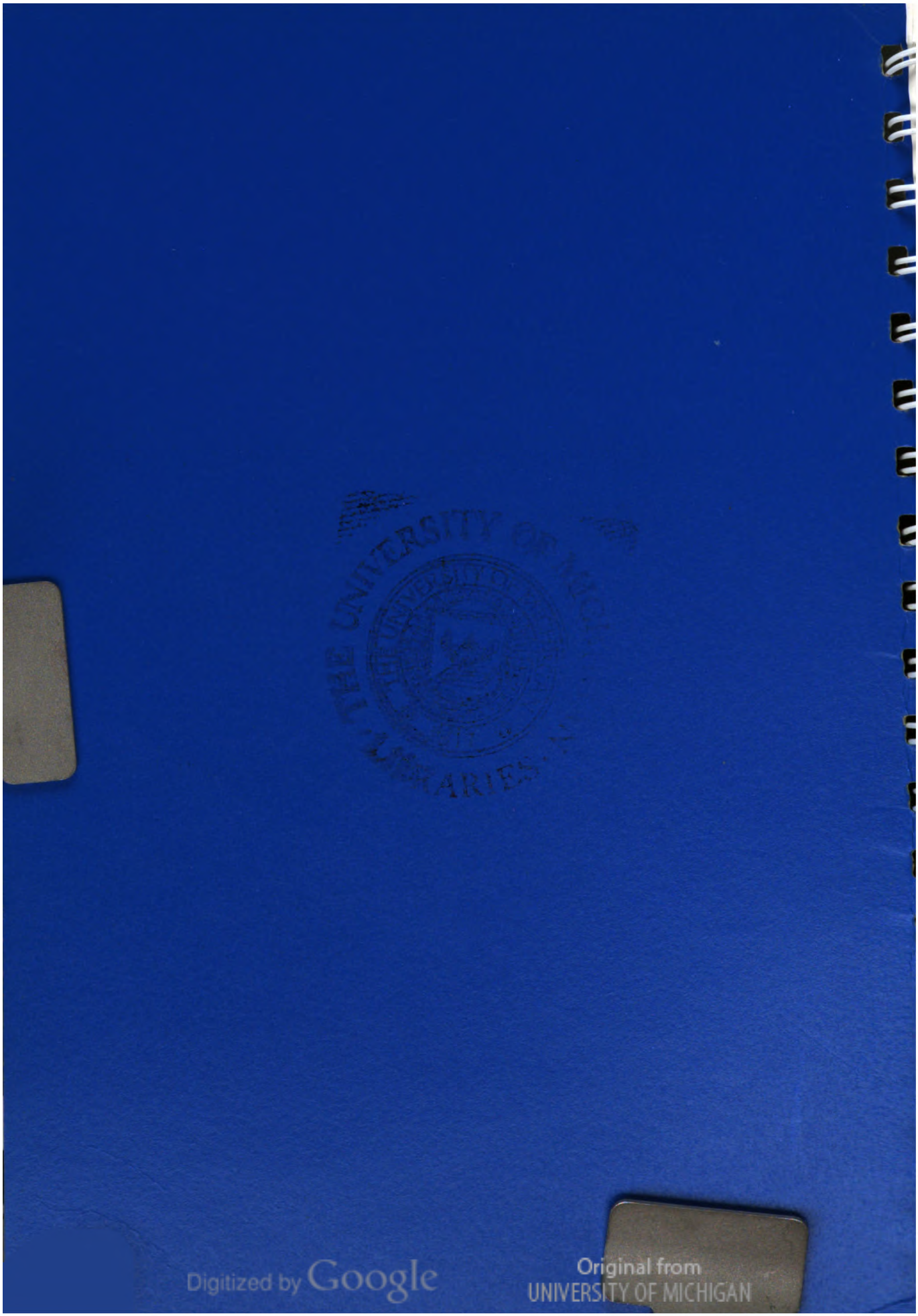
Armed Forces Institute of Pathology

Advanced Laboratory Methods in Histology and Pathology

Ulrika V. Mikel, Editor

Armed Forces Institute of Pathology
American Registry of Pathology
Washington, DC



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Preface

This book has been written for pathologists, medical researchers, and laboratory technicians. It covers specialized techniques in pathology, some of which are relatively new and therefore can be considered nonroutine in the average laboratory. The book is divided into seven chapters; each chapter covers one particular subject and its use in pathology. The techniques described and discussed are procedures in immunohistochemistry and in situ hybridization, the polymerase chain reaction, preparatory techniques for flow cytometry, quantitative staining methods for image cytometry, skeletal muscle enzyme histochemistry, and preparatory methods in cytopathology. The methods are presented in detail, with an introduction to and background material on each subject. Principles of the techniques and other useful information are also included.

During the last decades, exciting events in biochemistry and molecular biology have taken place that have had an impact on pathologic diagnosis and prognosis of disease. This book combines these new techniques, together with others that have been around for some time, under one cover. Because the subjects covered are continuously developing, the progress within each field should be followed closely and users should update, simplify, and modify procedures as advances occur. This is especially important in the field of molecular biology,

This book has been written for the experienced technician. The procedures are detailed, work well, and are effectively used at the Armed Forces Institute of Pathology (AFIP). They have been published elsewhere and are basically standard. When possible, our laboratories have modified and simplified them. We aim for the best results in the simplest and most direct way. Troubleshooting and problem-solving advice is included.

The manufacturers of the compounds listed under the "Materials and Solutions" section of each chapter are usually stated. This does not mean the authors endorse these particular products, only that we have found them to work well. As a rule, products from other manufacturers will work just as well.

A chapter on preparatory techniques in cytology has been included. We believe this information will be useful in laboratories where histotechnologists assist with cytologic preparations. It is a condensed version of preparatory techniques in cytology and is not intended for cytotechnologists. The procedures are described in enough detail, however, to prove useful to the experienced technician.

I gratefully acknowledge the help and advice I have received from Dr. Timothy J. O'Leary, Chairman, Department of Cellular Pathology, Dr. Robert L. Becker, Jr., Chief, Division of Quantitative Pathology, and Dr. Leslie H. Sobin, Associate

Director, Center for Scientific Publications, AFIP. Their support and suggestions are much appreciated.

Very special thanks go to Fran Card, for layout and organization of the text and the illustrations; to JoAnn Mills, for editing the manuscript; to Cathy Hemelt, for her photographic assistance; to Ken Stringfellow, for processing the transparencies for printing; and to Lena Hohmann, for her technical support and assistance in the development of the two chapters "Preparation of Nuclei for Flow Cytometry" and "Quantitative Techniques for Image Cytometry."

Ulrika V. Mikel

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Immunohistochemistry: *Antigen Detection in Tissue*

Gary L. Bratthauer and Lila R. Adams

INTRODUCTION

Immunohistochemistry involves the detection of specific chemical substances within tissue by the use of derived antibodies to the substances. Antibodies are applied to tissue sections and allowed to bind to their corresponding antigen. A detection system is then employed to identify the location of these antibodies using marker molecules that can be visually recorded.

Antibodies are produced from the introduction of a specific chemical substance to the immune system of an unrelated species. The immune system has the innate ability to recognize, via specific receptor molecules, virtually any combