by the nations; the disease remains endemic owing to its quasi-species nature. Although methods based on virus isolation or the demonstration of FMD viral antigen or nucleic acid in samples of tissue or fluid or culture products are sufficient for a positive diagnosis, in general, the ELISA [1, 2] using type-specific serological reagents is the preferred procedure for the detection of FMD viral antigen and identification of viral serotype in the early stages of research. Because it is more specific, sensitive and efficient, and it is not impacted by pro- or anti-complement factors [3], the ELISA has access to better development and even replaced complement fixation (CF) in most laboratories in the early research phase of FMD. Contrast to complement fixation test and virus isolation, almost the equivalent, even the higher of sensitivity was achieved in ELISA [4–6].

Enzyme-linked immunosorbent assay (ELISA) method was pioneered largely by the Swiss scientists Eva Engvall, and Peter Perlmann in the year 1971 by modifying the radioimmunoassay method used by Yalow and Berson in the 1960s. The ELISA technique was devised by conjugating antibody with enzymes rather than radioactive iodine 125 radioisotopes in radioimmunoassay to determine the levels of IgG in rabbit serum [7]. Van Weeman and Schuurs in the same year 1971 [8], a different research team, succeeded in quantifying human chorionic gonadotropin amounts in the urine by using horseradish peroxidase enzyme with the EIA method and also applied for a patent both in the USA and Europe.

Subsequent to the invention of ELISA, a number of researchers used it: Carlson and colleagues in 1972 [9], Holmgren and Svennerholm in diagnostic microbiology in 1973 [10], Ljungstrom and colleagues to identify the presence of trichinosis in parasitology in 1974 [11], and Voller et al. to diagnose malaria in 1975 [12]. Bishai and Galli, Leinikki et al., and Ukkonen et al. made use of the ELISA method to identify infections caused by influenza, parainfluenza, and mumps viruses in 1978, 1979, and 1981, respectively [13–16].

In the year 1980, Siegle et al. modified the ELISA test and incorporated microtitration plates to identify the concentrations of various hormones, peptides, and proteins apart from its application in diagnosis of infectious diseases [17]. At present, ELISA has transcended to newer level and is an indispensable assay in different formats in several fields of biology in research and diagnosis laboratories.

1.2 Timeline of Invention of Different Types of ELISA

1971—Engvall and Perlmann—Direct ELISA

1976—Yorde and his coworkers—Competitive ELISA

1977—Kato and his co-workers—Sandwich ELISA

1978—Lindström and Wager—Indirect ELISA.

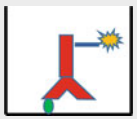
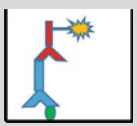
The principle of the basic ELISA procedure, direct ELISA is briefed at this juncture. The antigen utilized in the ELISA method is bound to a solid phase like tubes or microplates made of rigid polystyrene, polyvinyl, and polypropylene. These solid phases appropriately adsorb the antigen and the antibody primarily through hydrophobic interactions or hydrophobic/ionic interactions between the biomolecules and the surface. The enzymes that can be employed in ELISA include beta galactosidase, glucose oxidase, peroxidase, and alkaline phosphatase. Alkaline phosphatase on interaction with its substrate P-nitro-phenyl phosphate (pNPP) produces a yellow color in positive reactions at 405 nm. The horseradish peroxidase on interaction with its substrate 3,3′,5,5-′-tetramethylbenzidine (TMB) or 2,2′-azino-di-[3-ethylbenzthiazoline-6-sulfonicacid] (ABTS) or orthophenylenediamine hydrochloride (OPD) produces a brown color in a positive reaction at 492 nm. The beta galactosidase on interaction with its substrate ortho-nitrophenyl-β-galactoside (ONPG) produces a yellow color at 420 nm. The catabolic effects of enzymes determine both the acceleration and the specificity of the immunological reaction during the enzyme-substrate reaction [18]. The enzyme-substrate reaction is usually completed within 30–60 min. The reaction can be stopped using sodium hydroxide (NaOH), hydrochloric acid (HCl), or sulfuric acid (H_2SO_4) [19].

ELISA is a heterogeneous immunoassay technique involving washing procedure to remove unbound biomolecules and used to detect specific antibodies and soluble antigens. ELISA has been designed in different formats to determine the quantity of substances to be measured since each of them differs in its structure and biochemical characteristics. Each of them differs by which component is immobilized, how it is recognized, and what is detected (Table 1).

1.3 Direct ELISA

Direct ELISA has the simplest format, requiring antigen to be adsorbed to the plate and then bound by a labeled “detection” antibody followed by washing which removes the unbound antibodies. Subsequent addition of appropriate substrate produces a signal through color development depending on the concentration of analyte. “Direct” refers to the first and only antibody acting as both the antigen recognition molecule and signal delivery molecule. It is suitable for determining the amount of high molecular weight antigens.

Table 1
Features of and the differences between ELISA types

S. no.	Types of ELISA	Infographics	Detects	Advantages	Disadvantage
1.	Direct ELISA		Antibodies	• Faster than other ELISA. • Less prone to error.	• False-positive. • Antigen immobilization is not specific: may cause higher background noise. • Less flexible: each target protein needs a specific conjugated primary antibody. • No signal amplification: reduces assay sensitivity.
2.	Indirect ELISA (iELISA)		Antigen/antibodies	• Highly sensitive. • Economical. • Greater flexibility: different primary antibodies can be used with a single labeled secondary antibody.	• Possibility of background noise: secondary antibody may be cross-reactive. • Longer procedure than direct ELISA technique: additional incubation step for secondary antibody needed.
3.	Competitive/blocking/ inhibition ELISA (cELISA)		Antigen/antibodies	• Very highly sensitive. • No sample processing is required and crude or impure samples can be used. • More robust—less sensitive to sample dilution and sample matrix effects than the sandwich ELISA.	

S. no.	Types of ELISA	Infographics	Detects	Advantages	Disadvantage
				• More consistent—less variability between duplicate samples and assays. • Maximum flexibility—it can be based on direct, indirect, or sandwich ELISA. • Suitable for detecting small antigens.	
4.	Sandwich ELISA		Antigens	• High sensitive: 2–5 times more sensitive than direct or indirect ELISA. • High specificity: two antibodies are involved in capture and detection. • Analysis of complex samples possible: the antigen does not need to be purified prior to measurement. • Flexibility: both direct and indirect detection can be used.	
Antigen Substrate		Unknown Antibody	Primary antibody	Labeled antigen specific antibody	Conjugate (Enzyme-labeled detection antibody)

1.4 Indirect ELISA

Indirect ELISA as the name implies allows for the amplification of signal by using a secondary antibody. The indirect ELISA procedure starts with adsorption of antigen to the well of an ELISA plate. Following standard blocking and washing steps an unlabeled primary antibody (test serum sample to determine the concentration) binds to the specific antigen. The antigen and antibody complex formed during incubation is recognized by addition of an enzyme conjugated species-specific secondary antibody. The color development upon suitable substrate addition depends on the concentration of the primary antibody present in the serum sample.

1.5 Sandwich ELISA

The distinguishing feature of a sandwich ELISA is the adsorption of a "capture" antibody to the plate. Antigen is bound or captured by the plated antibody and then "sandwiched" between the capture and a detecting antibody which recognizes a distinctly different epitope on the antigen. Each antibody is therefore specific for a different and non-overlapping region or epitope of the same antigen. A major benefit of a sandwich ELISA is the ability to specifically measure antigen from impure samples. The capture antibodies provide the assay specificity by adsorbing the antigen of interest on the contrary the crude sample to the plate. The opportunity for indirect detection is also available in a sandwich ELISA, wherein the detection antibody would not carry the signal but rather be targeted by yet a third antibody which would impart the signal to the assay.

The procedure for sandwich ELISA involves coating the well of an ELISA plate with a capture antibody. The analyte or sample is then added, followed by a detection antibody. The detection antibody can be enzyme conjugated, which is referred to as a *direct sandwich ELISA*. In contrast, in an *indirect sandwich ELISA* a secondary enzyme-conjugated detection antibody is needed if the detection antibody is unlabeled.

1.6 Competitive/ Inhibition ELISA

The competitive/inhibition ELISA is predominantly used to measure the concentration of an antigen or antibody in a sample by detecting interference in an expected signal output. In this format, the sample antigen or antibody competes with a reference for binding to a limited amount of labeled antibody or antigen, respectively. The higher the sample antigen concentration, the weaker the output signal, indicating that the signal output inversely correlates with the amount of antigen in the sample.

The procedure involves coating a known antigen to a multiwell plate. Following standard blocking and washing steps, samples containing unknown antigen are added. Labeled detection antibody is then applied for detection using appropriate substrates. The intensity of color development is inversely related to the concentration of antigen in the sample.

2 Materials

2.1 Solid-Phase Competitive ELISA for the Diagnosis of FMD [20]

Solid-phase competitive ELISA (SPCE) has replaced complement fixation in most laboratories as it is more specific and sensitive [21]. The method described by Paiba et al. [21, 22] can be used for the detection of antibodies against each of the seven serotypes of FMDV. Moreover, SPCE has the advantage that the test is rapid (the result can be read in 1 day vs. waiting 3 days in VNT) and easier to perform [23].

1. Trapping/Capture Antibody: Serotype-specific antiserum raised in rabbit/guinea pig/monoclonal antibody.
2. Coating buffer: Carbonate/Bicarbonate buffer:

Sol. A:	0.2 M sodium carbonate solution:
Na_2CO_3	21.198 g
Distilled water	1000 ml
Sol. B:	0.2 M sodium bicarbonate solution
$NaHCO_3$	16.8 g
Distilled water	1000 ml
Working solution	Store at 4 °C
Solution A	16 ml
Solution B	32 ml
Triple distilled water	up to 200 ml

3. Washing Buffer: Phosphate buffer saline (PBS)—10×:

NaCl	80 g
KCl	2 g
Na_2HPO_4	14.4 g
KH_2PO_4	2.45 g
Triple distilled water	to 1000 ml

Adjust pH of the solution to 7.4 with HCl and sterilize by autoclaving for 20 min at 15 psi.

4. Blocking buffer:

PBS	Volume required
Tween 20	0.05% [w/v]
NBS—New born serum/Fetal calf serum	10% [v/v]
Normal rabbit serum	5% [v/v]

5. Antigen: 146S antigen (*see* **Note 1**).
6. Diluent: Blocking buffer with phenol red indicator.
7. Test sera: Diluted in PBST blocking buffer.
8. Detection antibody: Guinea-pig antisera (*see* **Note 2**).
9. Rabbit (or sheep) anti-guinea-pig immunoglobulin conjugated to horseradish peroxidase at a predetermined optimum concentration in PBSTM blocking buffer.
10. Substrate solution: Citrate buffer (pH 5.0). Store at 4 °C.

Citric acid	7.30 g
$Na_2HPO_4 \cdot 2H_2O$	11.87 g
Distilled water	up to 1000 ml

11. Stopping solution: 1 M sulfuric acid.

Conc. H_2SO_4	5.56 ml
Distilled water	1000 ml

Stored at room temperature.

12. Multichannel pipette and disposable pipette tips.
13. 96-well ELISA plates (polystyrene, medium to high binding).
14. ELISA reader.

2.2 Liquid Phase Blocking ELISA for Diagnosis of FMD [20]

- Antigen: 146S antigen (*see* **Note 3**).
- Diluent.
- Rabbit antiserum homologous to the antigen.
- Coating buffer: Carbonate/Bicarbonate buffer (*see* Subheading 2.1).
- Blocking buffer: (*see* Subheading 2.1).
- Test sera: Diluted in PBST blocking buffer.
- Detection antibody: Guinea-pig antisera against 146S antigen of one of the seven serotypes of FMDV.
- Rabbit (or sheep) anti-guinea-pig immunoglobulin conjugated to horseradish peroxidase at a predetermined optimum concentration in PBSTM blocking buffer (*see* Subheading 2.1).
- Substrate solution: Citrate buffer (pH 5.0) (*see* Subheading 2.1).
- Stopping solution: 1 M sulfuric acid (*see* Subheading 2.1).

2.3 Indirect ELISA to Detect Non-structural Protein (NSP ELISA) [20]

The non-structural proteins of foot and mouth disease virus are nothing but the enzymes involved in the viral replication. The presences of non-structural proteins or antibodies to non-structural proteins in the clinical specimens are clear indication of the animal

being infected or exposed to the live virus. The NSPs, unlike structural proteins, are highly conserved and therefore are not serotype specific and as a consequence, the detection of these antibodies is not serotype restricted. Hence, demonstrations of antibodies to non-structural proteins (L^{pro}, 2A, 2B, 2C, 3A, 3B, $3C^{pro}$ and 3D) [24, 25] are yardstick to differentiate clinically infected animals from the vaccinated ones (DIVA—Differentiation of infected from vaccinated animals) since animals are vaccinated only with inactivated viruses.

Enzyme-linked immunosorbent assay (ELISA) based assays can be used to detect anti-NSPs antibodies in carrier cattle [26]. Several recombinant NSPs of FMDV viz. 2B, 2C, 3A, 3AB, 3B, 3ABC, and 3D have been used in NSP ELISAs [27, 28]. These ELISAs either use purified antigens absorbed directly to microplates or use PAbs or MAbs to trap specific antigens from semi-purified preparations [29–32]. NSP ELISA although not considered as a suitable test for certifying animals prior to movement, NSP ELISAs may be a valuable adjunct in circumstances where the serotype or subtype of virus in the originating country is not known.

- Recombinant 3ABC antigen [33] (*see* **Note 4**).
- Coating buffer: Carbonate/Bicarbonate buffer (*see* Subheading 2.1).
- Washing buffer.

PBS (pH 7.2)	Volume required
Tween 20	0.05% [w/v]

- Blocking buffer.

PBS	Volume required
Tween 20	0.05% [w/v]
Nonfat dry milk	5% [w/v]
Equine	10% [v/v]
Escherichia coli lysate	0.1%

- Serum from suspected swine.
- Conjugate: Anti-porcine enzyme-linked IgG dilution as per the supplier instruction.
- Substrate solution: Citrate buffer (pH 5.0) (*see* Subheading 2.1).

- Stopping solution: 0.5 M Sulfuric acid (*see* Subheading 2.1).

Conc. H_2SO_4	2.78 ml
Distilled water	1000 ml

Stored at room temperature.

- *See* **Note 5**.

3 Methods [20]

3.1 Solid-Phase Competitive ELISA for the Diagnosis of FMD

- Coat the ELISA plates with 50 μl/well rabbit antiserum homologous to the antigen being used, diluted in carbonate/bicarbonate buffer, pH 9.6, and leave it overnight in a humid chamber at 4 °C.
- Wash the ELISA plates thrice with PBS.
- Add 50 μl of the FMDV antigen diluted in blocking buffer to each well of the ELISA plates. Cover the plates and place it on an orbital shaker at 37 °C for 1 h, with continuous shaking.
- After washing thrice with PBS, add 40 μl of blocking buffer to each well, followed by 10 μl of test sera (or control sera), giving an initial serum dilution of 1/5.
- Add 50 μl of guinea-pig serotype specific anti-FMDV antiserum diluted in blocking buffer immediately to give a final serum dilution of 1/10.
- Cover the plates and incubate on an orbital shaker at 37 °C for 1 h.
- After washing thrice with PBS, add 50 μl of anti-guinea-pig immunoglobulin conjugate (preblocked by incubation for 1 h at room temperature with an equal volume of NBS) diluted in blocking buffer. Cover the plates and incubate for 1 h at 37 °C on an orbital shaker.
- After washing thrice with PBS, add 50 μl of substrate solution containing 0.05% H_2O_2 plus orthophenylene diamine or a suitable alternative chromogen to each well.
- Stop the reaction after 10 min by adding 50 μl of 1 M sulfuric acid.
- Read the plates at 492 nm on a spectrophotometer linked to a computer.
- *Controls*: On each plate.
  - Conjugate control (two wells)—No guinea-pig serum.
  - Strong and weak positive sera (four wells each).

  - Negative sera (two wells).
  - 0% competition (four wells)—Without test sera.
- *Interpretation of the results*:
  The percentage of inhibition is calculated for each well either manually or using a suitable computer program.

$$\text{Percentage of inhibition} = \frac{100 - \text{Optical density of each test or control value}}{\text{Mean optical density of the 0\% competition}} \times 100$$

It represents the competition between the test sera and the guinea-pig anti-FMDV antisera for the FMDV antigen on the ELISA plate (*see* **Note 6**).

3.2 Liquid Phase Blocking ELISA (LPBE) for Diagnosis of FMD

- Coat the ELISA plates with 50 μl/well rabbit antiserum homologous to the antigen being used and leave overnight in a humid chamber at room temperature.
- Wash the ELISA plates thrice with PBS.
- Prepare 50 μl of duplicate, twofold series of each test serum in U-bottomed multiwell plates (carrier plates) starting at 1/8. Add 50 μl of a constant dose of viral antigen that is homologous to the rabbit antisera used to coat the plates to each well and leave the mixtures overnight at 4 °C, or incubated at 37 °C for 1 h. The addition of the antigen increases the final serum dilution to 1/16.
- Transfer 50 μl of serum/antigen mixtures from the carrier plates to the rabbit serum coated ELISA plates and incubate at 37 °C for 1 h in an orbital shaker.
- After washing thrice with PBS, add 50 μl of guinea-pig antiserum homologous to the viral antigen used in the previous step (preblocked with NBS and diluted in PBST containing 5% skimmed milk powder) to each well and incubate at 37 °C for 1 h on a rotary shaker.
- Wash the plates thrice and add 50 μl of rabbit anti-guinea-pig immunoglobulin conjugated to horseradish peroxidase (preblocked with NBS and diluted in PBST containing 5% skimmed milk powder) to each well and incubate at 37 °C for 1 h in an orbital shaker.
- Wash the plates again thrice and add 50 μl of substrate solution, containing 0.05% H_2O_2 plus orthophenylene diamine or a suitable alternative chromogen to each well.
- Stop the reaction after 15 min by adding 50 μl of 1 M sulfuric acid.

- Read the plates at 492 nm on a spectrophotometer linked to a computer.
- *Controls*: Include on each plate a minimum of four wells each of bovine reference sera at a final dilution of 1/32.
  - strong positive.
  - weak positive.
  - negative.
  - Reaction (antigen) control wells containing antigen in diluent alone without serum.
  - For end-point titration tests, duplicate twofold dilution series of positive and negative homologous reference sera should be included on at least one plate of every run.
- *Interpretation of the results*:

  Antibody titers are expressed as the 50% end-point titer, i.e., the dilution at which the reaction of the test sera results in an optical density equal to 50% inhibition of the median optical density of the reaction (antigen) control wells [33]. The median is calculated as the mean of two mid-values of the reaction control wells, eliminating from the calculation the highest and lowest values (*see* **Note 7**).

3.3 Indirect ELISA to Detect Non-structural Protein (NSP ELISA)

- Coat microplates with 1 μg/ml of the recombinant fusion antigen 3ABC in carbonate/bicarbonate buffer, pH 9.6 (100 μl per well) for overnight at 4 °C.
- Wash the plates six times with washing buffer (PBST).
- Add 100 μl of 1/20 dilution test sera in blocking buffer per well and incubate the plates for 30 min at 37 °C and wash six times in PBST.
- Add 100 μl optimally diluted horseradish-peroxidase-conjugated rabbit anti-species (swine) IgG in the blocking buffer per well and incubate the plates for 30 min at 37 °C.
- After six washings, fill each well with 100 μl of 3′3′, 5′5-′-tetramethylbenzidine plus 0.004% (w/v) H_2O_2 in phosphate/citrate buffer, pH 5.5.
- Stop the reaction after 15 min of incubation at room temperature by adding 100 μl of 0.5 M H_2SO_4.
- Read the absorbance at 450 nm and at 620 nm for background correction.
- *Interpreting the results*:

  Test results are expressed as percent positivity relative to the strong positive control

$$\text{Percent positivity} = \frac{\text{Optical Density of Testor Control wells}}{\text{Optical Density of Strong Positive Control}} \times 100$$

or alternatively as a test to control (T/C) index relative to a cut-off (i.e., threshold positive) control. Profiling the NSP antibody reactivity levels in herds along with age/vaccination stratification aids interpretation of herd infection status in vaccinated populations [34].

4 Notes

1. The 146S antigen needs to be prepared by propagating the serotype of interest in cell culture and inactivating viruses with ethyleneimine as described for vaccine manufacture (The final dilution chosen is that which, after addition of an equal volume of diluent, gives an absorbance on the upper part of the linear region of the titration curve) (optical density approximately 1.5).
2. Prepared by inoculating guinea-pigs with FMDV 146S antigen of serotype of interest. Predetermined optimal concentrations to be prepared in blocking buffer PBS containing 0.05% Tween 20, and 5% dried, nonfat skimmed milk (PBSTM).
3. Antigens are prepared from selected strains of FMDV grown on monolayers of BHK-21 cells. The unpurified supernatants are used and pretitrated in a twofold dilution series but without serum. The final dilution chosen is that which, after addition of an equal volume of diluent (*see* below), gives an absorbance on the upper part of the linear region of the titration curve (optical density approximately 1.5).
4. (a) The five bioengineered FMDV NSPs 3A, 3B, 2C, 3D, and 3ABC are expressed in *E. coli* C600 by thermo-induction. The 3D polypeptide is expressed in its complete form [35], whereas the rest of the proteins are obtained as fusions to the N-terminal part of the MS-2 polymerase gene [36].

   (b) The expressed polymerase is purified over phosphocellulose, followed by poly(U) Sepharose columns. The fused proteins 3A, 3B, 2C, and 3ABC are purified by sequential extraction of the bacterial extracts with increasing concentrations of urea. The 7M fraction containing the fusion proteins is further purified on a preparative 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). The fusion protein band is excised from the gel and electroeluted [35].

5. (a) Includes each plate a set of strong and weak positive and negative controls calibrated against the International Standard Sera
   (b) Test cut-off values, with or without suspicious zones, need to be determined with consideration to the purpose of testing and the intended target population. Inconclusive results may be followed up using confirmatory tests, retesting with EITB or a second NSP ELISA (taking account of the conditional dependence of the two tests). Although not a suitable test for certifying animals prior to movement, NSP ELISAs may be a valuable adjunct in circumstances where the serotype or subtype of virus in the originating country is not known.
6. The assay should be validated in terms of the cut-off value above which sera should be considered positive in relation to.
   (a) the particular serotypes and strains of virus under investigation
   (b) the purpose of testing
   (c) the population under test

   The reference values may be as per the OIE Reference Laboratory at Pirbright, for serotype O, for all species, for the purposes of demonstrating freedom from infection in a naïve population, *greater than 60% inhibition is considered positive* [21]. For maximum sensitivity, for example when certifying individual animals for international trade, *an inconclusive range may be set between 40 and 60%.*
7. In general sera with titers greater than or equal to 1/90 are considered to be positive. A titer of less than 1/40 is considered to be negative. For certification of individual animals for the purposes of international trade, titers of greater than 1/40, but less than 1/90 are considered to be doubtful, and further serum samples may be requested for testing; results are considered to be positive if the second sample has a titer of 1/40 or greater. For the purposes of herd-based serosurveillance as part of a statistically valid serological survey, a cut-off of 1/90 may be appropriate. Cut-off titers for evaluating immunological protection afforded by vaccination have to be established from experience of potency test results with the relevant vaccine and target species.

References

1. Ferris NP, Donaldson AI (1992) The World Reference Laboratory for Foot and Mouth Disease: a review of thirty-three years of activity (1958-1991). Rev Sci Tech 11(3):657–684
2. Roeder PL, Smith PLB (1987) Detection and typing of foot-and-mouth disease virus by enzyme-linked immunosorbent assay: a

sensitive, rapid and reliable technique for primary diagnosis. Res Vet Sci 43(2):225–232

3. Ferris NP, Dawson M (1988) Routine application of enzyme-linked immunosorbent assay in comparison with complement fixation for the diagnosis of foot-and-mouth and swine vesicular diseases. Vet Microbiol 16(3):201–209
4. Hamblin C, Armstrong RM, Hedger RS (1984) A rapid enzyme-linked immunosorbent assay for the detection of foot-and-mouth disease virus in epithelial tissues. Vet Microbiol 9(5):435–443
5. Have P, Lei JC, Schjerning-Thiesen K (1984) An Enzyme-Linked Immunosorbent Assay (ELISA) for the primary diagnosis of foot-and-mouth disease characterization and comparison with complement fixation. Acta Vet Scand 25(2):280–296
6. Smitsaart EN, Saiz JC, Yedloutschnig RJ, Morgan DO (1990) Detection of foot-and-mouth disease virus by competitive ELISA using a monoclonal antibody specific for the 12S protein subunit from six of the seven serotypes. Vet Immunol Immunopathol 26(3):251–265. https://doi.org/10.1016/0165-2427(90)90095-A
7. Engvall E, Perlmann P (1971) Enzyme-linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G. Immunochemistry 8(9):871–874
8. Van Weemen B, Schuurs AHWM (1971) Immunoassay using antigen—enzyme conjugates. FEBS Lett 15(3):232–236
9. Carlsson HE, Lindberg AA, Hammarström S (1972) Titration of antibodies to Salmonella O antigens by enzyme-linked immunosorbent assay. Infect Immun 6(5):703–708
10. Holmgren J, Svennerholm AM (1973) Enzyme-linked immunosorbent assays for cholera serology. Infect Immun 7(5):759–763
11. Ljungström I, Engvall E, Ruitenberg EJ (1974) Proceedings: ELISA, enzyme linked immunosorbent assay-a new technique for sero-diagnosis of trichinosis. Parasitology 69(2):xxiv
12. Voller A, Huldt G, Thors C, Engvall E (1975) New serological test for malaria antibodies. Br Med J 1(5959):659–661
13. Bishai FR, Galli R (1978) Enzyme-linked immunosorbent assay for detection of antibodies to influenza A and B and parainfluenza type 1 in sera of patients. J Clin Microbiol 8 (6):648–656
14. Leinikki PO, Shekarchi I, Tzan N, Madden DL, Sever JL (1979) Evaluation of enzyme-linked immunosorbent assay (ELISA) for mumps virus antibodies. Proc Soc Exp Biol Med 160(3):363–367
15. Ukkonen P, Penttinen K, Granström ML (1981) Mumps-specific immunoglobulin M and G antibodies in natural mumps infection as measured by enzyme-linked immunosorbent assay. J Med Virol 8(2):131–142
16. Siegle RL, Jennings BR, Adams PL, King LP (1980) Development of a model using polypeptide antibodies for scintigraphy of the pancreas. Investig Radiol 15(5):457–461
17. Engvall E (2010) The ELISA, enzyme-linked immunosorbent assay. Clin Chem 56(2): 319–320
18. Hornbeck P (1992) Enzyme-linked immunosorbent assays. Curr Protoc Immunol 1(1):2-1. https://doi.org/10.1002/0471142735.im0201s01. (Chapter 2: Unit 2.1)
19. Ma LN, Zhang J, Chen HT, Zhou JH, Ding YZ, Liu YS (2011) An overview on ELISA techniques for FMD. Virol J 8(1):1–9
20. OIE Terrestrial Manual (2018) Chapter.3.1.8. Foot and mouth disease (infection with Foot and Mouth disease virus). OIE
21. Paiba GA, Anderson J, Paton DJ, Soldan AW, Alexandersen S, Corteyn M, Donaldson AI (2004) Validation of a foot-and-mouth disease antibody screening solid-phase competition ELISA (SPCE). J Virol Methods 115(2): 145–158
22. Sevik M, Ozturk FF (2013) Comparative evaluation of liquid-phase blocking ELISA and solid-phase competition ELISA methods for the detection of antibodies to the structural proteins of foot-and-mouth disease types O and A viruses. Turk J Vet Animal Sci 37(5): 523–528
23. Belsham GJ (1993) Distinctive features of foot-and-mouth disease virus, a member of the picornavirus family; aspects of virus protein synthesis, protein processing and structure. Prog Biophys Mol Biol 60(3):241–260
24. Klump W, Marquardt O, Hofschneider PH (1984) Biologically active protease of foot and mouth disease virus is expressed from cloned viral cDNA in Escherichia coli. Proc Natl Acad Sci 81(11):3351–3355. https://doi.org/10.1073/pnas.81.11.3351
25. Moonen P, van der Linde E, Chénard G, Dekker A (2004) Comparable sensitivity and specificity in three commercially available ELISAs to differentiate between cattle infected with or vaccinated against foot-and-mouth disease virus. Vet Microbiol 99(2):93–101
26. Clavijo A, Wright P, Kitching P (2004) Developments in diagnostic techniques for

differentiating infection from vaccination in foot-and-mouth disease. Vet J 167(1):9–22

27. Brocchi E, Bergmann IE, Dekker A, Paton DJ, Sammin DJ, Greiner M et al (2006) Comparative evaluation of six ELISAs for the detection of antibodies to the non-structural proteins of foot-and-mouth disease virus. Vaccine 24(47–48):6966–6979
28. Bergmann IE, Malirat V, Neitzert E, Beck E, Panizzutti N, Sanchez C, Falczuk A (2000) Improvement of a serodiagnostic strategy for foot-and-mouth disease virus surveillance in cattle under systematic vaccination: a combined system of an indirect ELISA-3ABC with an enzyme-linked immunoelectrotransfer blot assay. Arch Virol 145(3):473–489
29. De Diego M, Brocchi E, Mackay D, De Simone F (1997) The non-structural polyprotein 3ABC of foot-and-mouth disease virus as a diagnostic antigen in ELISA to differentiate infected from vaccinated cattle. Arch Virol 142(10):2021–2033
30. Mackay DKJ, Forsyth MA, Davies PR, Berlinzani A, Belsham GJ, Flint M, Ryan MD (1998) Differentiating infection from vaccination in foot-and-mouth disease using a panel of recombinant, non-structural proteins in ELISA. Vaccine 16(5):446–459
31. Sørensen KJ, Madsen KG, Madsen ES, Salt JS, Nqindi J, Mackay DKJ (1998) Differentiation of infection from vaccination in foot-and-mouth disease by the detection of antibodies to the non-structural proteins 3D, 3AB and 3ABC in ELISA using antigens expressed in baculovirus. Arch Virol 143(8):1461–1476
32. Neitzert E, Beck E, de Mello PA, Gomes I, Bergmann IE (1991) Expression of the aphthovirus RNA polymerase gene in Escherichia coli and its use together with other bioengineered nonstructural antigens in detection of late persistent infections. Virology 184(2): 799–804
33. Karber G (1931) 50% end point calculation. Archiv Exp Pathol Pharma 162:480–483
34. Bergmann IE, Neitzert E, Malirat V, Ortiz S, Colling A, Sanchez C, Melo EC (2003) Rapid serological profiling by enzyme-linked immunosorbent assay and its use as an epidemiological indicator of foot-and-mouth disease viral activity. Arch Virol 148(5):891–901
35. McCullough KC, Bruckner L, Schaffner R, Fraefel W, Müller HK, Kihm U (1992) Relationship between the anti-FMD virus antibody reaction as measured by different assays, and protection in vivo against challenge infection. Vet Microbiol 30(2–3):99–112
36. Strebel K, Beck E, Strohmaier K, Schaller H (1986) Characterization of foot-and-mouth disease virus gene products with antisera against bacterially synthesized fusion proteins. J Virol 57(3):983–991

Chapter 23

SDS-PAGE and Western Blotting: Basic Principles and Protocol

Mukesh Bhatt, Vishal Rai, Ashok Kumar, Kiran, Ajay Kumar Yadav, Kaushal Kishor Rajak, Vikas Gupta, Vishal Chander, and R. K. Avasthe

Abstract

Western blotting is an important analytical technique used in cell and molecular biology for last four decades. It involves separation of proteins in SDS-PAGE and then transfer of proteins to a membrane followed by detection. By using a western blot, one can identify specific protein from a complex mixture of proteins. Along with its use as a diagnostic aid, it can also be used to verify proteins of interest in exploratory proteomic studies to identify different disease mechanisms. The ease of performing the technique, low cost, and accessibility further support the use of western blot in proteomic research. However, a good understanding, initial training, and optimization are of utmost importance because being a multi-step technique, it is prone to false results and incorrect interpretation. This chapter attempts to explain the technique and theory behind western blot along with some ways to troubleshoot.

Key words SDS-PAGE, Western blot, Protein blotting, Immunoblot

1 Introduction

The term "blotting" refers to the transfer of biological samples from a gel on to a membrane and their subsequent detection on the surface of the membrane. The western blot (often called as the protein immunoblot because an antibody is used to specifically detect the antigen) is a widely used analytical technique to detect specific proteins in a given sample of tissue homogenate or extract. The technique was initially described by Towbin et al. in 1979 [1] and the name was given by Burnette in 1981 [2] to match similar techniques used for detection of DNA in Southern blotting [3] and RNA in Northern blotting [4].

The first step in a Western blotting procedure is to separate the macromolecules using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis, the separated molecules

Rajib Deb et al. (eds.), *Protocols for the Diagnosis of Pig Viral Diseases*,
Springer Protocols Handbooks, https://doi.org/10.1007/978-1-0716-2043-4_23,

are transferred or blotted onto a second matrix, generally a nitrocellulose or polyvinylidene difluoride (PVDF) membrane [5]. Next, the membrane is blocked to prevent any nonspecific binding of antibodies to the surface of the membrane. The transferred protein is then complexed with an enzyme-labeled antibody as a probe. An appropriate substrate is then added to the enzyme and together they produce a detectable product such as a chromogenic or fluorogenic precipitate on the membrane for colorimetric or fluorometric detection, respectively. This technique is very useful to detect the presence of a given protein in a sample, to assess posttranslational modifications such as phosphorylation and to characterize protein complexes. Although the western blotting protocol for specific purpose can be optimized, here we discuss step-by-step, the general principle and protocol followed for western blotting procedure in virology.

2 Sample Preparation

The first step for western blotting is sample preparation. Protein samples from cultured cells can be extracted either by direct dissolution in a denaturing buffer or by homogenization in a buffer containing protease inhibitors. Sample denaturing buffers contain the powerful anionic detergent sodium dodecyl sulfate (SDS), which linearizes the proteins, and a reducing agent such as 2-mercaptoethanol to break disulfide bonds. The ionic buffers are supposed to provide higher yield of proteins as compared to the nonionic buffers; however, this may lead to the loss of some functional properties of proteins [5]. The viscosity of the sample treated with ionic buffers is also higher due to the release of the chromatin and thus may need sonication.

The other agents used for sample preparation are nonionic detergents which generally include Triton X-100, Nonidet P-40, Tween, etc. and zwitter-ionic (CHAPS) detergents. Although these agents are less harsh and less denaturing and keep the proteins relatively intact, the protein yield is comparatively low especially from subcellular organelles and membranes [6]. As the cell lysis leads to the release of proteases and phosphatases, the samples must be kept at 4 °C and protease inhibitor cocktail must be used to prevent any further protein degradation. It is useful to know the protein concentration in the lysates to control the amount loaded on the gel and to allow comparison between experiments. It is also important to have an idea of the protein concentration in the extracted sample using a suitable method [7–9] so as to ensure that the total protein amount in each sample under analysis is equivalent.

The final step is to mix the samples with a loading buffer or sample treatment buffer composed of (a) glycerol to increase the

density of the sample facilitating its loading on the gel, (b) a coloring dye to make the sample visible (bromophenol or Coomassie Blue), (c) the agents reducing disulfide bonds (β-mercaptoethanol or dithiothreitol, DTT), (d) SDS in case of denaturing conditions, and (e) Tris-HCl. To ensure complete denaturation of the proteins, samples are usually boiled at 95 °C for 5–10 min before loading.

3 Electrophoresis

The electrophoresis can be optimized for the specific proteins and based on the purpose of the study. The two common methods used for electrophoresis are discussed here.

4 SDS-PAGE

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is the most widely used method for analyzing protein mixtures qualitatively [10]. Denatured proteins in individual samples are separated by SDS-PAGE. Polyacrylamide gels are formed by the polymerization of two compounds, acrylamide and the crosslinking agent N, N-methylene bisacrylamide, in a buffered solution containing 1% SDS in presence of TEMED (tetramethylethylenediamine) and APS (ammonium persulfate). The polymerized gels form a 3-D network of long hydrocarbons cross-linked by methylene groups forming a sieve of pores that allows the movement of proteins. The pore size is inversely proportional to the concentration of the acrylamide in the gel (i.e., higher percentage gel allows better resolution of smaller proteins while the low conc. Gels allow better resolution of high molecular weight proteins) (Table 1). The negative charge to the proteins is rendered by binding of SDS molecule to the denatured proteins, thereby swamping the original charge on protein molecules. Generally, one molecule of SDS binds every two amino acids in a protein. Two vertical gels are used in SDS-PAGE procedure, i.e., stacking gel, which is having lower pH (6.8) and larger pore size and the resolving gel which is having a pH of 8.8 and different pore size depending on the target protein size and acrylamide concentration [11]. The composition of stacking and resolving gels is given in Table 2.

In stacking gel, proteins move at a faster speed sandwiched between chloride ions at top and glycinate ions at bottom to form a layer at the junction of stacking and resolving gel. After reaching the resolving gel, the glycinate becomes fully ionized and moves at a faster speed leaving behind the protein-SDS complexes which move towards the anode [11]. As protein-SDS complexes have the same charge per unit length, they travel into the separating gel under the applied electric field with the same mobility.

Table 1
Suggested acrylamide concentration in resolving gel for separation of proteins based on molecular weight

Protein mol. weight (kDA)	Acrylamide conc. in resolving gel (%)
10–43	15
12–60	12
20–80	10
30–95	8
50–200	6

Table 2
Composition of stacking and resolving gel for SDS-PAGE

		Resolving gel (10 ml)		
Component	**Stacking gel (5 ml)**	**15%**	**12%**	**10%**
Water	3.4	2.3	3.3	4.0
Acrylamide: bisacrylamide (29:1)	0.83	5.0	4.0	3.3
Stacking gel buffer (1.0 M pH 6.8)	0.63	–		
Resolving gel buffer (1.5 M pH 8.8)	–	2.5	2.5	2.5
10% SDS	0.05	0.1	0.1	0.1
10% APS	0.05	0.1	0.1	0.1
TEMED	0.005	0.004	0.004	0.004

5 Native PAGE

Although SDS-PAGE is the most frequently used gel system for studying proteins, it is not a suitable method to study the proteins (enzymes) based on their biological activity as the proteins are denatured by the SDS–PAGE procedure [11]. In such cases, the non-denaturing conditions are used where the polyacrylamide gels but without SDS are used and no sample treatment is done before sample loading. Since all the proteins carry their native charge at the gel pH (8.7), proteins are separated based on their electrophoretic mobility and difference in charge and size of the proteins in a given mixture. The biological properties of proteins under study can be measured using a suitable substrate solution which may produce detectable signal at the position of protein in the gel.

6 Protocol for SDS-PAGE

6.1 Materials Required

- Acrylamide:bisacrylamide (29:1).
- SDS (10%).
- Ammonium persulfate (10%).
- TEMED.
- SDS-PAGE apparatus.
- Resolving and stacking gel buffers.
- Gel loading buffer.
- Electrophoresis buffer.
- 2× gel loading buffer.
- Staining solution.
- Destaining solution.

6.2 Procedure

6.2.1 Casting of Gel

Determine the volume of the gel to be prepared. Prepare the required volume of solution containing the desired concentration of acrylamide for resolving gel as given in table (Table 1).

6.2.2 Details of Gel Preparation

1. Mix the components in the order shown. Polymerization will begin as soon as the TEMED has been added. Without delay, swirl the mixture rapidly and proceed to next step.
2. Pour the acrylamide solution between the glass plates using a pipette. Overlay with water or water saturated butanol.
3. After polymerization is complete, pour off the overlay and wash the top of gel several times with deionized water to remove any unpolymerized acrylamide.

6.2.3 Stacking Gel

Pour the stacking gel solution directly on the surface of the polymerized resolving gel. Immediately insert a clean Teflon comb in the stacking gel solution being careful to avoid trapping air bubble.

6.2.4 Sample Preparation and Loading

1. While the stacking gel is polymerizing, prepare the samples by boiling for 3 min in 1× SDS gel loading buffer to denature the protein.
2. After polymerization is complete, remove the Teflon comb carefully and mount the gel in the electrophoresis apparatus. Add Tris-glycine electrophoresis buffer to the top and bottom reservoirs.
3. Load upto 15 μl of each of the sample in a predetermined order.
4. Attach the electrophoresis apparatus to an electric power supply and run the gel for 1–2 h at 100 V constant voltage.
5. After completion of run, take apart the glass plates and carefully remove the gel into a staining tray.

6.2.5 Staining of Polyacrylamide Gel with Coomassie Brilliant Blue

Proteins separated by SDS-PAGE can be simultaneously fixed with methanol: glacial acetic acid and stained with Coomassie brilliant blue.

1. Dissolve 0.25 g of Commassie Brilliant Blue R250 in 90 ml of methanol: H_2O (1:1 v/v) and 10 ml of glacial acetic acid. Filter the solution through a Whatman No.1 filter.
2. Immerse the gel in 5 volume of staining solution, keep at room temperature for 4 h.
3. Remove the stain.
4. Destain the gel in destaining solution for 12 h (Methanol: Acetic Acid:Water = 30:10:60).

After destaining, gels may be stored indefinitely in water.

In case, the gel is to be used for western blotting, the staining step is not performed and the gel is used for transfer of separated proteins on to a nitrocellulose (NCM) of PVDF (polyvinylidene difluoride) membrane.

6.2.6 Electrotransfer

For transferring the separated proteins, gel is kept over either the nitrocellulose or PVDF membrane and sandwiched between the tissue papers followed by application of electromagnetic filed in perpendicular direction (Fig. 1). The efficient transfer of protein bands can be confirmed by using the protein-staining dyes like Ponceau S which do not interfere with the subsequent immunological detection [6]. Additionally, the prestained protein molecular weight markers also facilitate the monitoring of transfer of proteins on the membrane. The efficiency of transfer depends on a number of factors including size of protein, method of transfer, buffer composition, and type of membrane used. Generally, the transfer buffer is a Tris and glycine solutions supplemented with methanol and SDS (Table 3). The balance between SDS and methanol is crucial for efficient transfer of proteins as SDS improves the solubility and the migration of proteins but reduces their binding to membranes, while methanol precipitates proteins reducing their migration but improves protein-binding to membranes.

There are two methods for transfer of separated proteins: wet and semidry [12]. For a wet transfer, the gel-membrane sandwich system is kept submerged in the transfer buffer for the entire duration and is more favorable for larger proteins of size more than 100 kDa. However, this method is time-consuming and requires more volume of buffers. On the other hand, in a semidry transfer (Fig. 1), the gel-membrane sandwich is placed directly between electrodes and only the filter paper is soaked with the transfer buffer. This method is comparatively fast which completes between 10 min to 1 h and requires less volume of buffer. However, the transfer is sometimes less effective and poses difficulty in visualizing the transferred proteins especially when the proteins are of

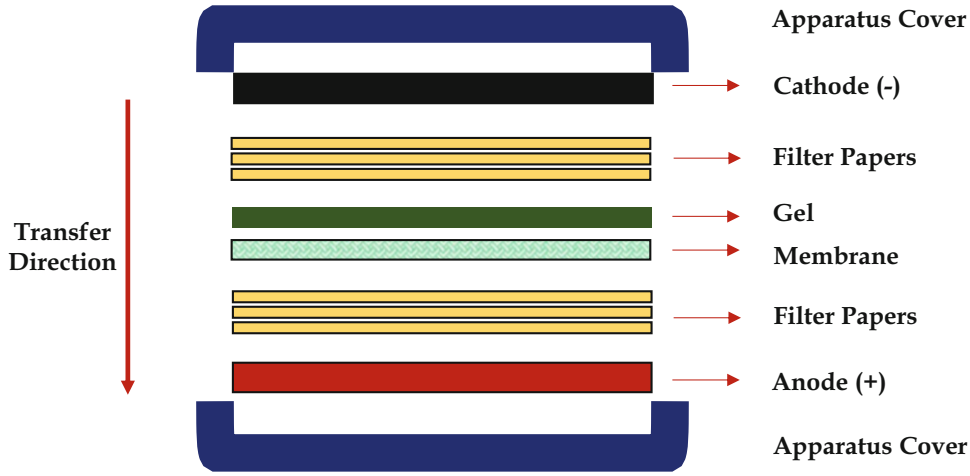


Fig. 1 Representation of semidry system for transfer of proteins from gel to membrane

Table 3
Transfer buffer components and their uses

Component	Example	Use
Buffer	Tris, CAPS, Carbonate	Support conduction, maintain appropriate pH
Alcohol	Methanol, ethanol	Increase binding of proteins to membrane
Detergent	SDS	Promote migration of proteins out of gel
Tris-tricine	Tris-tricine up to 9.5	Tricine separates low MW proteins from free SDS

high molecular weight. Nevertheless, due to its convenience, semidry method is the most commonly used method for the transfer of separated proteins.

Out of the nitrocellulose and PVDF membranes, nitrocellulose membrane is charged and binds with the hydrophilic part of proteins while the PVDF membrane binds with hydrophobic part. Although the nitrocellulose membrane binds very quickly and efficiently with the proteins, the bonding is mechanically weaker than that of PVDF membrane and hence the NCM is not suitable when re-probing is required. In contrast to NCM, the PVDF membrane produces high background noise and requires activation with methanol.

6.2.7 *Blocking*

As the membranes used for the transfer of proteins have characteristics for nonspecific protein binding, it may lead to background development in subsequent processing. Therefore, blocking is done by using the solution containing irrelevant proteins such as 1–5% nonfat dry milk or bovine serum albumin. The nonionic detergent like 0.01% Tween 20 can also be included to reduce nonspecific binding without disrupting specific binding of antibody to the target proteins [5].

6.2.8 Probing

After blocking, the protein of interest is probed with an antibody specific to that protein which may require extensive optimization owing to the variable strength and specificity of different antibodies to proteins. The optimum antibody dilution must be determined using different conc. of detector antibody so as to obtain better sensitivity in the assay. Probing can be performed in two ways: one-step or two-step probing. In one-step probing, the antibody conjugated to an enzyme [such as horseradish peroxidase (HRP), or alkaline phosphatase (AP)], radio-isotope or fluorophore is used to detect the target protein [6]. In contrast, in two-step probing, two different antibodies are used; one against the protein of interest (primary antibody) and secondly the secondary anti-species antibody against the primary antibody conjugated with an enzyme or fluorophore. In addition to anti-species antibodies, other proteins like protein A and G, which have the affinity for immunoglobulin heavy chains, can also be used as secondary reagents. These reagents can be radiolabeled (e.g., with 125I) or, more commonly, conjugated to biotin, fluorescein, or to an enzyme such as horseradish peroxidase (HRP) [5, 6]. As more than one secondary antibodies can bind to the one primary antibody leading to higher signal production and sensitivity, the two-step procedure is the most commonly used method for probing of target proteins in western blot. In some cases, the antibody can be replaced by other proteins like avidin and streptavidin which can be used to detect biotinylated proteins on the membrane.

The excess unbound antibodies may produce the background during development and hence must be removed through washing step. Generally, a mild detergent solution like phosphate buffered saline with 0.05% Tween 20 (PBST) is used for washing of unbound antibody on the membrane before addition of substrate solution.

6.2.9 Detection of Bound Antibody

The bound primary antibody on the membrane can be quantified by three means: colorimetry, chemiluminescence, and fluorescence [1, 5, 6]. Out of these three, colorimetry is the cost-effective though less sensitive than other two methods and can be used for the procedures where the amount of target protein is sufficiently large to be detected. Colorimetric detection is based on the generation of colored product that is formed by the reaction of enzyme on conjugated antibody and the substrate which is deposited on the membrane. No specialized equipment is required in colorimetry for visualization of colored product and the color produced is also quite stable.

Fluorometric detection (Fig. 2b) is based on the use of an antibody which is labeled with a fluorophore. A light source is then used to excite the fluorophore, which subsequently produces a transient light emission as it returns to its ground state. The light

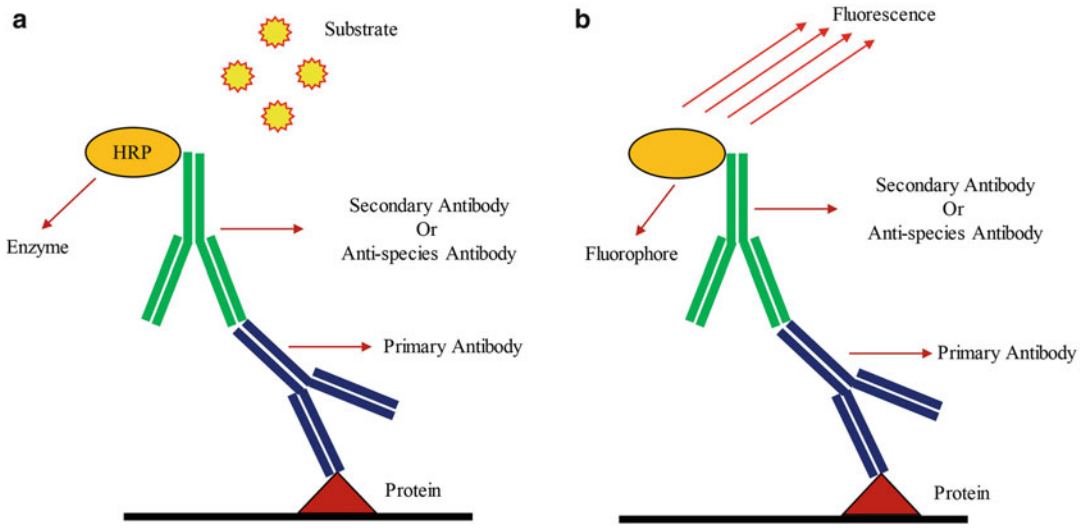


Fig. 2 Detection of target protein. (**a**) Chemiluminescence method and (**b**) Fluorescent detection

emitted at a higher wavelength than that was used for excitation is detected by a fluorescence reader. The advantage of this method is that it can be used for detection of more than one protein at the same time using fluorophores that produce different color signal.

In chemiluminescence method (Fig. 2a), the enzyme labeled secondary antibodies (e.g., HRP labeled antibody) are visualized using a substrate solution containing luminol. The results are recorded in a digital imager. Although chemiluminescence method of detection is quite sensitive with an ability to detect proteins in femtolitres, substrate acts as a limiting reagent and as the substrate gets exhausted, the light production decreases and eventually stops. However, a well-optimized tests are reported to produce a stable light output for several hours, allowing consistent and sensitive protein detection.

6.2.10 Stripping and Re-probing

Stripping is an optional step in the western blotting when more than one protein is analyzed by different antibodies. The procedure of removing the bound antibody is called stripping and is performed before the re-probing. In mild stripping, generally low pH buffers (e.g., pH = 2.2) with low concentration of detergent (e.g., 0.1% SDS) are used while for harsh stripping, a high incubation temperature (e.g., 50 °C) with high concentration of detergent (e.g., 2% SDS) is used [5]. After stripping, the membrane is blocked again and subjected to probing as per standard protocol. However, it is to mention that the stripping is not consistent throughout the membrane and hence the downstream results must be used with caution for semiquantification.

7 Protocol for Western Blot

7.1 Reagents and Materials Required for Blotting

1. Electroblotting transfer buffer for 1000 ml.

Tris Base 25 mM	3 g
Glycine 192 mM	14.4 g
Methanol 20% (v/v)	20 ml
SDS (sodium dodecyl sulfate) 0.1%	1 g

2. Phosphate buffered saline solution (PBS) pH 7.4 for 1000 ml.

KH_2PO_4 (anhydrous) 1.4 mM	0.20 g
Na_2HPO_4 (anhydrous) 8 mM	1.14 g
NaCl 140 mM	8.0 g
KCl 2.7 mM	0.2 g

3. Phosphate buffered saline with 0.05% Tween 20 (PBST).
4. Bovine serum albumin (BSA)—3% in PBST (Blocking solution).
5. Nitrocellulose (NC) membrane 0.45 μm pore size. 0.2 μm pore size nitrocellulose membrane may be used for low molecular weight molecules.
6. Primary antibody.
7. HRPO-conjugated secondary antibody.
8. Minigel system.
9. Power pack.
10. Semidry blotter.
11. DAB (3,3′ Diaminobenzidine) solution (prepared freshly before use with 0.05% DAB, 0.05% Nickel Chloride and 0.015% H_2O_2 in PBS, pH 7.2).

7.2 Procedure

Carry out SDS-PAGE (cassette size approx. 100 × 100 mm). Load 5–20 μg of protein per well. Apply constant voltage at 50–100 V.

7.2.1 Protein Blotting (Semidry Method) and Developing

1. Build the transfer "sandwich" onto the anode (+) plate as follows: 3–4 sheets blotting papers soaked in blotting buffer (roll out air bubbles), NC membrane pre-wet with deionized water, Slab gel, 3–4 sheets blotting papers soaked in blotting buffer (avoid trapping air bubbles between gel and membrane).
2. Carry out the transfer at a constant current of 1.9–2.5 mA per cm^2 of the gel area for 1.5 h at room temperature.

3. Remove the NC membrane from the apparatus and air-dry the NC blot thoroughly.
4. NC membrane is then incubated in 3% w/v BSA in PBST (blocking solution) at room temperature for 2 h or overnight at 4 °C. The choice of blocking reagent depends on the type of probe that will be subsequently used in the overlay procedure and should be chosen accordingly.
5. Remove the blocking buffer by decantation and wash the NC membrane 3–4 times with 10–15 ml of PBST for 5 min each.
6. Overlay the blot with 10 ml of primary antibody at an appropriate dilution (generally 1:50–1:500). Incubate for 1–3 h at 37 °C.
7. Wash the NC membrane 3–4 times for 5 min each, with sufficient PBST.
8. Incubate the NC membrane for 1 h at 37 °C in HRPO-conjugated secondary antibody at an appropriate dilution (1:2000 to 1:10,000) in PBST.
9. Wash the NC membrane 3–4 times for 5 min each, with sufficient PBST.
10. The membrane is developed by adding 10 ml of DAB solution for 5–10 min or until the color development.
11. Stop the reaction by washing the NC membrane in several changes of distilled water.
12. Air-dry the strips and store in the dark in a plastic sleeve between two sheets of blotting paper.

7.2.2 Precautions

- Avoid touching the surface of the membrane. Wear clean gloves and handle the blot only with clean forceps.
- Do not use skimmed milk in blocking when using avidin/biotin detection systems. Endogenous biotin in milk can cause high background.
- Do not use sodium azide as a preservative for buffers when using an HRP detection system. Sodium azide inhibits HRPO enzyme activity.

8 Applications of PAGE and Western Blotting in Virology

1. Native PAGE can be employed to study the electrophoretic mobility of segmented viruses like porcine rotaviruses. For this, the extracted RNA is loaded into the wells of a polyacrylamide gel and the genomic segment migration can be visualized by silver staining procedure [13]. In some cases, the animal species may be carrying more than one species of rotaviruses which can

be differentiated based on the electropherotype pattern of RNA isolated from fecal samples [14].

2. Polyacrylamide gel electrophoresis can be employed to study the differential protein expression in virus-infected and non-infected cells [15].
3. Western blotting procedure using virus specific antiserum can be used to study the difference in protein migration between the parental and mutant viruses in which some part of the genome is missing or deleted [16].
4. The expression of different viral proteins intended for development of vaccines and diagnostics can be studied with the help of western blotting.
5. Western blot can be utilized to assess the posttranslational modifications. The target protein is first immunoprecipitated and then probed with antibodies against ubiquitin or small ubiquitin-like modifier (SUMO) or vice versa. Similarly, western blot can also be used to study the protein–protein interactions by testing the presence of a potential interactor using co-immunoprecipitation. Here, one protein (the bait) is immunoprecipitated using a specific antibody against the bait and the immunocomplex is allowed to run onto an SDS-PAGE. The membrane carrying the immobilized immunocomplexes is then subjected to WB using antibodies specific to potential interactors (targets) [5].
6. Western blot can also be used in diagnostic virology for detection of expressed proteins in body fluids [17] especially in the prion diseases to detect prion proteins in body tissues. Although there are no reports of prion disease in pigs following natural infection, the western blot is commonly used to study the prion proteins in experimentally infected pigs [17, 18].
7. Western blotting is also a useful tool in analytical virology to study the antibodies in the serum of infected animals or to assess the diagnostic potential of expressed proteins for development of different immunoassays [19–21].

9 Limitations of Western Blot

Although western blot is a useful analytic technique, there are some caveats which are discussed here that must be considered.

1. *Quantification*: Western blot does not provide the quantitative results for the protein measurements but can be used with caution to study the relative expression of proteins. Although the digital imaging devices like charged coupled devices (CCD) have an advantage over the X-ray imaging techniques, it is

always recommended to use an internal control to improve the accuracy. In such cases, the immunoreactivity of the target antigen is normalized to the immunoreactivity of the housekeeping protein in each sample for quantitative analysis. This makes it important to carefully determine the equivalent protein loading volume in each well so as to increase the accuracy which makes the test time-consuming and more laborious.

2. *Specificity*: The specificity of the primary antibody should be meticulously characterized to rule out the concerns over cross-reactivity with nontarget proteins. The structurally similar proteins of similar size may move to the same position in the gel and the cross-reactive antibody may result in false results. Therefore, control experiments should always be run and further the membrane should also be probed with secondary antibody alone to ensure that the immunoreactive bands are specific for primary antibody only.
3. *Sensitivity*: Although the western blot is quite sensitive and the detection methods like chemiluminescence can detect protein levels up to femtograms, due to some intrinsic factors like insufficient transfer, decreased affinity to antibodies, changes in buffer conditions, etc. the detection limit is generally higher. Therefore, the proteins in very low concentration are generally missed and hence the negative results cannot be interpreted as complete lack of target protein in the sample.

10 Troubleshooting

Sr. no.	Problem	Possible causes	Interventions needed
1.	No signal	Insufficient amount of a sample	Make sure to measure the concentration of lysates to ensure that the proper amount of total proteins is loaded on the gel (20–50 μg of cell lysate per lane or 25–100 ng purified proteins). If the target protein is expected to be at low level, increase the amount of lysate loaded to the gel.
		Incomplete transfer	Make sure that the transfer is uniform and complete. A prestained protein ladder is an easy way to assess protein transfer but staining the membrane with Ponceau S or the gel with Coomassie Blue is more thorough. If

(continued)

Sr. no.	Problem	Possible causes	Interventions needed
			the transfer is incomplete, extend the time and/or increase electromagnetic field (voltage or current) or consider performing a wet transfer. Buffer composition should also be checked to meet the requirements.
		Poor detection	There are two main reasons for poor detection: (1) weak recognition and/or binding of a target protein by the antibody or (2) problems with detection system. Validate the antibody by taking along a purified target protein or a sample with heterologously expressed target protein. Carefully standardize the optimum concentration of the antibody or extend the incubation time (overnight at 4 °C). Monitor the wash buffer composition also. Use less stringent washing conditions. In case of a chemiluminescent detection system, use a more sensitive substrate (e.g., ECL femto) and increase the exposure time.
2.	High back ground	The high background usually comes from the (a) binding of the antibody to nonspecific targets, (b) overloaded samples, (c) insufficient washings, or (d) poor selectivity of the antibody used.	1. Decrease the amount of proteins loaded per lane. 2. Optimize the number and/or extend time of washings after antibody incubation. 3. Ensure sufficient blocking of the membrane. 4. Increase stringency of an antibody incubation by using a blocking solution or increase detergent concentration. 5. Optimize the concentration of the antibody. 6. Dry the membrane before imaging.
		Degradation or posttranslational modifications of the target protein may result in the detection of multiple bands.	Minimize degradation by using appropriate inhibitors and handle samples at 4 °C.
		Membrane not fully stripped	Ensure adequate buffer volume and stripping time
3.	Misshaped or missing bands	Usually this indicates problems with the electrophoresis or the transfer due to excessive heat or uneven transfer.	Run the gel and transfer on ice or decrease the voltage/current. Make sure that all the air is "rolled out" from the blot sandwich.

(continued)

Sr. no.	Problem	Possible causes	Interventions needed
		Bulky bands and migration artifacts may indicate gel overloading or buffer problems ("smiling gel").	Decrease loading or optimize the loading protein concentration.
		Cellular debris in samples	Sonicate and filter to remove the debris. For DNA in samples, shear samples with syringe or add DNase

11 Conclusion

Western blot has been in practice for more than four decades now and has proved to be a very useful analytic method to study the relative protein expression, posttranslational modifications, and protein–protein interactions. Although it is not a quantitative technique, it has been adapted for semiquantitative analysis of proteins. The low cost, ease to perform and accessibility has made this method a widely used technique in research labs for studying the proteins. Western blotting can also be used to verify proteins of interest in exploratory proteomic techniques such as two-dimensional gel electrophoresis as well as to identify the potential mechanisms underlying aberrant tissue functions or disease. It is also used routinely to help in diagnosis of some important viral diseases especially the prion diseases. However, a good understanding, initial training, and optimization are of utmost importance because being a multi-step technique, it is prone to false results and incorrect interpretation.

References

1. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 76(9):4350
2. Burnette WN (1981) "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate—polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal Biochem 112(2):195–203
3. Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98 (3):503–517
4. Alwine JC, Kemp DJ, Stark GR (1977) Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. Proc Natl Acad Sci U S A 74(12):5350–5354
5. Gwozdz T, Dorey K (2017) Western blot. In: Basic science methods for clinical researchers. Academic, Boca Raton, pp 99–117
6. Counts SE (2010) Western blot. In: Encyclopedia of movement disorders, vol 2010. Academic, pp 323–326 . ISBN 9780123741059. https://doi.org/10.1016/B978-0-12-374105-9.00297-5
7. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254
8. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193(1):265–275
9. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD et al (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150(1):76–85

10. Kurien BT, Scofield RH (2015) Western blotting: an introduction. Methods Mol Biol 1312: 17–30. https://doi.org/10.1007/978-1-4939-2694-7_5. PMID: 26043986; PMCID: PMC7304528
11. Wilson K, Walker JM, Hofmann A, Clokie S (2018) Wilson and Walker's principles and techniques of biochemistry and molecular biology. Cambridge University Press, New York, NY
12. Kurien BT, Scofield RH (2009) Protein blotting and detection: methods and protocols. Humana Press, New York
13. Kattoor JJ, Saurabh S, Malik YS, Sircar S, Dhama K, Ghosh S, Bányai K, Kobayashi N, Singh RK (2017) Unexpected detection of porcine rotavirus C strains carrying human origin VP6 gene. Vet Q 37(1):252–261. https://doi.org/10.1080/01652176.2017.1346849
14. Todd D, McNulty MS, Allan GM (1984) The use of polyacrylamide gel electrophoresis of virus RNA in the study of rotavirus infections. In: McNulty MS, McFerran JB (eds) Recent advances in virus diagnosis. Current topics in veterinary medicine and animal science, vol 29. Springer, Dordrecht. https://doi.org/10.1007/978-94-009-6039-8_11
15. Urzainqui A, Tabarés E, Carrasco L (1987) Proteins synthesized in African swine fever virus-infected cells analyzed by two-dimensional gel electrophoresis. Virology 160(1):286–291. https://doi.org/10.1016/0042-6822(87)90076-6
16. Bhatt M, Mohapatra JK, Pandey LK, Mohanty NN, Das B, Prusty BR, Pattnaik B (2018) Mutational analysis of foot and mouth disease virus nonstructural polyprotein 3AB-coding region to design a negative marker virus. Virus Res 243:36–43. https://doi.org/10.1016/j.virusres.2017.10.010
17. Greenlee JJ, Kunkle RA, Smith JD, Greenlee MHW (2016) Scrapie in swine: a diagnostic challenge. Food Saf 4(4):110–114. https://doi.org/10.14252/foodsafetyfscj.2016019. PMID: 32231914; PMCID: PMC6989210
18. Hedman C, Otero A, Douet JY, Lacroux C, Lugan S, Filali H, Corbière F, Aron N, Badiola JJ, Andréoletti O, Bolea R (2018) Detection of PrPres in peripheral tissue in pigs with clinical disease induced by intracerebral challenge with sheep-passaged bovine spongiform encephalopathy agent. PLoS One 13(7):e0199914. https://doi.org/10.1371/journal.pone.0199914
19. Ameri M, Zhou EM, Hsu WH (2006) Western blot immunoassay as a confirmatory test for the presence of anti-mycoplasma hyopneumoniae antibodies in swine serum. J Vet Diagn Investig 18(2):198–201. https://doi.org/10.1177/104063870601800210
20. Plotzki E, Keller M, Ivanusic D, Denner J (2016) A new western blot assay for the detection of porcine cytomegalovirus (PCMV). J Immunol Methods 437:37–42. https://doi.org/10.1016/j.jim.2016.08.001
21. Yang S, Li L, Yin S et al (2018) Single-domain antibodies as promising experimental tools in imaging and isolation of porcine epidemic diarrhea virus. Appl Microbiol Biotechnol 102: 8931–8942. https://doi.org/10.1007/s00253-018-9324-7

Chapter 24

Immune Assays as Diagnostic for Pig Viral Diseases

Prabhakar Maurya, Jupi Talukdar, Sarmistha Debbarma, Monuj Kumar Doley, and Luit Barkalita

Abstract

In the present era of globalization, there is a constant threat of infectious diseases with the potential of cross-species transmission, hence mitigation of infectious diseases is a one-health problem which needs to be addressed using collaborative research and resources. Correct and quick diagnosis is central to any infectious disease management strategy. Among different available diagnostic options, immunological and new generation molecular biology-based tests have become more popular than the rest. Immuno-diagnostic assays are widely used for routine diagnosis and surveillance of viral and bacterial infections in human and animals. Immunoassays are frequently used in various livestock diseases, including diseases of pig because of its specificity, sensitivity, ease of operation, relatively low-cost involvement, and advantage of being usable for population screening. In recent decades, increased popularity of pork in different cuisines has contributed to growth of organized swine farming in many states in India. As a collateral effect, increased swine population and human–pig interaction have exacerbated the circulation of viruses and challenged our ability to prevent, control, and/or eliminate impactful pig diseases, making early and efficient diagnosis of pig viral disease a need of the day. Here, we highlight different methods for diagnosis of pig viral diseases with a special focus on immuno-diagnostics.

Key words Pig, Infectious disease, One-health, Immuno-diagnostic assays, Fluid-phase immunoassay

1 Introduction

The occurrence of diseases puts one to think of the state of health and how being healthy is different from being diseases. At initial thoughts the answer to "What is a disease?" is simple but a look through any medical dictionary soon shows that articulating a satisfactory definition of disease is surprisingly difficult. Not only there are various types of diseases in various species but the reasons of occurrence of diseases are different with different manifestations in different species and even may differ from individuals within the same species though the etiology may be same. The World Health Organization's claim that health is "a state of complete physical, mental and social well-being, not merely the absence of disease or

Rajib Deb et al. (eds.), *Protocols for the Diagnosis of Pig Viral Diseases*,
Springer Protocols Handbooks, https://doi.org/10.1007/978-1-0716-2043-4_24,

infirmity" [1] has been praised for embracing a holistic viewpoint, and equally strongly condemned for being wildly utopian: the historian Robert Hughes remarked that it was "more realistic for a bovine than a human state of existence" [2, 3].

The emergence of sudden diseases, escalating the spread to a small geographical region or over the whole world, now considered as possible events especially after the present pandemic of COVID-19, but a peek in the history reveals that such events have been reported since antiquity. In modern times we have had the devastating pandemics of influenza, cholera, Ebola, SARS, and recent COVID-19. The source of infection has always been regarded as an utmost factor in epidemiology. Although many diseases are species specific, meaning that they can only occur in one animal species, many other diseases can be spread between different animal species. These are infectious diseases, caused by bacteria, viruses, or other disease causing organisms that can live as well in humans as in other animals. There are different methods of transmission for different diseases.

Communicable diseases can be classified according to the source of infection as anthroponoses (when the source is an infectious human; interhuman transfer is typical), zoonoses (the source is an infectious animal; interhuman transfer is uncommon), and sapronoses (the source is an abiotic substrate, nonliving environment; interhuman transfer is exceptional) [4]. Many of these diseases are caused by viruses, particularly those with RNA genomes [5]. In some cases, zoonotic diseases are transferred by direct contact with infected animals, much as being near an infected human can cause the spread of an infectious disease. The source of infection is often the reservoir or, in ecologic terms, the habitat where the etiologic agent of the disease normally thrives, grows, and replicates. A characteristic feature of most zoonoses and sapronoses is that once transmitted to humans, the epidemic chain is usually aborted, but the clinical course might be sometimes quite severe, even fatal. An ecologic rule specifies that an obligatory parasite should not kill its host to benefit from the adapted long-term symbiosis, whereas an occasionally attacked alien host, such as a human, might be subjected to a severe disease or even killed rapidly by the parasite because no evolutionary adaptation to that host exists [4].

When considering the factors that lead to the emergence of viral diseases, it becomes very apparent that there is a high degree of interrelatedness. Human encroachment in a virgin ecosystem provides the opportunity for a previously unencountered virus to infect human beings or their domestic animals. Viral mutability could enable a virus to adapt to the new host. Subsequent trade and migration could allow spread of the new virus to susceptible individuals and the large populations found in urban centers could ensure that the virus is maintained. The interplay of all these factors

provides a rational framework and explanation for virus emergence. However, to gain an appreciation of the components of this process it is necessary to focus on each in itself before linking them together [6]. By its nature, the emergence of unknown diseases is impossible to predict [7].

2 Viral Diseases of Pigs and Their Public Health Importance

Between 1968 and 2018, the worldwide swine inventory increased from 550 to 981 million pigs (+78%), with the most marked growth in the developing regions of the world, i.e., Africa + 504%, Asia + 137%, and South America + 59% [8]. Over the same period, albeit with regional variations, the majority of pig production moved from smaller, farrow-to-finish enterprises into larger, multi-site production systems that are highly dependent upon the interchange of animals, people, equipment, and sundries between production sites; a process that connects farms and moves infectious agents between them [9, 10].

The majority of emerging human pathogens are zoonotic. Frequently changing husbandry practices and environmental factors (e.g., large-scale domestic animal production, urbanization, interaction between wild and domestic swine populations with humans, population increases, etc.) may predispose humans and pigs to pathogens common to other species, or may allow for the adaptation of these organisms to humans or swine. Being omnivorous and having the anatomy and physiology similar to that of man, pigs are a good medium for the adaptation and increase in virulence of organisms that have so far not been identified as human pathogens [11].

During recent times, much emphasis has been focused on human emerging infectious diseases (EID) caused by pathogens of animal origin. All these zoonotic threats and events have emphasized the need for a "One Health" approach, which has been summarized in the so-called 12 Manhattan principles. The "One Health" approach integrates communication, collaboration, and coordination between public health, animal health, and other communities at multiple levels to prevent, detect, and control emerging or re-emerging infectious diseases at the animal–human–environment interface [12].

Given the sheer number of swine present worldwide, and the large percentage of the population that consume pork, swine represent a significant reservoir of confirmed or potential zoonoses. Swine represent a potential reservoir for many novel pathogens and may transmit these to humans via direct contact with live animals (such as swine farmers and large animal veterinarians), or to the general human population via contaminated meat [13]. Over the last 30 years, diseases caused by emerging swine viruses (ESV) have acquired great relevance, more than in other species [14].

Major viral disease outbreaks occur continually in the pig industry (Table 1) [17]. The global understanding of viral disease dynamics requires to account for all interactions at all levels, from within-host to between-herd, to have all the keys for development of control measures [18]. The intensification and globalization of the swine industry has contributed to the emergence and global spread of pathogens of swine, driven in part by frequent movements of pigs, feed, and pork products at local, national, and international scales [19]. Effective prevention and clinical management of infectious diseases are intimately linked to early and accurate screening of pathogens, not only by detecting the infectious particles in the organism but also by elucidating the aspects that confer resistance to therapy and immune escape profiles, including mutations and genotype disparity. Therefore, rapid diagnosis benefits in allowing timely therapy to prevent complications; and benefits public health by collecting data for epidemiological studies, to prevent outbreaks and spreading of diseases [20].

3 Diagnostic Methods for Viral Diseases: Needs and Challenges

Infectious diseases represent a global threat to humans, livestock and wildlife animals, and plants with potential cross-species transmission. Mitigating infection is therefore a one-health problem, which needs to be addressed using all materials in hands, both in terms of research and resources. When referring to such problems, one commonly thinks about biological analysis, i.e., virological and immunological diagnostics, which are essential for the understanding of host–pathogen interactions (Table 2) [18]. Major viral disease outbreaks occur continually in the pig industry [17]. From serious pandemics and highly contagious infections to common influenza episodes, clinical prognosis often relies on early detection of the infectious agent. Thus, effective identification of viral pathogens is needed to help prevent transmission, set up appropriate therapy, monitor response to treatment and lead to efficient disease management and control [20].

Diagnostic assays for detection and monitoring of virus infections have become more and more important and are widely used in routine diagnostics. In particular, the detection of newly emerging infectious diseases is challenging; therapeutic treatment regimens and prevention strategies can also influence the need for diagnostic assays [21]. An important first step in choosing an appropriate diagnostic approach is to decide the objectives of the tests to be performed. These objectives might include detection of infection in at least one animal in a herd, determining the prevalence of a virus within the herd, confirming exposure to virus or vaccine, or assessing the timing of an infection. These factors will determine which animals to test, the number of samples required, and the

Table 1
Major viral diseases of pigs (Adapted from [15, 16])

Sl. no.	Virus	Disease(s) caused	Zoonotic concern
1	African swine fever virus	African swine fever	No
2	Aujeszky's disease virus	Aujeszky's disease, also known as pseudorabies	No
3	Classical swine fever virus	Classical swine fever, also known as hog cholera	No
4	Coronaviruses	Primarily causing diarrhoea with similar treatment and control but immunologically are very distinct from each other (i.e. no cross-protection between these different viruses)	Not established properly
5	Foot-and-mouth disease virus	Foot-and-mouth disease	No
6	Influenza A virus	Respiratory infections	Yes
7	Porcine circovirus type 2 virus	Cause variety of systemic diseases in pigs including wasting, pneumonia, late-term abortions, stillbirths, porcine dermatitis, nephropathy syndrome and diarrhoea	Not established properly
8	Porcine reproductive and respiratory syndrome virus	Primarily causing diarrhoea with similar treatment and control but immunologically are very distinct from each other (i.e. no cross-protection between these different viruses).	No
9	Rotaviruses	Major cause of diarrhoea in neonatal and young pigs	No
10	Swine vesicular disease virus	Swine vesicular disease	No

Table 2
Summary of main viral diagnostic methods (Adapted from [20])

Diagnostic technique	Principle	Variants
Immunoassay	Formation of Ag–Ab through recognition and binding	RIA EIA (FPIA, MEIA, CLIA)
Nucleic Acid Amplification Tests (NAAT)	Amplification and detection of Sequences from the viral genome (DNA or RNA)	RT-PCR qPCR NASBA TMA
NGS: Next-Generation Sequencing	Polymerization of DNA template by incorporation of labeled dNTPs, and terminate the extension	Pyrosequencing Fluorescently labelled dNTP Detection of released hydrogenion (H+)
MS: Mass Spectrometry	Ionization of the sample, then separation and detection of the particles according to their mass-to charge ratio (m/z)	MALDI-TOF MS ESI MS Often combined with other methods: PCRMS

CLIA chemiluminescent immunoassay, *dNTP* deoxyribonucleotide triphosphate, *EIA* enzyme immunoassay, *ESI* electrospray ionization, *FPIA* fluorescence polarization immunoassay, *MALDI-TOF* matrix-assisted laser desorption ionization time-of-flight, *MEIA* micro-particle enzyme immunoassay, *MS* mass spectrometry, *NGS* next-generation sequencing, *NAAT* nucleic acid amplification test, *NASBA* nucleic acid sequence-based amplification, *qPCR* quantitative polymerase chain reaction, *RIA* radio-immunoassay, *RT-PCR* real-time polymerase chain reaction, *TMA* transcription-mediated amplification

appropriate tissues to use. Furthermore, interpreting individual test results may not reflect the status of the entire herd, as the timeline of infection will vary for each individual animal—so appropriate measures need to be taken to compensate for the cases of delay in diagnostic procedure or statistically insignificant results.

Molecular and serological techniques should be used in a complimentary fashion for the diagnoses of virus infections. Knowledge of the stage of infection, viral pathogenesis, and epidemiology help to make decisions regarding the correct test to choose for appropriate diagnosis. Advances in specificity and sensitivity and capacity to test for a range of viruses through multiple platforms make accurate diagnosis and rapid identification of circulating viruses for real-time clinical relevant data more feasible. Virus isolation and collaboration with specialist laboratories make newer techniques for identification of emerging and re-emerging viruses possible [22].

Prominent swine viral diseases such as swine fever, porcine reproductive and respiratory syndrome, classical swine fever, food and mouth disease, swine vesicular disease, influenza A virus

disease, African swine fever, porcine respiratory disease complex, etc. have not only clinical importance but also great economic importance [23]. Therefore, the primary requirement of diagnosis of suspected viral diseases in pigs is of rapid diagnosis and with maximum accuracy. Most of the pig farms are commercial or industrial in nature and are intensive farms mostly located at a certain distance from the populated areas [24], hence the access of veterinary and laboratory care is not immediate. Currently, there are many diagnostic tests for the detection of viral diseases, but many challenges are faced on the field in the diagnostic procedures:

- Sample collection and transportation from field to laboratory: Most of the diagnostic tests use serum samples which require blood collection and then centrifugation. Such facilities are not available at the farm and require preservation and then transport of sample to appropriate laboratory settings which increases the time duration from sample collection to diagnostic results.
- Sample processing: Complex diagnostic tests require exhaustive sample processing procedures which can further increase the duration for diagnostic results. Improper sample processing can provide inaccurate results.
- Location and capacity of laboratories: Specialized laboratory settings are required for viral diagnostics and such laboratory settings are not readily accessible to farm settings in semi-urban or rural areas.
- False positive of false negative results: Many diagnostic tests can display false positive or false negative results due to external or technical factors.
- Cost benefit ratio: Pig farming is carried out on a commercial scale in most places, but for accurate diagnostic results, a large number of samples are to be processed from suspected farm, which increases the cost of diagnostics.

Citing the above factors, efficient but cost-effective and rapid diagnostic tests are required for detection of viral diseases of swine. Blood collection from multiple animals is itself a challenging task in the field setting which further increases the chances of exposure of research personnel to susceptible animals. Saliva-based viral diagnostic tests are most practical in terms of logistics as well as time required for collection, oral fluid-based surveillance reflects the adaptation of conventional testing methods to an alternative diagnostic specimen [25]. Most of the diagnostic tests discussed above do not effectively meet the cost benefit requirements of pig farmers expecting large commercial farms or research studies. Hence, the most effective and cost-effective diagnostic tests are radioimmunoassay and enzyme immunoassays.

4 Recent Methods in the Diagnosis of Viral Infections

In a rapidly growing world of technology, the industry is continuously delivering up-to-date instruments but many factors are limiting their implementation in healthcare and field settings which unfortunately delays global benefit. Diagnostic tests are direct measures of exposure or vaccination, and indirect measures of immunity. Depending on the specific diagnostic assay, laboratory tests detect antibodies or proteins that are produced in large quantities by the pig in an immune response to an infection or the presence of an infectious agent [26]. Broadly the following diagnostic methods are available for detection of viral infections [20, 27].

4.1 Immunoassay-based Tests

Immunoassays are bioanalytical methods in which the quantitation of the analyte depends on the reaction of an antigen (analyte) and an antibody (Table 3). Automated immunoassay-based methods are among the most frequently used for testing, and are effective because of the high specificity and binding affinity between antigen and antibody. Therefore, the principle of the test relies in the formation of an immuno-complex between antibody present in

Table 3
Classification of various immunoassays and their characteristics (Adapted from [27])

Diagnostic technique	Labels (Reporter Groups)	Bound vs free separation	Signal detection	Sensitivity
Precipitation immunoassays	Not required	Not required	Naked eye	≈10 μg/mL
			Turbidity Nephelometry	
Particle immunoassays	Artificial particles (gelatin, particles, latex, etc.)	Not required	Naked eye Pattern analyzer Spectrophotometry Particle counting	≈5 ng/mL
Radioimmunoassays	Radioisotopes (I 125, H 3)	Required	Photon counting	≈5 pg/mL
Enzyme immunoassays	Enzymes	Required	Spectrophotometry	≈0.1 pg/mL
			Fluorometry Photon counting	
Fluorescent immunoassays	Fluorophores	Required	Photon counting	≈5 pg/mL
Chemiluminescent immunoassays	Chemiluminescent compounds	Required	Photon counting	≈5 pg/mL

the sample and synthetic antigen present in the reagent or vice versa, to generate a measurable signal.

Immunoassays use labels conjugated to synthetic antibodies or antigens which are linked to a solid phase, and used to capture corresponding antigens or antibodies present in sera samples. These labels could be radioactive isotopes, enzymes that cause change in color or light-generating substances. Consequently, this principle has generated several methodologies for the testing.

4.1.1 Radio-immunoassay (RIA)

It uses radioisotopes (such as Iodine 125) to label antigen or antibody. The amount of substance to analyze is determined by the amount of the generated radioactivity. RIA is a highly sensitive method but the main drawback is the handling and disposal of hazardous radioactive substances. Fluid-phase radio binding assays (RBAs) provide a more efficient alternative. In this method, the autoantigens are tested in solution rather than being immobilized on a solid surface.

Various competitive RIA methods have been developed to measure a plethora of different biological compounds. Initially, a known amount of labeled antigen and an antigen from a biological specimen are combined and reacted with a known amount of antibody that is usually coated on a solid phase such as sepharose beads or on the inner wall of plastic tubes. After the mixture equilibrates, it is washed to remove unreacted antigens, and the immune complex containing both labeled and unlabeled antigen is trapped in the solid phase. The washing step is referred to as B/F (bound versus free) separation.

RIAs can offer a number of advantages over other immunoassays in that they are highly sensitive and precise, although the latest chemiluminescent-based methods typically surpass RIA limits of detection. Disadvantages include the fact that radioisotopes are highly regulated, may have a short half-life, and that RIAs are heterogeneous assays.

4.1.2 Enzyme Immunoassays

Immunoassays employing enzyme-mediated reactions as labels were developed as alternatives to RIAs. Commonly used techniques include the enzyme-linked immunosorbent assay (ELISA), the EIA, and the enzyme-multiplied immunoassay (EMIT).

Heterogenous EIAs are essentially the same as RIAs except that enzymes are used as labels rather than radioisotopes. Unlike RIAs, however, homogenous assays can be developed that eliminate washing steps for separation of bound and free molecules. EIAs have a number of advantages compared to other immunoassay methodologies in that highly sensitive assays can be developed because of the ability of enzymatic reactions to amplify antigen–antibody interactions. In addition, reagents are cheap and have a longer shelf life than RIAs. Further, no radiation hazards exist. Finally, a number of different types of assays have been developed.

Disadvantages of EIAs include the fact that assays can be more complex, and enzyme activity may be affected by various substances in biological fluids.

An important advantage of EIAs over RIAs is that the former can be developed as homogenous assays in which the tedious washing step to remove free antigen is eliminated, although homogenous EIAs are frequently less sensitive than RIAs or heterogeneous EIAs.

The main variants of EIA are as follows:

1. *Fluorescence polarization immunoassay (FPIA)*: uses fluorescent label and polarized light.
2. *Micro-particle enzyme immunoassay (MEIA)*: widely used and relies on alkaline phosphatase enzyme and a corresponding fluorescent substrate.
3. *Chemiluminescent immunoassay (CLIA)*: uses chemiluminescent or light-emitting labels.

4.1.3 Precipitation and Particle Immunoassays

These methods, unlike other immunoassays, are often qualitative in nature. They have also been in use longer than other assay types. Precipitation assays are used to measure immunoprecipitation reactions, which form when large complexes of antigens and antibodies combine to generate insoluble complexes. Detection of complexes can be afforded using light scattering instrumentation and is termed nephelometry. The lower limit of sensitivity using these methods is about 10 mg/mL. Common assays that use these techniques include the measurement of many major serum proteins.

A related method of immunoassay is particle agglutination in which either antibodies or antigens are detected in biological fluids using corresponding antigens or antibodies, respectively, bound to various particles. Commonly used particles are latex beads and gelatin particles. These assays have wide applicability and can measure biological molecules as diverse as human chorionic gonadotropin or antibodies to HIV.

4.1.4 Multiplex Immunoassays

Technological advances in the analysis of materials have created a situation where hundreds of candidate biomarkers can be generated in the discovery phases of biomarker research. Each of the candidate markers needs to be confirmed in the verification/validation phase.

4.2 Amplification-based Assays

The most widely used variants of conventional amplification are real-time PCR (quantitative PCR) and reverse transcription-PCR (RT-PCR). Other amplification-based tests such as nucleic acid sequence-based amplification (NASBA) and transcription-mediated amplification (TMA) are suited for detection of RNA viruses by amplification of the mRNA instead of conversion to cDNA.

4.3 Next-Generation Sequencing

Next-generation sequencing (NGS) is one of the greatest achievements of the modern era. Beyond genome sequencing from known organisms, it allowed discovery of novel viruses responsible for unknown human diseases, and tracking of outbreaks and pandemics such as influenza to understand their emergence and transmission profiles.

4.4 Mass Spectrometry

Mass spectrometry (MS) is nowadays a benchmark of laboratory qualitative and quantitative investigation, particularly in bacteriology. The principle of MS relies on converting the sample into charged particles (ions) by ionization process. These ions are separated according to their mass-to-charge ratio (m/z) and analyzed by a detector. The result obtained is compared to a reference database (library), existing within the system and delivered as an interpretive spectrum.

In clinical laboratories, matrix-assisted laser desorption ionization (MALDI) and electrospray (ES) are the most used ionization methods because they allow processing of considerable amounts of analyte. These approaches have extensively been evaluated experimentally and provided excellent results, either used alone or combined with other molecular methods, such as PCR, in order to enhance sensitivity. The combination (RT-PCR/ESI-MS) was able to detect viral pathogens usually undetected by regular testing methods, and provided rapid and detailed data (types and subtypes) within a short time.

5 Immune Assays in Swine Viral Diseases

Diagnostic assays either measure the immune response induced by infection or detect the pathogen itself. Most common diagnostic assays are based on detection of antibodies specific to the pathogen. Typically, serum is used as the sample, although thoracic fluid and colostrum are also used. Diagnostic assays or tests are tools used by producers and veterinarians to assess the disease and immune status of pigs. Immunity is essentially the pig's resistance to infection. Diagnostic assays do not measure immunity. Rather, they look for evidence of disease exposure in response to vaccination, antibodies, or infectious agents. In terms of immunity, the underlying assumption is that prior infection or vaccination has stimulated an immune response that will provide some level of protection from disease symptoms in the future. For these reasons, diagnostic tests are direct measures of exposure or vaccination, and indirect measures of immunity [26].

Immunoassays represent the most frequently used and perhaps simplest approach to the analysis of biological materials for the translational researcher. Enzyme immunoassay (EIA) and enzyme-linked immunoassay formats are the easiest to utilize and are widely

commercially available. More sensitive methods of immunoassay (electrochemiluminescence, RIA, fluorescence polarization immunoassay) require specialized instrumentation for use [27]. Antibody detection assays have distinct advantages in detection of a pathogen. The sample (usually blood) is easy to collect, handle, and store; antibodies are abundant in blood and can usually be detected for a long time after infection. With most diseases, antibodies are detectable long after the pathogen is not. The pathogen may be detected by isolation (growing it in the laboratory), staining and visualization by microscopy [26].

6 Immunoassays and Immunochemistry

Immunoassay methodologies represent, perhaps, the most frequently used approach to measure biological compounds in translational and clinical research. Assays exist, from either commercial or research sources, for both the qualitative and quantitative measurement of a plethora of naturally occurring small molecules such as lipid mediators and hormones as well as larger peptides and proteins that are present in human body fluids and tissues [28]. In addition, a number of therapeutic agents can be measured by immunoassays. It is important to note that immunoassays can not only measure antigens but antibodies as well. Many immunoassays are extremely sensitive and can detect as little as 0.1 pg of compound per milliliter of body fluid [29].

The first immunoassays resulted from the pioneering work of Yalow and Berson in the late 1950s and utilized antibodies labeled with a radioisotope like 125I. Regardless of the method used, all immunoassays rely upon the interaction of an antigen with an antibody [28]. The extent to which this interaction occurs (the amount of antigen that is bound to antibody versus free) allows one to measure, either qualitatively or quantitatively, the amount of that particular antigen that is present in a biological fluid or tissue. Detection methods for particular assays vary and depend on the approach used to detect the antigen-antibody complex.

Antigens are defined as any substance that possesses antigenic sites (epitopes) which produce corresponding antibodies [28]. Antigens can be small molecules such as haptens, hormones, etc. or they can be very large compounds such as glycolipids and proteins. Antibodies that are generated in response to antigens can be one of the five types and include IgG, IgM, IgA, IgE, and IgD. Antibodies consist of heavy chains and either k or l light chains and possess constant and variable regions. The hypervariable region can be assembled to recognize a wide variety of epitopes [29].

Although antibodies can serve as antigens, for purposes of immunoassays, they are reactants used to detect antigens. Different types of antibodies can be obtained from several sources. Polyclonal antibodies are generated by immunizing an animal with an antigen.

In this case, multiple antibodies are generated, which recognize different epitopes. As a consequence, the affinity of polyclonal antibodies for a complex antigen is usually stronger than that of a monoclonal antibody. Monoclonal antibodies are generated using somatic cell fusion and hybridoma selection [30].

The resulting established cell line generates a homogeneous antibody population that represents a single epitope [29]. While specific for a certain epitope, it should be kept in mind that monoclonal antibodies may cross-react with different antigens that possess the same epitope. Nonetheless, the development of monoclonal antibodies has revolutionized immunoassay methodologies because monoclonal antibodies are well defined, and specific reagents and their production can yield a nearly limitless supply of an antibody [31]. Further, they can be prepared through immunization of a nonpurified antigen. A more recent approach to the development of antibodies for use in immunoassays is phage display, in which antibody fragments of predetermined binding specificity are encoded in a phage and expressed in bacteria [32].

7 Swine Viral Diseases and Major Diagnostics Immunoassays

7.1 African Swine Fever

African swine fever is one of the most important viral diseases in pigs caused by an *Asfivirus*. There are different strains with different virulence. It is a systemic disease and is notifiable on most countries.

Alternative names: ASF.

Diagnosis:

- It presents postmortem changes with hemorrhagic lymph nodes, necrotic areas in the spleen, multiple small hemorrhages in kidneys, and button ulcers in the intestine.
- In all suspected cases, the diagnosis should be confirmed by laboratory analysis.
- Laboratory analysis include the identification of virus via PCR, isolation of the virus and the presence of antibodies in serum. In most countries, the ASF is a notifiable disease.

7.2 Aujeszky's Disease

The Aujeszky's disease is caused by a herpesvirus that can remain latent and causes respiratory, reproductive, and nervous problems.

Alternative names: Pseudorabies (PRV).

Diagnosis:

Serology analysis confirms the diagnosis.

7.3 Blue Eye Disease

It is a viral disease that produces nervous symptoms, reproductive failure, and corneal opacity that develops a bluish color caused by a paramyxovirus.

Alternative names: blue eye disease, BE, paramyxovirus.

Diagnosis:

- In severe/acute outbreaks, a presumptive diagnosis can be made based on clinical signs.
- Confirm diagnosis by serological tests.
- Isolation of virus from brain tissue.
- Histopathologic lesions especially in the brain are highly suggestive.

7.4 Bovine Viral Diarrhea Virus

Disease caused by two pestiviruses in the same group as the swine fever virus. These viruses mainly affect cattle and sheep, but can enter pig farms causing reproductive problems.

Alternative names: Border Disease Virus, BDV, Border disease.

Diagnosis:

Laboratory analysis. A cross reaction (false positive) with antibodies against classical swine fever is possible, which may cause problems for classical swine fever surveillance in epidemiology and eradication.

7.5 Classical Swine Fever

Classical swine fever is one of the most important viral diseases in pigs caused by a pestivirus related with bovine viral diarrhea and with border disease. There are several strains with different virulence. It is a systemic disease and it is notifiable in most countries.

Alternative names: CSF.

Diagnosis:

- They present typical postmortem changes with hemorrhagic lymph nodes, dead zones in the spleen, multiple small hemorrhages in kidneys and so-called "button ulcers" in the intestine.
- In all suspicious cases, the diagnosis should be confirmed by laboratory analysis.
- Laboratory analysis include the identification of viral antigen, virus isolation, and the presence of antibodies in serum. In most countries, CSF is reportable.
- Infections with bovine viral diarrhea and with border disease may give false positive.

7.6 Delta Coronavirus

The diarrhea caused by deltacoronavirus is similar to porcine epidemic diarrhea (PED) but at a very low severity.

Alternative names: Deltacoroavirus, Delta corona virus.

Diagnosis:

Suspected by clinical signs, but cannot be differentiated from TGE and PED. The presence of the organism is confirmed by PCR.

7.7 Ebola Reston Virus

Ebola is a very significant infection in humans. Of the five species of Ebola virus, Ebola Reston virus does not infect humans but can infect pigs.

Alternative names: Ebola, Ebola Reston, Ebolavirus, REBOV.

Diagnosis:

Serological tests or identification of the virus by PCR.

7.8 Encephalomyocarditis

Infection caused by Encephalomyocarditis virus, found globally. Usually it is of no clinical importance, but there are some myocarditis cases with high mortality or reproductive problems.

Alternative names: EMC.

Diagnosis:

To make a definitive diagnosis, the virus must be isolated and identified or an increase in blood antibodies must be demonstrated in two samples taken in 2 weeks apart. Encephalomyocarditis can be confused with Aujeszky, parvovirus, and PRRS virus, although there are symptoms that differentiate these four infections.

7.9 Enteroviruses

Swine enteroviruses are found in the intestine, although their clinical significance is questionable.

Alternative names: picornavirus, SMEDI.

Diagnosis:

It has no clinical importance. Virus isolation and serology must be performed to diagnose.

7.10 Foot-and-Mouth Disease

Foot-and-mouth disease is one of the most important vesicular diseases. Foot-and-mouth disease belongs to the *Picornaviridae* virus family of which there are more than 60 strains classified into seven serotypes.

Diagnosis:

Serological and PCR tests are needed. Foot-and-mouth disease does not differentiate clinically from the rest of the vesicular diseases. Laboratory samples must include blood, vesicular tissue and fluid, when possible.

7.11 Hepatitis E Virus

Hepatitis E has been identified in pigs with uncertain clinical significance, but it is a very important viral infection in humans, mostly seen in third world countries of Asia and Africa. Hepatitis E virus is prevalent in pigs worldwide.

Alternative names: HEV.

Diagnosis:

- Identification of histological lesions in the liver.
- Identification of the virus by PCR.
- Serology by ELISA.

7.12 Influenza

Influenza is a respiratory disease of high importance due to its fast transmission and zoonotic potential. Porcine influenza is caused by several Influenza Type A viruses closely related, characterized to have the ability to change the antigenic structure and create new strains.

Each serotype is identified through surface proteins called "H" and "N". The three most common serotypes affecting pigs are H1 N1, H1 N2, and H3 N2.

Alternative names: Porcine Influenza, influenza type A.

Diagnosis:

In the acute disease, a reliable diagnosis can be done based on clinical signs, due to the fact that there are no other diseases with such a dramatically quick transmission and clinical effects. Blood samples taken from sows at the beginning of the disease and 2–3 weeks after, show an increase in antibody titers. Influenza virus can be identified from nasal swabs analyzed by PCR.

7.13 Japanese B Encephalitis

Japanese B encephalitis is caused by a virus found in South Asia. It is transmitted by mosquitos and presents as reproductive problems. Pigs are a significant source of infection.

Diagnosis:

- Lab tests of stillbirths and of testes from affected boars.
- Definitive diagnosis requires tissue culture virus isolation, and serum antibodies from stillbirths, usually using ELISA.

7.14 Nipah Virus Disease

The Nipah virus is zoonotic causing respiratory disease in pigs and mild to severe symptoms or even death in humans caused by paramyxovirus.

Alternative names: paramyxovirus.

Diagnosis:

- In the postmortem examination, the predominant sign is the consolidation of the lungs.
- The diagnosis is made by serological testing, virus isolation and identification. On infected farms, high levels of antibodies are detected in the sows.

7.15 PRRS

The Porcine Reproductive and Respiratory Syndrome (PRRS) is the viral infection caused by an arterivirus. The virus causes reproductive problems and affects the respiratory system.

Alternative names: Porcine respiratory and reproductive syndrome, Blue ear disease.

Diagnosis:

This is based on the clinical signs, postmortem examinations, and the known presence of the virus by PCR. There are several tests available, but ELISA is the standard test used. Currently, oral fluids

are used to monitor farms. Virus sequencing can only be used epidemiologically to investigate the presence of a new strain and possibly its origin.

7.16 Parainfluenza

Porcine Parainfluenza virus type-1 of the *Paramyxoviridae* family is a novel agent suspected of causing mild respiratory disease in pigs.

Alternative names: PPIV1.

Diagnosis:

- PCR is currently used to confirm the presence of the virus in trachea, lung, nasal swabs, and nasal turbinates. ELISA serology can also be used to confirm exposure to the virus.

7.17 Porcine Circovirosis

Porcine circovirus is caused by porcine circovirus type 2 (PCV2). Clinically it is presented as a disease which deteriorates animals from the weaning to the finishing period producing a high mortality rate.

Alternative names: Porcine circovirosis type 2 (PCV2), PMWS.

Diagnosis:

- Due to the fact that most farms have antibodies for porcine circovirus, tests to detect antibodies in blood do not help with the diagnosis.
- Clinical signs are not specific, so in order to have a diagnosis, it is often necessary to perform postmortem examinations in several pigs.
- The diagnosis is based on three factors:
  - Lymph depletion.
  - High amount of PCV2 present in tissue (immunohistochemical tests).
  - The clinical picture corresponds to PCV2 infections.

7.18 Porcine Cytomegalovirus

Cytomegalovirus is a herpes virus common and its consequences are often insignificant; it is characterized by rhinitis which causes sneezing.

Alternative names: Porcine cytomegalovirus infection, PCMV, inclusion bodies rhinitis.

Diagnosis:

It can be confirmed by serology, fluorescence antibody tests, and demonstrating the presence of inclusion bodies in histological sections of tissue.

7.19 Porcine Epidemic Diarrhea

Porcine epidemic diarrhea is caused by a coronavirus leading to vomiting and diarrhea with mortality up to 100% within susceptible piglets under 2 weeks of age.

Alternative names: PED.

Diagnosis:

Clinical signs might help, but cannot be differentiated from TGE. The presence of the organism is confirmed by PCR. Histological lesions are characteristic of PED and TGE, thus immunochemistry (IHC) or PCR is used to confirm the disease.

7.20 Porcine Parvovirus Infection

Parvovirus affects mainly non-vaccinated primiparous sows, causing reproductive problems such as mummies.

Alternative names: PPV.

Diagnosis:

Antibodies fluorescent tests and PCR can be performed to mummified piglets in order to confirm the parvovirus infection. Serology with very high titers is an indicative of exposition to the field virus because vaccination does not produce high titers.

7.21 Rotavirus Infection

Rotavirus infections present clinically as diarrhea in nursing piglets or in the first 2 weeks after weaning.

Alternative names: Rotavirus, rotavirosis.

Diagnosis:

As with any diarrhea problem in pigs 10–14 days old, it is important to make sure—through histological changes and PCR—that rotavirus is the main factor causing the diarrhea.

7.22 Senecavirus A

Senecavirus A (SVA) is a non-enveloped RNA virus from genus Senecavirus and family *Picornaviridae* which also includes foot-and-mouth disease (FMD) virus and swine vesicular disease virus. Vesicular disease is indistinguishable from foot-and-mouth disease. Senecavirus A can also cause high transient mortality in piglets up to 7 days of age.

Diagnosis:

- Timely confirmation of diagnosis is critical to rule out FMD.
- Presence of vesicles especially on snout and feet.
- Detection of virus via PCR from vesicular fluid.
- New ELISA assays have been developed to confirm exposure.

7.23 Teschen Disease

Commonly Teschovirus does not produce clinical cases, but there are very virulent strains that affect the central nervous system.

Alternative names: Teschen disease, Talfan disease, benign enzootic paresis, poliomyelitis suum, Teschovirus encephalomyelitis, Teschen/Talfan.

Diagnosis:

Identification of the virus is by isolation or by PCR.

7.24 Transmissible Gastroenteritis

Transmissible gastroenteritis is a very important and highly infectious disease in pigs caused by a coronavirus, with severe impact in reproduction, where diarrhea can cause a 100% mortality in piglets younger than 2 weeks of age.

Alternative names: TGE.

Diagnosis:

The clinical picture of the acute disease is almost pathognomonic. Except for the epidemic porcine diarrhea, there are no other enteric diseases so rapidly transmitted, and that cause high mortality in piglets. Final diagnosis must be made in the lab from samples of the intestine of a recently death pig using fluorescent antibodies test. PCR in feces can also be used. Serology interpretation is complicated due to a crossed reaction with respiratory coronavirus antibodies, which is a common disease that only affects the respiratory system.

7.25 Vesicular Exanthema

Vesicular exanthema is clinically indistinguishable from foot-and-mouth disease and therefore is of great importance.

Diagnosis:

Same diagnosis as for suspected FMD and swine vesicular disease and requires laboratory tests for its identification. Serological and PCR tests are needed.

7.26 Vesicular Stomatitis

Vesicular stomatitis produces a disease clinically indistinguishable from foot-and-mouth disease (FMD) and therefore is of great importance.

Alternative names: VSVs.

Diagnosis:

Paired blood samples can be taken (i.e., a sample during the initial phase of the disease and at 10–14 days). Virus neutralization, complement fixation, and ELISA are the usual tests used. In pigs, isolated positive samples would be strongly indicative of active infection. The disadvantage of blood tests and serology is that it involves a long delay of at least two weeks to get results.

8 Conclusion

From an animal health perspective, larger pig populations combined with the extensive movement of pigs, people, and material between production sites produce conditions that promote the circulation of infectious diseases within/between farms. These circumstances challenge our ability to prevent, control, and/or eliminate impactful swine pathogens. Disease management based on clinical observations is neither timely nor accurate; we require an active and systematic process that achieves the timely detection of pathogens and produces useful data that can guide management decisions. Viral infectious diseases represent an important portion of global public health concerns with thousands of deaths annually. From serious pandemics and highly contagious infections to common influenza episodes, clinical prognosis often relies on early detection of the infectious agent. Thus, effective identification of

viral pathogens is needed to help prevent transmission, set up appropriate therapy, monitor response to treatment, and lead to efficient disease management and control. Recent technological advances in viral identification, including polymerase chain reaction, mass spectrometry, and next-generation sequencing are applied in the diagnosis and management of viral infections. These powerful tools combine rapidity and efficiency in detecting viral pathogens and have revolutionized the field of clinical diagnostics. However, a number of drawbacks such as high cost have limited their use in many laboratories, particularly in resource-limited settings. On the contrary, the advent of microfluidic technology coupled with immunoassay has attracted increasing interest from biomedical research groups, and could represent a challenging alternative to diagnose viral infections at lower cost.

References

1. WHO (1946) Preamble to the constitution of the World Health Organization. WHO, New York
2. Hudson RP (1993) In: Kiple KF (ed) The Cambridge world history of human disease. Cambridge, Cambridge University Press, pp 45–52
3. Scully JR (2004) What is a disease? EMBO Rep 5(7):650–653. https://doi.org/10.1038/sj.embor.7400195
4. Hubálek Z (2003) Emerging human infectious diseases: anthroponoses, zoonoses, and sapronoses. Emerg Infect Dis 9(3):403–404. https://doi.org/10.3201/eid0903.020208
5. Taylor LH, Latham SM, Woolhouse ME (2001) Risk factors for human disease emergence. Philos Trans R Soc Lond Ser B Biol Sci 356(1411):983–989. https://doi.org/10.1098/rstb.2001.0888
6. Johnson N (2014) A short introduction to disease emergence. In: The role of animals in emerging viral diseases. Academic, pp 1–19. https://doi.org/10.1016/B978-0-12-405191-1.00001-6
7. Tesh R (1994) The emerging epidemiology of Venezuelan haemorrhagic fever and Oropouche fever in tropical South America. Ann N Y Acad Sci. https://doi.org/10.1111/j.1749-6632.1994.tb19863.x
8. FAO (2020) Food and Agriculture Organization of the United Nations. FAOSTAT statistical database. FAO. http://www.fao.org/faostat/en/#data/QA
9. Boender GJ, Van Den Hengel R, Van Roermund HJW, Hagenaars TJ (2014) The influence of between-farm distance and farm size on the spread of classical swine fever during the 1997-1998 epidemic in the Netherlands. PLoS One 9:95278
10. Kinsley AC, Perez AM, Craft ME, Vanderwaal KL (2019) Characterization of swine movements in the United States and implications for disease control. Prev Vet Med 164:1–9
11. Khan SU, Atanasova KR, Krueger WS, Ramirez A, Gray GC (2013) Epidemiology, geographical distribution, and economic consequences of swine zoonoses: a narrative review. Emerg Microb Infect 2(1):1–11. https://doi.org/10.1038/emi.2013.87
12. Segalez J (2015) Safepork 2015 conference; keynote lecture: emerging swine diseases and infections: an increasing zoonotic threat. pp 97–100
13. Smith TC, Harper AL, Nair R, Ferguson DD, Wardyn SE, Hanson BM, Dressler AE (2011) Emerging swine zoonoses. Vector Borne Zoonotic Dis 11:9. https://doi.org/10.1089/vbz.2010.018
14. Perfumo CJ, Pereda A, Jongkaewwattanna A, Chen Z, Perez DR, Ma J (2020) Editorial: emerging swine viruses. Front Vet Sci 7:132. https://doi.org/10.3389/fvets.2020.00132
15. Murcia P, Donachie W, Palmarini M (2009) Viral pathogens of domestic animals and their impact on biology. Medicine and Agriculture. Encyclopedia of Microbiology 80–819. https://doi.org/10.1016/B978-012373944-5.00368-0
16. Ramirez (2018) Chapter-1: diseases affecting pigs: an overview of common bacterial, viral and parasitic pathogens of pigs. In: Wiseman J (ed) Achieving sustainable production of pig meat: animal health and welfare, vol

3. Burleigh Dodds Science Publishing, Cambridge, pp 1–29. https://doi.org/10.19103/AS.2017.0013.14
17. Kedkovid R, Sirisereewan C, Thanawongnuwech R (2020) Major swine viral diseases: an Asian perspective after the African swine fever introduction. Porcine Health Manag 6:20. https://doi.org/10.1186/s40813-020-00159-x
18. Andraud M, Rose N (2020) Modelling infectious viral diseases in swine populations: a state of the art. Porcine Health Manag 6:22. https://doi.org/10.1186/s40813-020-00160-4
19. VanderWaal T, Deen J (2018) Global trends in infectious diseases of swine. PNAS 115(45): 11495–11500
20. Souf S (2016) Recent advances in diagnostic testing for viral infections. Biosci Horizon 9: 1–11. https://doi.org/10.1093/biohorizons/hzw010
21. Verheyen J (2015) Challenges in the diagnosis and prevention of viral infections. Lab Med 38(2). https://doi.org/10.1515/labmed-2014-0020
22. Pretorius M, Venter M (2017) Diagnosis of viral infections. Viral Infect Child 1:151–182. https://doi.org/10.1007/978-3-319-54033-7_6
23. Straub OC (1993) The important viral infections of pig. Swine Health Product 2(4):15–18
24. Zanon S (2020) Research links industrial pig farming and virus outbreaks. Mongabay
25. Henao-Diaz A, Giménez-Lirola L, Baum DH, Zimmerman J (2020) Guidelines for oral fluid-based surveillance of viral pathogens in swine. Porcine Health Manag 6:28, pp 1–12. https://doi.org/10.1186/s40813-020-00168-w
26. Zimmerman J, Thacker E (2003) Monitoring immunity through diagnostics. National Hog Farmer
27. Kellogg MD (2017) Chapter 8: measurement of biological materials. Clin Transl Sci:137–155. https://doi.org/10.1016/B978-0-12-802101-9.00008-9
28. Davies C (1994) Principles. In: Wild D (ed) The immunoassay handbook. Stockton Press, New York, pp 3–47
29. Ashihara Y, Kasahara Y, Nakamura RM (2001) Immunoassays and immunochemistry. In: Henry JB (ed) Clinical diagnosis and management by laboratory methods. W.B. Saunders, Philadelphia, pp 821–849
30. Koehler G, Milstein C (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256:495–497
31. Zola H (1987) Monoclonal antibodies: a manual of techniques. CRC Press, Boca Raton, FL
32. Winter G (1994) Making antibodies by phage display technology. Annu Rev Immunol 12: 433–455

Chapter 25

Production of Virus-like Particles Using the Baculovirus Expression System and Their Application in Vaccines and Viral Disease Diagnosis

Hemanta Kumar Maity, Rajib Deb, Sinéad Lyons, and Ian M. Jones

Abstract

Over many years, the baculovirus expression vector system (BEVS) has grown in popularity as a platform for expressing recombinant proteins. In addition to high levels of expression, the recombinant protein is near authentic as the insect cell is capable of posttranslational modifications such as glycosylation, oligomerization, disulfide bond formation, acylation, and phosphorylation.

The BEVS method has been particularly successfully employed to produce virus-like particles (VLP) from a variety of enveloped and non-enveloped viruses. VLPs are recombinant viral structural proteins which assemble spontaneously into a virus-like structure which is structurally similar to the authentic virus but devoid of any genomic material, providing a safe choice compared to the risk associated with the handling of live virus. Currently, many experimental vaccine prototypes have adopted the BEVS as their choice production VLP based platform as well as for the development of diagnostics for viral diseases. Following immunization of laboratory animals, insect cell generated VLPs have produced antigen-specific antibody consistent with a protective capacity equivalent to commercially available live or inactivated vaccines.

In this chapter we provide a detailed protocol for the purification of VLPs of an FMDV O serotype using the baculovirus expression vector system. The process illustrated by this method may be effectively used for the development of vaccine candidate and diagnostics against other burdensome viral diseases such as PCV2, PRRS, or Avian influenza.

Key words Baculovirus expression vector system (BEVS), Virus-like particles (VLP), Vaccine, FMDV, PCV 2

1 Introduction

Since the first expression of human beta interferon in insect cells by infection with a recombinant *Autographa californica multiple nuclear polyhedrosis virus* (AcMNPV), the baculovirus expression vector system (BEVS) has become a very popular platform for the expression of recombinant proteins [1, 2]. In addition to high expression levels, the insect cell successfully allows posttranslational modifications such as glycosylation, oligomerisation, disulfide bond

Rajib Deb et al. (eds.), *Protocols for the Diagnosis of Pig Viral Diseases*,
Springer Protocols Handbooks, https://doi.org/10.1007/978-1-0716-2043-4_25,

formation, acylation, and phosphorylation [3, 4] so that recombinant proteins are produced in a near authentic state. AcMNPV is a rod shaped double-stranded DNA virus, predominantly infecting insect larvae in the order *Lepidoptera* such as Moth and Butterfly. The non-essential *polh* gene which expresses the polyhedrin protein (which normally protects the virion particle from environmental exposure) is replaced by a foreign gene of interest under *polh* promoter control for the expression of recombinant proteins in the very late phase of the virus replication cycle [5]. Other non-essential genes such as p10, cathepsin and chitinase may be replaced for the expression of multiple recombinant proteins in a similar way [6, 7]. AcMNPV produces two types of virus particle, budded virus (BV) and occlusion derived (polyhedra based) baculovirus (ODV), the former of which continue to replicate when genes for the latter phenotype are deleted or replaced, making a helper independent expression system. The combination of high-level expression and the ability to express multiple recombinant proteins has made the system a natural choice for the production of VLPs for a number of enveloped and non-enveloped viruses [8, 9]. VLPs produced in insect cells have shown specific antibody responses in vaccinated animals that are protective with an efficacy similar to commercially available inactivated vaccines suggesting that a commercial vaccine supply may be viable [10, 11].

VLPs assemble spontaneously following the expression of the viral structural proteins associated with virus capsid assembly. They are structurally similar to authentic virus but devoid of any genomic material, providing a safe choice compared to the risk associated with the handling live virus. In addition, they offer a solution for vaccine development in cases where emergent strains do not grow well in tissue culture and the possibility of further engineering, for example to improve stability or yield [12]. Notably, the three-dimensional conformational epitopes on the outer surface of VLPs are antigenically indistinguishable from those on authentic virus and induce strong humoral and cell mediated immunity that cannot be matched by the individual structural proteins alone [13–15].

Many experimental vaccine prototypes have adopted VLPs as their candidate immunogen including FMDV, HFMD, poliovirus [16–19], EV71 [20], PCV 2 [21], Duck hepatitis A virus [22], and Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) [23].

However, a concern associated with VLP production in the BEVS system is that the final vaccine preparation contains baculovirus particles released by the infected cells. These may have to be inactivated to comply with legislative requirements on the release of genetically modified organisms which can be an issue in vaccine production even if the risk from the baculoviruses themselves is minimal [7]. In this chapter we provide a detailed protocol for purification of VLPs of an FMDV O serotype from baculovirus infected cells. The method for the purified VLPs described here can be used similarly for other VLP vaccine and diagnostic candidates [24].

2 Materials

1. *Insect cell*: Sf9 cells (*Spodoptera frugiperda*), Tnao38 *cell line (Ascalapha odorata*).
2. *Medium*: Insect cell lines were cultured in Insect-XPRESS protein-free cell medium (Lonza, Switzerland).
3. Fetal calf serum (FCS).
4. T25, T75, T175 vented tissue culture flasks (Greiner Bio-One).
5. 125 ml, 250 ml, 500 ml, 1 l vented conical flasks (Corning).
6. Lipofectin Transfection Reagent (Fisher).
7. Benzonase® Nuclease (Sigma).
8. TAE buffer (50×) (Geneflow).
9. TBS (10×) Tris buffer saline (Geneflow).
10. SuperBlock™ (TBS) Blocking Buffer (Thermo Scientific).
11. Complete protease inhibitor cocktail (Roche).
12. Vivaspin column, 2 ml, MWCO 100,000 kDa (GE healthcare).
13. HRP-conjugated anti-rabbit antibody (Agilent Technology, USA).
14. Carbon coated formvar 200 square mesh copper grids (Agar Scientific).
15. Beckman Tube, Thinwall, Ultra-Clear™, 38.5 ml, 25 × 89 mm (Beckman).
16. Beckman Tube, Thinwall, Ultra-Clear™, 14 ml, 14 × 95 mm (Beckman).

3 Methods

3.1 Cell Culture and Media Requirement

3.1.1 Bacterial Strain and Insect Cell Lines

Insect cell lines *Spodoptera frugiperda* (*Sf9*) were developed from the ovarian tissue of the Fall Armyworm [25] and *Ascalapha odorata* (Ao38), originally thought to derive from eggs of the black witch moth [26], were later shown to be from *Trichoplusia ni* and renamed Tnao38 [27], were used for virus stock production and empty capsid protein expression, respectively.

3.1.2 Culture Conditions and Stock Preparation

Insect cell lines Sf9 and Tnao38 were cultured in Insect-XPRESS protein-free cell medium (Lonza, Switzerland) supplemented with 2% FCS. The Sf9 cells were cultured as monolayers at 27 °C for virus stock preparation, and Tnao38 cells were cultured as suspension cultures in a 27 °C shaker incubator at 100 rpm. The Tnao38 cells were used for expression and purification. For preservation of

baculovirus stocks, infected insect cells were harvested and centrifuged at 3000 × *g* for 10 min and the supernatant containing baculovirus was stored at 4 °C in the dark.

3.2 Generation of Recombinant Baculovirus Expressing Gene of Interest

Recombinant plasmid vectors with the target gene encoding one or more viral structural proteins were constructed as described [11] and co-transfected into Sf9 cells with suitable baculovirus DNA (such as flashBAC™ GOLD (FBG, Mirus Bio) or ProEasy (AB Vector LLC, San Diego, USA)) to produce a recombinant baculovirus via homologous recombination as described [11]. Briefly, 1 ml of Sf9 cells were seeded into a 6-well tissue culture plate at a concentration of 0.9×10^6 cells/well and incubated at room temperature for 30 min to settle. The recombinant plasmid (100 ng/μl) 5 μl was mixed with 2 μl baculovirus DNA (100 ng/μl) and 5 μl nuclease free water (NFW) in a sterile Eppendorf tube and, separately, a lipofection mixture (12 μl) was prepared by mixing 8 μl lipofectin reagent and 4 μl sterile water. Both the lipofectin (12 μl) and plasmid DNA-baculovirus contents (12 μl) were mixed together and incubated at room temperature for 30 min. Following incubation, the DNA-Lipo mixture (24 μl) was mixed with 1 ml serum free media by gentle pipetting. The seeded *Sf*9 cells were washed with serum free media and the 1 ml of DNA-Lipo mix was layered gently onto the Sf9 monolayer and incubated at 27 °C for 24 h. The next day, 1 ml of complete growth media containing 2% fetal calf serum (FCS) was added per well and incubation continued at 27 °C for 4–5 days. After five days, the media were harvested and centrifuged at 2000 x g for 10 min. The supernatant was collected and labeled as the P0 passage virus. Similarly, 0.05 ml of P0 virus was used to infect additional Sf9 cells and kept for 4–5 days for the generation of the P1 virus stock and the same process repeated for P2 and P3 virus passages. The final virus titer was ~2.0×10^8 pfu/ml with a volume dependent on the volume of the passage and the intended use. A flowchart of the process is shown in Fig. 1.

3.3 Detection of Recombinant Baculoviral Titer by Plaque Assay

3.3.1 Baculoviral Titer by Plaque Assay

Obtaining an accurate virus titer is essential for determining the mutiplicity of infection (MOI) necessary for optimum protein production and the titer of recombinants expressing the P1 region of O Bangkok FMDV was measured by plaque assay. Sf9 cells seeded in a 6-well dish at a concentration 0.5×10^6/ml were incubated at 27 °C for 30 min to adhere and after attachment the cell growth media was removed and the monolayer washed with serum free media (Insect-XPRESS™ protein-free cell medium; Lonza, Switzerland). A series of tenfold serial dilutions of the virus to be titrated was prepared by adding 100 μl of viral sample into 900 μl of growth media to a final dilution of 10^{-6}. One milliliter of each viral dilution was added into the respective wells and incubated for 1 h at room temperature to allow virus adsorption. The supernatant

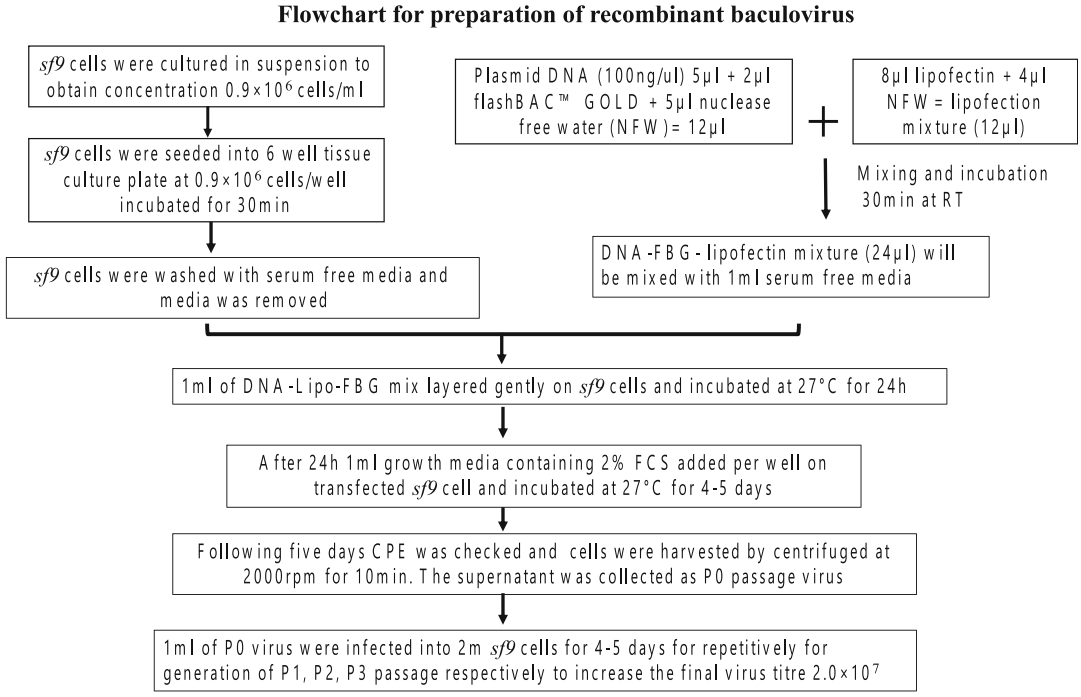


Fig. 1 Flowchart for preparation of recombinant baculovirus

was then carefully removed and the monolayer overlaid with plaque media (0.75% low melting point agarose in 50% serum free media) at a final temperature of approximately 33 °C. The plaque media was allowed to solidify and 1 ml of complete growth media was overlaid on top of the agarose and incubation continued at 27 °C for 5–6 days. After incubation, plates were stained with 1 ml of neutral red solution (Sigma-Aldrich) for 1 h at room temperature and then drained by inverting the plates. Plaque numbers of 3–30 per well were considered ideal for counting and an end point titer was evaluated by the formula below. Alternatively, a 50% cytopathic effect score was used and the TCID 50 determined by the Reed and Muench method [28].

$$\text{Virus titer(pfu/ml)} = \text{average plaque count} \times \text{dilution factor} \times 10.$$

3.3.2 Titration by Sf9 ET Cell

An alternative and quicker method of detection of viral titer was by use of the Sf9 ET cell line [29]. Sf9 ET cells were seeded into 96-well plates at a concentration 0.9×10^6/ml, 100 µl/well, in Insect-XPRESS™ medium supplemented with 2% FCS and allowed to attach for 30 min at 27 °C. The serially diluted baculovirus stock from 10^{-1} to 10^{-8} was incubated with the cells in respective wells. In the negative control, only insect cell media was added. The plate was incubated at 27 °C for 48–72 h until

green fluorescent foci had appeared when viewed under fluorescence microscopy. The foci were counted and the virus titre determined as before.

3.4 Purification of Empty Capsids from Insect Cell

100 ml cultures of Tnao38 suspension cell cultures (at 1×10^6 cells/ml) were infected with recombinant baculovirus (titer ~10^8/ml) at an MOI of ~3 and incubated in a 500 ml cell culture conical flask for 3 days with shaking. The viablity of the cells at the end of this period was typcially 50% when measured by trypan blue exclusion. Following incubation, the cell suspension was pelleted, 2000 × *g* for 10 min at 10 °C. Pelleted cells were lysed in a 1/20th volume of lysis buffer containing 50 mM HEPES (pH 8.0) supplemented with 400 mM NaCl, 1% NP40, benzonase 3 μl and one tablet of complete protease inhibitor cocktail (Roche). Lysates were incubated in ice and vortexed intermittently for approximately 3 h. After treatment, the cell lysate was centrifuged at 15,000 × *g* for 30 min at 10 °C. The clarified lysis supernatant was layered above a 30% sucrose cushion (in lysis buffer) and any VLPs present were sedimented through the cushion by ultracentrifugation at 100,000 × *g* for 2 h at 4 °C. The pellet was allowed to resuspend overnight at 4 °C and then loaded on top of a 15–45% sucrose velocity gradient and ultracentrifugation repeated at 100,000 × *g* for 16 h at 4 °C. The sucrose gradient samples were fractionated into eight equal fractions from the top, preserved at 4 °C, and all fractions assessed by ELISA, Western blot analysis, or TEM as described below.

3.5 Analysis of Empty Capsids

Expressed proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) on precast 4–12% Nu PAGE gels in 1× MES buffer (Invitrogen). All protein samples were prepared by adjusting to 1× loading buffer (Novex) and heated at 98 °C for 10 min before loading on the gel. The protein samples were loaded into the wells and the gels were electrophoresed at a constant voltage of 200 V for 30 min. The separation of the protein samples was compared with a prestained protein ladder (Geneflow). After completion of electrophoresis, the gels were removed from the gel cast and subjected to either Coomassie staining or transfer to PVDF membrane for Western blot analysis.

3.5.1 Analysis of Expression of Capsid Protein by Coomassie Staining

Protein bands were visualized by staining the electrophoresed gels with Coomassie brilliant blue stain (40% methanol v/v, 10% glacial acetic acid, and 0.5% Coomassie brilliant blue) at room temperature with constant rocking for 1 h. The stain was removed from the gel by incubating overnight with destaining solution (40% methanol v/v, 10% glacial acetic acid) at room temperature with continuous rocking.

3.5.2 Analysis of Expression of Capsid Protein by Western Blot

Proteins were transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes (Immobilon® Membranes, Merck, Germany) to detect protein by immunoblot. The PVDF membranes were pre-wetted with methanol and subsequently with Western blot transfer buffer. Electrophoresis was performed in semidry format at 90 V for 80 min in transfer buffer with 20% methanol. The complete transfer of the proteins was confirmed by the transfer of the prestained ladder into the PVDF membrane. Membranes were blocked with SuperBlock™ Blocking Buffer (Fisher Scientific) and incubated with primary antibody rabbit anti-VP0 (available in the host laboratory) at 1:400 dilution followed by washing three times and incubation with polyclonal goat HRP-conjugated anti-rabbit antibody (Agilent Technology, USA). The protein bands were developed by addition of ECL Western blot Detection Reagent (GE Healthcare Life Sciences) for 2 min incubation and imaged by a Syngene G-Box.

3.5.3 Analysis of Capsids Protein Assembly by Transmission Electron Microscopy (TEM)

The assembly of FMDV capsid proteins into virus-like particles (VLP) were analyzed by transmission electron microscopy (TEM). The purified capsid protein containing fraction was concentrated by spin dialysis prior to absorption to the grids. Firstly, 100 μl of the peak western blot positive gradient factions were diluted threefold in TBS to reduce the sucrose concentration and re-concentrated tenfold using a microspin column (500 μl Vivaspin column, MWCO 100,000, GE Healthcare) dialysis by tabletop centrifugation at 4 °C. The concentrated final capsid sample was absorbed to the grid (carbon-coated formvar 200 square mesh copper grids, Agar Scientific) by floating it, glossy side down, on a 10 μl droplet of the concentrated sample for 10 min at room temperature. Followed two washes with distilled water each of 2 min duration, the grids were stained by two 30 s incubations on a droplet of 2% uranyl acetate. Excess stain was removed by blotting and the grid examined on a Joel transmission electron microscope (JEOL USA, Inc.) operating at 200 kV. The empty FMDV capsid particles expressed in insect cells were visualized with an approximate diameter of 30–35 nm (Fig. 2).

3.5.4 Detection of Antigenicity by Immunogold Staining

The antigenicity of the visualized capsid particles was analyzed by immunogold staining. For labeling the particle grids were prepared as described up to the wash stage. The grids were then blocked by floating on drops of SuperBlock™ TBS blocking buffer for 10 min and then incubated with a 10 μl drop of 1:10 diluted VP0 polyclonal rabbit antibody for 10 min. Following washing the grid was then incubated with 10 μl of diluted anti-rabbit Ig (1:100) conjugated with 10 nm gold. Finally, the grids were washed and stained as before. The nanogold particle immunologically attached with the empty capsid particle as seen in Fig. 3.

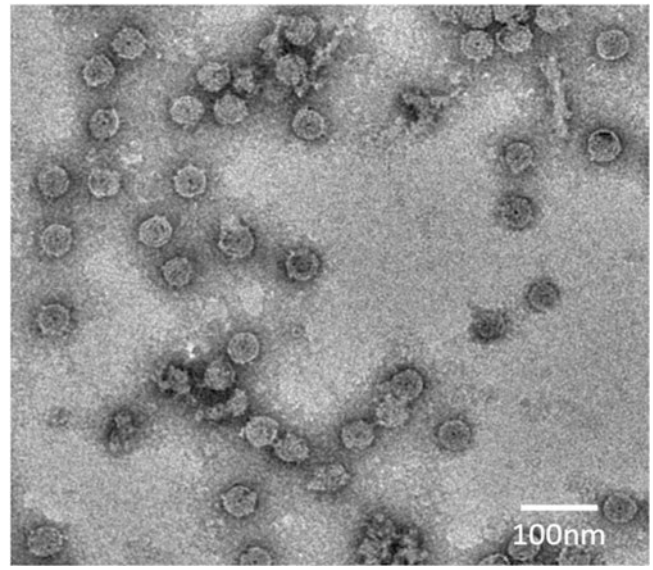


Fig. 2 The empty capsid sample of FMDV O Bangkok expressed in insect cell (A038), prepared from 15–45% peak sucrose gradient visualized under transmission electron microscopy by negative staining with 2% uranyl acetate

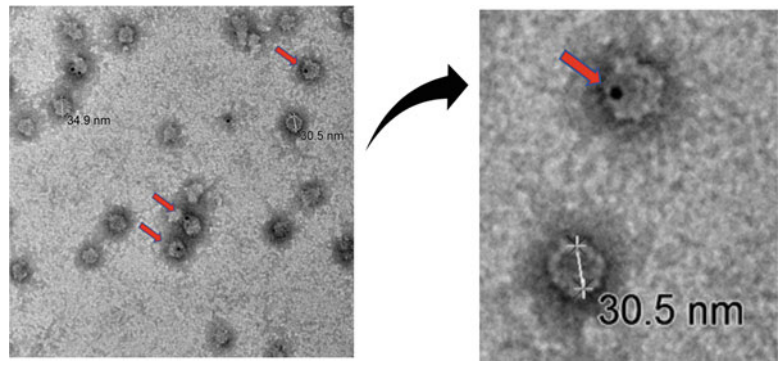


Fig. 3 The antigenicity of the assembled capsid particle analyzed by immunogold staining with the anti-VP0 polyclonal antisera raised in rabbit, followed by incubation with anti-rabbit IgG conjugated with 10 nm gold. The specific attachment of the gold particle to the capsid structure is indicated

3.6 Detection of Yield of Empty Capsid by ELISA

ELISA assay components were provided by the FMD World Reference Laboratory at The Pirbright Laboratory, UK. Briefly, polystyrene plates were coated in triplicate with 50 μl of capture antibody O1BFS polyclonal rabbit sera at 1:100 dilution (in sodium bicarbonate buffer, pH 9.6) and incubated overnight at 4 °C. The plate was washed three times with PBST (1× PBS with 0.1% Tween-20) and blocked with SuperBlock™ PBS blocking buffer (Thermo

Scientific) for 1 h at 37 °C. After blocking 50 μl of purified antigen was diluted 1:2 in the original well and then further diluted in a twofold serial dilution and incubated for 1 h at 37 °C. After washing, the plates were probed with 50 μl per well of O1 Manisa polyclonal guinea pig sera diluted (1:100) in PBS and incubated for 1 h at 37 °C. After washing three times, 50 μl of 1:1000 diluted HRP-conjugated anti-guinea pig antibody (Abcam, UK) was added per well and incubated 1 h at 37 °C. The plates were finally washed three times with PBST and 50 μl of TMB substrate (Thermo Scientific) was added to each well and incubated for 20 min in the dark. Then 50 μl 0.03 M sulfuric acid was added per well as stop solution and the absorbance was measured at 420 nm in a 96-well plate reader (Tecan). The data was analyzed by GraphPad Prism8 software.

3.7 Use of Empty Capsid to Detect Serum Antibody by ELISA

The empty capsid can be also used to detect serum antibody by ELISA. The seroconversion of different serum sample was detected by ELISA by collecting sera samples, pre-bleed and final bleed from immunized animals and assay can be performed by ELISA as described in column 6 except that the probing layer was formed of the test sera, not the control serum provided by the kit and the detecting layer was an anti-mouse HRP conjugate.

4 Notes

1. To prepare virus stocks in *Sf9* cells, the cells need to be seeded at low confluency, no more than 50%, checked every day following virus addtion for cytopathic effect (CPE). Virus stocks can be harvested when more than 50% cells showed CPE, by gently tapping in the flask to dislodge the monolayer and then clarification by centrifugation as described.
2. Purification of protein was performed by infecting Tnao38 cell line with recombinant baculoviruses in suspension culture by incubation at 27 °C in shaker incubator. The progress of the infection be checked every day by routine microscopy and trypan blue viability. Generally, cells will look intact on the first day post infection but will become slightly granular after 48 or 72 h. Cell counting is the best measure to check the density of the cells and infection is normally done as a density of approximately ~1.0×10^6 ml. Cell counts may rise for 1 more day but will then plateau and decline such that a viability of <50% is apparent after 72–96 h. This is the best time to harvest the cells for VLP purification as cell debris is not excessive and the clarified culture supernatent is clear. Virus titration in Sf9 cells with each recombinant baculovirus is needed to ensure adequate titres for expression experiments, as is preliminary

epression analysis to verify recombinant protein expression with an expected pattern. It is not practical to check expression during a large scale infection study.

3. Despite the generally robust natue of virus capsids purification of empty capsids (VLPs) from insect cell is best performed as quickly as possible, with all necessary manisplation steps performed at ambient temperature and all storage steps kept below 10 °C to minimise protein degradation. Highly purified VLPs are less prone to degradation and working stocks can be stored at 4 °C with longterm storage at −20 °C in 5% glycerol.
4. The purification of empty capsids from the baculovirus vector is very useful to remove the background in TEM. A method that uses sucrose gradient fractionation naturally achieves this as the middle fractions (sucrose density 30–35%) contain empty capsid, whereas the lower fractions (sucrose density 40–45%) contains mostly baculovirus. Simple concetration of the VLPs, for example by size exclusion chromatography may not achieve the same removal of baculovirus background.

Acknowledgments

This review has been promoted and supported by Felix Trust and The Department of Biotechnology, Government of India.

References

1. Smith GE, Summers MD, Fraser MJ (1983) Production of human beta interferon in insect cells infected with a baculovirus expression vector. Mol Cell Biol 3:2156–2165
2. Chambers AC, Aksular M, Graves LP et al (2018) Overview of the baculovirus expression system. Curr Protoc Protein Sci 91:541–546
3. Liu F, Wu X, Li L et al (2013) Use of baculovirus expression system for generation of virus-like particles: successes and challenges. Protein Expr Purif 90:104–116
4. Qu Z, Li M, Guo Y et al (2020) Expression, purification, and characterisation of recombinant ferritin in insect cells using the baculovirus expression system. Biotechnol Lett 42:57–65
5. Gwak WS, Lee SN, Choi JB et al (2019) Baculovirus entire ORF1629 is not essential for viral replication. PLoS One 14(8):e0221594
6. López-Vidal J, Gómez-Sebastián S, Sánchez-Ramos I et al (2013) Characterization of a Trichoplusia ni hexamerin-derived promoter in the AcMNPV baculovirus vector. J Biotechnol 165:201–208
7. Van Oers MM, Pijlman GP, Vlak JM (2015) Thirty years of baculovirus-insect cell protein expression: from dark horse to mainstream technology. J Gen Virol 96(Pt 1):6–23
8. Rohrmann G (2019) The baculovirus replication cycle: effects on cells and insects. Baculovirus Molecular Biology [Internet]. 4th edition. National Center for Biotechnology Information (US); 2019
9. Li Y, Shen S, Hu L et al (2018) The functional oligomeric state of tegument protein GP41 is essential for baculovirus budded virion and occlusion-derived virion assembly. J Virol 92(12):e02083-17
10. Mohana Subramanian B, Madhanmohan M, Sriraman R et al (2012) Development of foot-and-mouth disease virus (FMDV) serotype O virus-like-particles (VLPs) vaccine and evaluation of its potency. Antivir Res 96:288–295
11. Porta C, Xu X, Loureiro S et al (2013) Efficient production of foot-and-mouth disease virus empty capsids in insect cells following down regulation of 3C protease activity. J Virol Methods 187:406–412

12. Le DT, Müller KM (2021) In vitro assembly of virus-like particles and their applications
13. Noad R, Roy P (2003) Virus-like particles as immunogens. Trends Microbiol 11:438–444
14. Syomin BV, Ilyin YV (2019) Virus-like particles as an instrument of vaccine production. Mol Biol 53:323–333
15. Qian C, Liu X, Xu Q et al (2020) Recent progress on the versatility of virus-like particles. Vaccine 8(1):139
16. Gong M, Zhu H, Zhou J et al (2014) Cryo-electron microscopy study of insect cell-expressed enterovirus 71 and coxsackievirus A16 virus-like particles provides a structural basis for vaccine development. J Virol 88(11): 6444–6452
17. Xiao Y, Chen HY, Wang Y et al (2016) Large-scale production of foot-and-mouth disease virus (serotype Asia1) VLP vaccine in Escherichia coli and protection potency evaluation in cattle. BMC Biotechnol 16:56
18. Adeyemi OO, Nicol C, Stonehouse NJ et al (2017) Increasing type 1 poliovirus capsid stability by thermal selection. J Virol 91: e01586-16
19. Zhang W, Dai W, Zhang C et al (2018) A virus-like particle-based tetravalent vaccine for hand, foot, and mouth disease elicits broad and balanced protective immunity article. Emerg Microbes Infect 7:1–12
20. Wang X, Ku Z, Zhang X et al (2017) Structure, immunogenicity, and protective mechanism of an engineered enterovirus 71-like particle vaccine mimicking 80S empty capsid. J Virol 92: e01330-17
21. Li Y, Yi X, Zhuang Y et al (2016) Regulation of porcine circovirus type 2-like particles expressed in baculovirus expression system. Bioresour Bioprocess 3:37
22. Wang A, Gu L, Wu S et al (2018) Duck hepatitis A virus structural proteins expressed in insect cells self-assemble into virus-like particles with strong immunogenicity in ducklings. Vet Microbiol 215:23–28
23. García Durán M, Costa S, Sarraseca J et al (2016) Generation of porcine reproductive and respiratory syndrome (PRRS) virus-like-particles (VLPs) with different protein composition. J Virol Methods 236:77–86
24. Fabre LL, Arrías PN, Masson T et al (2019) Baculovirus-derived vectors for immunization and therapeutic applications. In: Emerging and reemerging viral pathogens: volume 2: applied virology approaches related to human, animal and environmental pathogens. Academic, pp 197–224
25. Vaughn JL, Goodwin RH, Tompkins GJ, McCawley P (1977) The establishment of two cell lines from the insect spodoptera frugiperda. In Vitro 13:213–217
26. Hashimoto Y, Zhang S, Blissard GW (2010) Ao38, a new cell line from eggs of the black witch moth, Ascalapha odorata (Lepidoptera: Noctuidae), is permissive for AcMNPV infection and produces high levels of recombinant proteins. BMC Biotechnol 10:50
27. Hashimoto Y, Zhang S, Zhang S et al (2012) Correction: BTI-Tnao38, a new cell line derived from Trichoplusia ni, is permissive for AcMNPV infection and produces high levels of recombinant proteins. BMC Biotechnol 12:12
28. Reed LJ, Muench H (1938) A simple method of estimating fifty per cent endpoints. Am J Epidemiol 27:493–497
29. Hopkins RF, Esposito D (2009) A rapid method for titrating baculovirus stocks using the Sf-9 Easy Titer cell line. BioTechniques 47: 785–788

Chapter 26

Good Laboratory Practices and Biosafety Containments in a Virology Laboratory

Yashpal Singh Malik, Anuradha Sharma, Niraj Kumar Singh, B. T. Naveen Kumar, and Naveen Kumar

Abstract

Infectious disease outbreaks keep challenging human and veterinary health worldwide since decades. Disease outbreaks such as smallpox, influenza, polio, SARS, Ebola, foot-and-mouth disease, African swine fever, and the most recent and devastating COVID-19, all point to the need for a more proactive approach to developing diagnostics and treatment methods for these deadly diseases. Because the pathogenic agents that cause these diseases are highly transmissible, careful containment of these agents within the laboratories is necessary, with little or no exposure to working personnel. Different regulatory authorities across the world provide guidelines and procedures to ensure that research and diagnostic laboratories operate safely. This chapter delves into the many events that occur as a result of lab-mediated disease spread, as well as the need for, importance of, and guidelines for good lab practices and biosafety.

Key words Biosafety, Lab-acquired infections, Containment, Good laboratory practices, Virology, Risk groups, Biosafety levels

1 Introduction

Clinical, biomedical, and research laboratories employ a vast number of laboratory personnel worldwide, and biosafety is a key concern in these environments. Laboratory personnel are exposed to a wide range of pathogenic microorganisms and are always at risk of contracting infection if basic biosafety measures such as personal protective equipment, engineering setup, standard operating procedures, and administrative controls fail or are not in place [1]. Viruses, bacteria, fungi, and parasites are the most common causative agents of lab-acquired infections (LAIs), which are not only dangerous to the laboratory personnel but also pose a substantial risk of community outbreaks if they go unnoticed. Such disease outbreaks also result in social and economic losses all across the world.

Rajib Deb et al. (eds.), *Protocols for the Diagnosis of Pig Viral Diseases*,
Springer Protocols Handbooks, https://doi.org/10.1007/978-1-0716-2043-4_26,

The infectious diseases have a complex epidemiology, and only a few diseases such as Corona virus disease 19 (COVID 19) and severe acute respiratory syndrome (SARS) share clinical symptoms [1]. Because identifying and distinguishing causative agents only based on clinical signs is difficult, laboratory methods/technologies have become a key aspect of disease diagnosis and subsequent clinical treatment. Good Laboratory Practices (GLPs) aid in the maintenance of international quality standards in laboratory operations by allowing control over laboratory protocols, resulting in precision, accuracy, and reproducibility of results. Furthermore, GLPs help to ensure the safety of laboratory personnel involved in infectious disease diagnosis and research [2]. Non-clinical research with environmental safety and quality drug development for infectious diseases around the world is also boosted by GLPs.

2 History of Lab-Escaped Pathogens

Individuals working in laboratories have long been aware that they can become infected by the pathogenic agent they are working with, either directly or indirectly, making their profession an occupational hazard. Dr. HT Ricketts, for example, died in 1910 after contracting Typhus infection while studying the disease with a less specific containment setup. In 2001, a researcher at the United States contracted anthrax after an antimicrobial disinfectant was switched incorrectly in a public health laboratory [3]. Some reports and surveys of LAIs around the world have been in the past [4–7] and the largest survey of LAIs was conducted by Pike [5], who reported 159 pathogenic agents caused 4079 LAIs, with ten major infectious agents accounting for more than half of the cases.

The following are the major incidents of viral escapes from the lab in the past.

2.1 Smallpox Virus in 1966–1978

The World Health Organization's (WHO) campaign to eradicate smallpox identified the virology labs as a potential source of infection and epidemic. Between 1963 and 1978, only 4 cases of smallpox infection were documented in smallpox endemic areas in the United Kingdom, yet 80 cases and three deaths were reported from three different lab escapes of smallpox virus during the same period. This notion was backed by the fact that smallpox lab escapes reported in three different years, 1966, 1972, and 1978, all were from the areas where smallpox virus was being handled for research purpose at that time. These infections were extremely similar, and the individuals who first became infected in 1966 and 1978 worked at the same location where Variola minor research was being conducted. Following the identification of these lab escapes, different investigations were conducted, and stringent guidelines for handling pathogens, personnel training, and sufficient rooms/

cabinets ventilation were established. These guidelines served as the foundation for biosafety protocols at all levels [8, 9].

2.2 Human Influenza H1N1 in 1977

The H1N1 influenza virus was found circulating in humans in 1977. It started in China and the Soviet Union and spread throughout the world. This pandemic was also known as Russian flu, and it primarily affected people under the age of 21 years. For more than three decades, scientists debated whether the pandemic was caused by lab release or by some other factor. The 1977 H1N1 influenza virus strain was found closely related to the H1N1 virus strain circulating in 1949–1950, based on extensive genomic and serological tests. The isolation of H1N1 RNA from Siberian Lake meltwater, which is frequently visited by migratory birds and could have frozen since long times, suggested a reason other than lab escape in a 2006 report [10]. However, in 2008, natural stasis was ruled out, and it was found that the H1N1 isolation from meltwater was caused by contamination in the lab, where the H1N1 (1977) strain was being used as a positive control [11]. The advent of flu was thus clearly proposed to be an escape from a viral sample lot frozen in a virology lab since 1950, and the reason for the virus's thawing out was the consequence of the US vaccine campaign against H1N1 influenza in 1976 [4].

2.3 Venezuelan Equine Encephalitis (VEE) in 1995

VEE is a hemorrhagic fever-causing viral epizootic disease that first appeared in South Armenia in 1969–1971. Veterinarians utilized the inactivated whole VEE virus as a vaccine against this lethal disease at that time, and inactivating VEE is notoriously difficult. The viral strain found in 1970 outbreak was similar to the one used for immunization in the 1930s, implying the incomplete inactivation of VEE virus and that the virus was able to escape [4, 12].

2.4 SARS (Severe Acute Respiratory Syndrome) Virus in 2002–2003

The SARS outbreak in 2002–2003 resulted 8000 infections and 774 deaths in 29 countries; however, the epidemic was contained in 2003 due to implementation of strict containment and quarantine procedures [13, 14]. In terms of transmission, SARS is a super spreader, and even a single LAI can cause the disease to spread [15]. After 2003, no natural outbreaks of SARS were recorded; nonetheless, six lab-escaped outbreaks (Singapore 1, Taiwan 1, China 4) were reported from the labs handling SARS virus for vaccine research [16, 17]. As a result, labs conducting SARS-CoV research and retaining specimens of SARS patients pose the greatest risk of virus exposure and transmission.

In addition to these incidents, lab escapes of the Ebola virus, and foot-and-mouth disease virus were also recorded in the early 2000s [4]. All of these events of viral lab escapes emphasize the significance of following stringent GLPs and biosafety guidelines.

3 Good Laboratory Practices

Good Laboratory Practices are the set of rules established to ensure the integrity and quality of laboratory studies conducted in support of government-sponsored research or marketing policies of products. GLPs were initially adopted in 1972 in Denmark and New Zealand and then, in 1979 in the USA [19, 20]. Later, the Organization of Economic Cooperation and Development (OECD) released GLP guidelines to help with various toxicological studies and the production of high-quality data for environmental and human risk assessment. Institute, personnel, experiment facility, quality assurance system, experiment system, experiment item, standard operating procedures (SOPs), experiment recording, and reporting of findings are all covered under the OECD GLP guidelines [19]. According to the OECD, GLP is "a quality system concerned with the organizational process and the conditions under which non-clinical health and environmental safety studies are planned, performed, monitored, recorded, archived and reported." Because non-validated data generated by various laboratories is not acceptable in various countries throughout the world, the OECD member countries took the initiative to create the national monitoring authorities to ensure that GLP is followed in their respective countries. GLP-compliant labs follow a set of standard protocols while conducting the experiment/studies/tests, and analyses. This helps in the planning, execution, monitoring, reporting, documenting, and validation of findings in order to deliver the data/products that are compliant with international standards. Because GLPs allow interchange of information, these practices result in the removal of technical and international trade barriers as well as a reduction in input/operational expenses. Henceforth, GLPs make it easier for international cooperation to work toward a common goal of protecting human health and the environment [19]. The International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH), an organization that oversees pharmaceutical quality, efficacy, and safety has proclaimed GLP to be a prerequisite for international pharmaceutical registration [20].

The GLP regulations include pre-established plan and standard operating procedures. These set the rules for good practices on the test site and help the technical or research personnel (s) in conducting the study according to a pre-designed and approved plan. The GLPs are based on the following fundamental principles:

1. Resources: organization, personnel, facilities, and equipment.
2. Characterization: test items and test systems.
3. Rules: study plans (or protocols) and written procedures.
4. Results: raw data, final report, and archives.

5. Quality Assurance: determine the quality of product/report generated from the non-clinical research independently [21, 22].

The GLP principles were developed by an OECD expert panel in 1978, and adopted by member countries in 1981. These principles were further amended in 1997 (Annex to the Council Decision on the Mutual Acceptance of Data in the Assessment of Chemicals; amended Annex II of the 1981 Council Decision) [2]. Table 1 summarizes the various GLP principles.

4 Biosafety Requirements While Working with Infectious Agents

Biosafety guidelines are developed to ensure the safety of individuals working in research and clinical labs by implementing various measures such as establishing primary and secondary barriers between personnel and biological hazards, some of which are supported by recent advances in engineering and material science. Strict regulations with appropriate protective equipment, practices, and facilities are required when handling pathogenic agents in order to prevent contracting infection by laboratory personnel. According to the World Health Organization (WHO), each country should identify and categorize the infectious agents found inside its borders into distinct risk groups, and imply the regulations accordingly. Authorities from various countries, including the USA, Canada, Europe, and Australia, have published biosafety guidelines, and infectious agents grouping is mostly the same (Standards Australia [25]; American Biological Safety Association [26]; Public Health Agency of Canada [27]; Directive 2000/54/EC of European parliament [28]).

In India, all biosafety regulatory framework activities are governed by the Rules for the Manufacture, Use, Import, Export, and Storage of Hazardous Microorganisms/Genetically Engineered Organisms or Cells are advised by the Ministry of Environment, Forest and Climate Change [29], Government of India, under the Environment (Protection) Act, 1986 [30]. The institutional biosafety committee, which was constituted with the Department of Biotechnology's approval, plays an important role in the implementation of biosafety principles, rules, regulations, and guidelines. This committee provides the biosafety regulatory framework to the institution/scientists/industry for handling dangerous microorganisms, genetically engineered (GE) organisms, and products derived from GE organisms [31]. Table 2 shows that competent authorities assigned to implement the biosafety regulations under Rules 1989. The Genetic Engineering Appraisal Committee (GEAC), Review Committee on Genetic Manipulation (RCGM), and Institutional Biosafety Committee (IBSC) have approval and

Table 1
A list of GLPs principles

S. no.	Principles	Details
1.	Test facility, organization, and personnel	• Test facility should ensure safety while performing experiments. • Each experiment should have test and references; responsibility of each individual should be defined and staff should be trained properly. • Study plans and sites should be approved by the director and project investigator (PI), and SOPs should be followed strictly, along with cooperation/communication between officials and working staff.
2.	Quality assurance program	• The experiment should be conducted as per GLP guidelines. • Quality assurance personnel should prepare the records of experiment including all details, and communicate to management, PI, and the director. • Quality assurance personnel should prepare the final reports detailing all the inspections and meetings that have taken place.
3.	Facilities	• The best location to satisfy the study's requirements with the least amount of disturbance should be selected and facility should be constructed in accordance with GLP guidelines. • Proper facility for collection, storage, handling, disposal, and transportation should be arranged while adhering to GLPs.
4.	Apparatus, material, and reagents	• Proper maintenance, calibration on regular basis, and corresponding records should be followed. • SOPs should be maintained and followed for preparing reagents/chemicals, proper arrangement and records with detailed expiry date, safety and storage instructions.
5.	Test systems	• Appropriate equipment with capacity and quality required for the study should be used and maintained as per industry standards. • Before starting the experiment, procured animals/plants should go through biosecurity, quarantine, and acclimatization procedures and all records pertaining to their source, strain, dosage, cleaning, health, and so on should be maintained.
6.	Test and reference items	• Cross-contamination of test and reference products should be strictly avoided and tracking facility for the samples should be available. • Proper records for all the test and reference products should be maintained.
7.	Standard operating procedures (SOPs)	• SOPs should be written in accordance with the study objectives and approved by the test facility management committee. • SOPs should be designed in such a way that a technical person can perform the experiment with little or no guidance.
8.	Performance of the study	• The study should be performed strictly as per approved SOPs, and records and experimental details should be maintained.
9.	Reporting of study results	• PI should maintain the raw data and report the findings to the study director. • After statistical analysis of data, a report should be prepared, and

(continued)

Table 1
(continued)

S. no.	Principles	Details
		any changes in report should be approved by the study director/director and the PI.
10.	Storage and retention of records and materials	• Records should be properly documented, organized in a timely manner, stored and made available to competent authorities as and when needed with restricted access.

Table 2
Various competent authorities assigned for implementation of biosafety regulations

Competent authorities	Mandate and functions
Recombinant DNA Advisory Committee (RDAC)	RDAC comes under Department of Biotechnology (DBT). Its role is to review the advances in biotechnology at national and international levels and recommend appropriate and suitable safety regulations for India.
Institutional Biosafety Committee (IBSC)	IBSC regulates research activities related to hazardous and genetically engineered microorganisms in institute according to the guidelines/manuals of RCGM.
Review Committee on Genetic Manipulation (RCGM)	RCGM comes under DBT. It functions to monitor the safety-associated aspects in on-going activities and research projects encompassing hazardous microorganisms/genetically engineered organisms. The RCGM has important functions of preparing manuals of guidelines postulating procedure for supervisory process concerned to activities linking to GE organisms in research activity and its industrial applications for safety of personal, laboratory, and environmental.
Genetic Engineering Appraisal Committee (GEAC)	The GEAC comes under the MOEF & CC. It is accountable for approval of activities related with bulk scale use of recombinants and hazardous microorganisms in research and industrial production. It is also concerned in preparation of proposals related to release of GE organisms and products obtained from GE organisms.
State Biotechnology Coordination Committee (SBCC)	SBCC related with the control measures and safety in the various installations/institutions in given state involves in handling hazardous microorganisms/genetically engineered organisms.
District Level Committee (DLC)	DLC related with the control measures and safety in the various installations/institutions in given district involves in handling hazardous microorganisms/genetically engineered organisms.

regulatory functions, whereas the Recombinant DNA Advisory Committee (RDAC) has advisory functions. At the district and state levels, the District Level Committee (DLC) and the State Biotechnology Coordination Committee (SBCC) are in charge of

monitoring operations involving genetically modified organisms (GMOs), respectively [31].

5 Risk Groups, Biosafety Levels, and Physical Containment Levels

Pathogenic agents are classified into four risk groups (RG) based on a variety of factors such as pathogenicity, ease and mode of transmission, host range, and local control and prevention measures. Pathogenic agents of risk group 1 (RG1) pose a low risk to individuals and communities, and comprise microorganisms that are already present in the environment and are unlikely to infect healthy hosts.

RG2 agents pose a moderate risk, and while they can cause disease, they are unlikely or difficult to transmit. The infection caused by RG2 agents can be prevented or treated. RG3 agents are of high risk for individuals and can cause serious infections to laboratory workers. These agents, while posing a limited risk to the community, can pose a major threat if the pathogen escapes into the environment; nonetheless, effective preventive and treatment measures are available for RG3 agents. Furthermore, RG4 pathogenic agents pose a high risk to individuals and communities, and because they are easily transmissible, they can cause serious infections among laboratory workers. For these agents, there are usually no effective preventive or treatment options [32]. Figure 1 shows some examples of microorganisms belonging to various risk groups.

Biosafety levels (BSL) and physical containment levels (PCL) are established for each risk group to ensure worker safety and prevent the escape of pathogenic microorganisms. RG, BSL, and PCL are interconnected to each other (Fig. 2).

For each biosafety laboratory, a competent supervisory committee is assigned, and a range of primary barriers, such as gloves, protective equipment, biosafety cabinets, and other containment equipment, are installed between workers and infectious pathogens. The Lab Incharge is primarily responsible for the lab's safe operation and biosafety guidelines, and his judgment and recommendations are critical for assessing risks and handling a specific agent. Recommended BSL offers an environment in which an infectious agent can be handled/modified without the risk of it escaping. As per the U.S. Centers for Disease Control and Prevention (CDC), BSLs are classified into four categories: BSL-1, BSL-2, BSL-3, and BSL-4; characteristics, practices, safety equipment (primary barriers), and facilities (secondary barriers) are summarized in Table 3, along with the allowed risk group and physical containment levels [4, 33].

Four distinct types of animal biosafety levels (ABSLs) are necessary for handling of vertebrate animals exposed to agents that

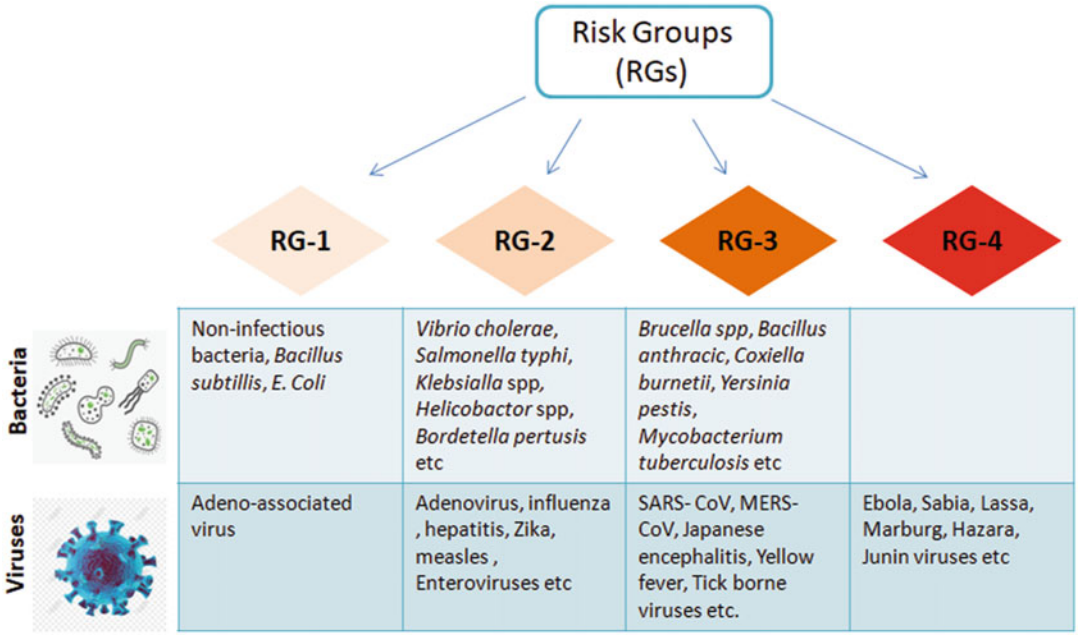


Fig. 1 Examples of biological agents from different risk groups

may infect humans, just like four types of BSL laboratories (required for microorganism handling). These ABSLs, which include ABSL-1, ABSL-2, ABSL-3, and ABSL-4, provide practices, equipment, and facilities that are comparable to the laboratory BSLs. The ABSL-1 facility is appropriate for working with well-characterized agents that are unlikely to cause adverse effects in humans and pose a low risk to the environment and personnel. ABSL-2 is suitable for working with experimental animals infected with human disease-causing agents and posing a modest hazard to the environment and personnel. ABSL-3 facility is suitable for working with experimental animals infected with aboriginal or exotic agents that can be transmitted through aerosols. Hazardous agents can induce serious or potentially deadly disease. Animals inoculated with a hazardous or exotic organism that can transmit via aerosol and poses a serious risk of death must be housed in an ABSL-4 facility. Attendants or animal care personnel essentially should have extensive and systematic training in handling of hazardous, infectious agents and inoculated animals [34, 35].

6 Working Safely in a Virology Laboratory

Different types of investigations are carried out in a basic virology research laboratory, including virus isolation, cell culture, DNA and RNA isolation, transfection, molecular biology and recombinant

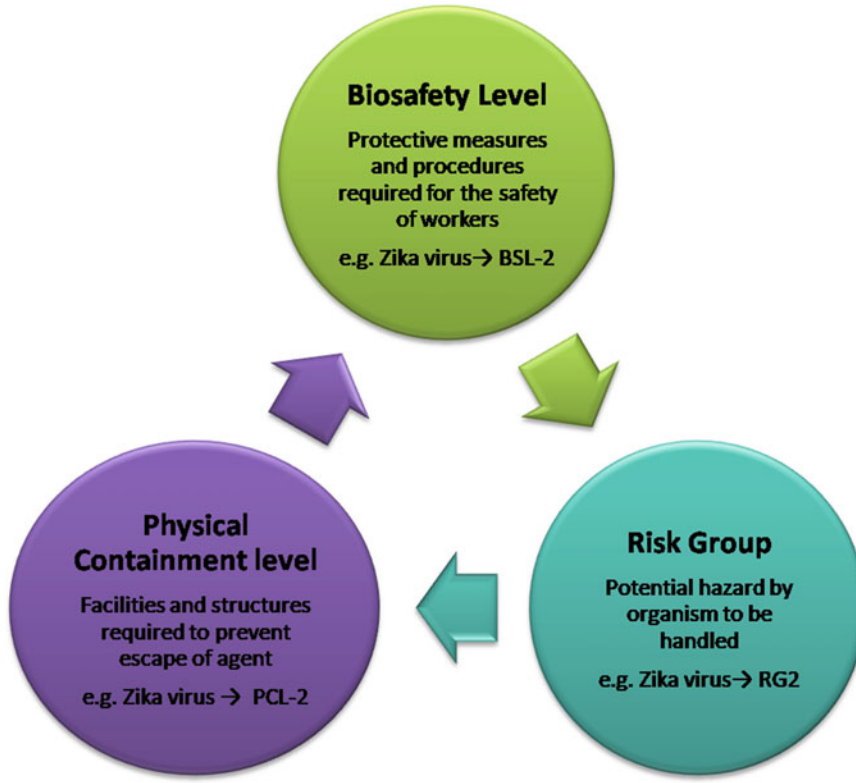


Fig. 2 Biosafety levels (BSL), physical containment levels (PCL), and risk groups (RG) of a particular infectious agent are interrelated

DNA techniques for genetic modifications, and so on. Some of the low to moderate risk viruses such as Adenovirus, Parvovirus, and Canine Distemper virus are utilized in research projects and graduate student dissertations, and require a Biosafety Cabinet-1 or Biosafety Cabinet-2 to handle them. Despite the fact that these viruses pose a low to moderate risk to healthy individuals with a low possibility of transmission, accidental contact with virus and viral genetic material may occur while performing experiments. Such mishaps could put laboratory workers' health at danger, and thus they must be avoided. A typical BSL-2 design is shown in Fig. 3.

While working in a virology laboratory, routine biosafety guidelines such as limited access to working areas, immediate and proper disinfection of work surfaces upon spills, no mouth pipetting, solid and liquid waste decontamination, proper disposal of waste and sharps, no eatables, smoking, cosmetics in laboratory areas, no food storage in laboratory refrigerators, washing hands after performing experiments, removing gloves and leaving laboratory, wearing appropriate protective equipments, minimization of aerosol generation while working with viable material, following proper guidelines while transferring or transporting the research

Table 3
Summary of biosafety levels for handling infectious biological agents (as per CDC guidelines)

	Description	Lab practices	Primary barriers	Secondary barriers	RG and PCL
BSL-1	For handling agents or toxins not known to cause disease to healthy individuals	• Standard microbiological practices • Work can be conducted on open working bench	• No special equipment required • Gloves, lab coat, etc. could be used as and when needed	• Work surface that can be easily disinfected • Open sink to wash hands	RG-1 PCL-1
BSL-2	For handling agents which pose a moderate health risk upon ingestion, injury, or exposure to mucous membrane	• Restricted access while work is in progress • Guidelines for sharps disposal, waste decontamination and limited biohazard signs	• Class I or II biosafety cabinets (BSCs) • Appropriate PPE is required • Face shield and eye protection gear required	• Open sink for washing hands and eyes • Autoclave to decontaminate the laboratory waste	RG-2 PCL-2
BSL-3	For handling agents which can cause lethal diseases, and are transmitted via aerosols, could be prevented or treated	• All BSL-2 practices • Decontamination of waste before disposal and clothing before laundry • Controlled access • Significant training of working individual	• All BSL-2 primary barriers • Respiratory protection is necessary	• Open and hands-free sink near exit • Self-closing, double door access, sealed windows • Direction flow of air from non-laboratory areas to laboratory areas • No recirculation of exhaust air	RG-3 PCL-3
BSL-4	For handling agents which can cause life-threatening disease conditions via aerosol transmitted lab escapes, no prevention or treatment therapy available	• All BSL-3 practices • Change of clothing before entering • Shower on exit • Each and every material needs to be decontaminated before exit • Immunizations of lab staff for the microbes they are working on (recommended)	• All work to be performed in BSC III or in BSC I or II while wearing full body protective gears with positive air pressure personnel suit	• All BSL-3 secondary barriers • Separated/isolated unit with dedicated supply, exhaust, vacuum and decontamination systems	RG-4 PCL-4

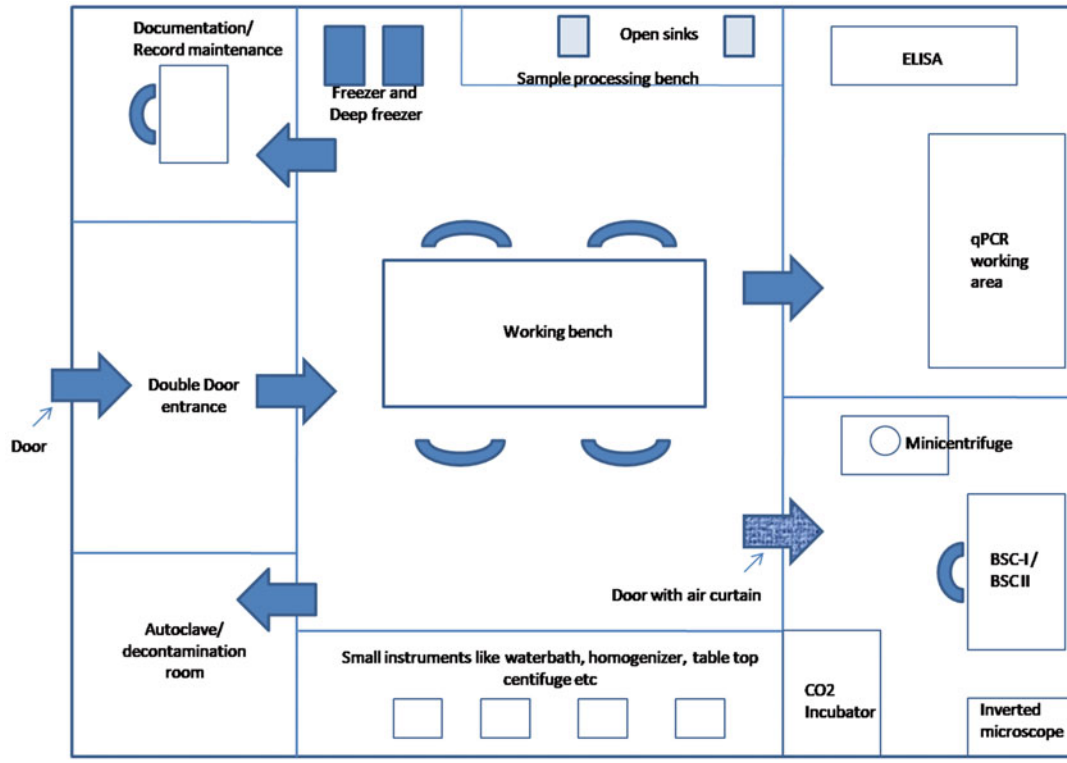


Fig. 3 Biosafety Level-II laboratory plan for a basic virology lab

items from one place to other, regular maintenance of records, and reporting of spills should be followed. The biological samples should only be handled by trained personnel. To avoid cross-contamination or unintentional thawing, which could lead to lab-acquired infections, storage of biological samples/viruses should be properly recorded and updated on a regular basis. Proper biohazard labels should be placed on storage units having potential pathogens and access should be limited to only those employees who are aware of and trained to handle these pathogens.

Even if all of these biosafety guidelines are followed, there is still a danger of mishaps or research interruptions, thus risk management strategies should be implemented immediately. Spills, exposures, power loss, fire, and other disruptions should be anticipated in advance, and an action plan of "what to do when" should be kept ready. Each floor should have its own fire safety devices and emergency exits. In the event of an accidental contact or inhalation of virus, first aid should be done immediately, followed by obtaining medical assistance. Every laboratory member should be informed of the risks posed by viruses being handled in the lab, as well as the minimal protection/response measures.

There is a long history of lab-acquired viral infections, some of which have been discussed previously. Sometimes infection

contracted from lab by an individual may not result in secondary infections, but if not reported promptly, it can cause anxiety among the entire research staff, chaos, wastage of time and money in tracing the possible contacts. For example, a scientist infected with the Sabia virus did not report the initial viral exposure and was only diagnosed after 5 days of illness, delaying the implementation of effective control measures. A total of 142 people were identified as potential contacts, with the bulk of them being laboratory workers. Although no secondary infections were identified, the fear and anxiety of contacts, the 6-days surveillance program, and cost of investigation by various agencies strongly suggest aggressive and compulsory reporting of mishaps/exposures in lab, immediate use of available medical facilities (identified in advance), and identification of surveillance or quarantine methods for such mishaps [36].

All the information on experiments, spills, and other minor to major incidents should be communicated to the lab supervisor. In most cases, poor communication is one of the main causes of laboratory mishaps. The lab incharge and biosafety officer should ensure that all staff and students receive mandatory training upon joining the facility, and that inspections are conducted on a regular basis to ensure that GLPs and biosafety guidelines are being followed effectively.

7 Conclusion

Because pathogens are emerging and re-emerging, research practices for prevention and treatment of these pathogens are in high demand, raising the risk of lab-acquired infections even with a minor negligence. Therefore, all laboratories should strictly adhere to GLPs and biosafety guidelines provided by their respective country's governmental authority. With the recent SARS-CoV2 pandemic, even the common person has realized the importance of proper disinfection and containment practices. One can assure safe working research practices with minimal risk of accidental infection spread by following guidelines, being aware of potential dangers, and utilizing our best judgment in the event of unanticipated mishaps.

References

1. Mourya DT, Yadav PD, Majumdar TD, Chauhan DS, Katoch VM (2014) Establishment of biosafety level-3 (BSL-3) laboratory: important criteria to consider while designing, constructing, commissioning & operating the facility in Indian setting. Indian J Med Res 140(2):171–183

2. Mohanty SK, Satapathy A, Naidu MM, Mukhopadhyay S, Sharma S, Barton LM et al (2020) Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) and coronavirus disease 19 (COVID-19)—anatomic pathology perspective on current knowledge. Diagn Pathol 15(1):1–17

3. OECD (1998) Principles on good laboratory practice. OECD. https://www.oecd.org/chemicalsafety/testing/good-laboratory-practiceglp.htm
4. Burnett LC, Lunn G, Coico R (2009) Biosafety: guidelines for working with pathogenic and infectious microorganisms. Curr Protoc Microbiol 13(1):1A.1
5. Furmanski M (2014) Laboratory escapes and "Self-fulfilling prophecy" epidemics. Center for Arms Control and Nonproliferation
6. Pike RM (1976) Laboratory-associated infections: summary and analysis of 3921 cases. Health Lab Sci 13(2):105–114
7. Weinstein RA, Singh K (2009) Laboratory-acquired infections. Clin Infect Dis 49(1): 142–147
8. Wurtz N, Papa A, Hukic M, Di Caro A, Leparc-Goffart I, Leroy E et al (2016) Survey of laboratory-acquired infections around the world in biosafety level 3 and 4 laboratories. Eur J Clin Microbiol Infect Dis 35(8): 1247–1258
9. Fenner F, Henderson DA, Arita I, Jezek Z, Ladnyi ID (1988) Smallpox and its eradication, vol 6. World Health Organization, Geneva. http://apps.who.int/iris/handle/10665/39485
10. Shooter RA (1980) Report of the investigation into the cause of the 1978 Birmingham smallpox occurrence. HMSO, London. https://www.nlm.nih.gov/nichsr/esmallpox/report_1978_london.pdf. Accessed Nov 2021
11. Zhang G, Shoham D, Gilichinsky D, Davydov S, Castello JD, Rogers SO (2006) Evidence of influenza A virus RNA in Siberian Lake ice. J Virol 80(24):12229–12235
12. Worobey M (2008) Phylogenetic evidence against evolutionary stasis and natural abiotic reservoirs of influenza A virus. J Virol 82(7): 3769–3774
13. Weaver SC, Pfeffer M, Marriott K, Kang W, Kinney RM (1999) Genetic evidence for the origins of Venezuelan equine encephalitis virus subtype IAB outbreaks. Am J Tropic Med Hygiene 60(3):441–448
14. Abraham T (2007) Twenty-first century plague: the story of SARS. JHU Press
15. Lim PL, Kurup A, Gopalakrishna G, Chan KP, Wong CW, Ng LC et al (2004) Laboratory-acquired severe acute respiratory syndrome. N Engl J Med 350(17):1740–1745
16. Shen Z, Ning F, Zhou W, He X, Lin C, Chin DP et al (2004) Superspreading sars events, Beijing, 2003. Emerg Infect Dis 10(2): 256–260
17. WHO Global Alert and Response (GAR) (2004) China reports additional SARS cases—update 23 April 2004. http://www.who.int/csr/don/2004_04_23/en/index.html
18. WHO Global Alert and Response (GAR) (2003) Severe acute respiratory syndrome (SARS) in Taiwan, China. http://www.who.int/csr/don/2003_12_17/en/. Accessed 17 Dec 2003
19. Baldeshwiler AM (2003) History of FDA good laboratory practices. The Quality Assurance Journal: The Quality Assurance Journal for Pharmaceutical, Health and Environmental Professionals 7(3):157–161
20. Alatgi AC, Chougule SB (2015) Good laboratory practice. J Evol Med Dent Sci 4(103): 16901–16906
21. Turnheim D (1994) The OECD policy for the implementation of the principles of good laboratory practice. Annali-Istituto Superiore Di Sanita 30:395–395
22. Cavero I (2009) Exploratory safety pharmacology: a new safety paradigm to de-risk drug candidates prior to selection for regulatory science investigations. Expert Opin Drug Saf 8(6):627–647
23. Akyar I (2011) GLP: good laboratory practice. Modern Appr Quality Control:37–56
24. Nahler G (2009) Good laboratory practice (GLP). In: Dictionary of pharmaceutical medicine. Springer, Vienna, p 82
25. Standards Australia (2010) Safety in lab part 3: microbiological safety and containment. Standards Australia. AS/NZS, Sydney, pp 22433–22010
26. American Biological Safety Association. Risk group database. https://my.absa.org/Riskgroups. Accessed Aug 2021
27. Public Health Agency of Canada. Pathogen safety data sheets and risk assessments. https://www.canada.ca/en/public-health/services/laboratory-biosafety-biosecurity/pathogen-safety-data-sheets-risk-assessment.html. Accessed Aug 2021
28. Directive 2000/54/EC of the European parliament. https://eur-lex.europa.eu/eli/dir/2000/54/oj. Accessed Aug 2021
29. MoEF & CC (1989) Ministry of Environment and Forests, Notification, New Delhi, the 5th December, 1989. https://www.indiacode.nic.in/bitstream/123456789/4316/1/ep_act_1986.pdf. Accessed Nov 2021
30. EPA (1986) The Environment (Protection) Act, 1989 (Act No. 29 of 1986). https://www.indiacode.nic.in/bitstream/123456789/4316/1/ep_act_1986.pdf. Accessed Nov 2021

31. Department of Biotechnology (ed) (2020) Handbook for institutional biosafety committees (IBSCs), 3rd edn. Department of Biotechnology. https://ibkp.dbtindia.gov.in/Content/FlashPDF/IBSC%20Handbook.pdf
32. Kaufer AM, Theis T, Lau KA, Gray JL, Rawlinson WD (2020) Laboratory biosafety measures involving SARS-CoV-2 and the classification as a Risk Group 3 biological agent. Pathology 52(7):790–795
33. Department of Biotechnology (2017) Regulations and guidelines for recombinant DNA research and biocontainment. Department of Biotechnology. https://rcb.res.in/upload/Biosafety_Guidelines.pdf
34. World Health Organization (2004) Laboratory biosafety manual, 3rd edn. WHO. https://www.who.int/publications/i/item/9241546506
35. Sewell DL (1995) Laboratory-associated infections and biosafety. Clin Microbiol Rev 8(3):389–405
36. Kortepeter MG, Martin JW, Rusnak JM, Cieslak TJ, Warfield KL, Anderson EL, Ranadive MV (2008) Managing potential laboratory exposure to Ebola virus by using a patient biocontainment care unit. Emerg Infect Dis 14(6):881–887

INDEX

Rajib Deb et al. (eds.), *Protocols for the Diagnosis of Pig Viral Diseases*,
Springer Protocols Handbooks, https://doi.org/10.1007/978-1-0716-2043-4,

G

H

I

L

M

N

O

P

Q

R

S

T

V

W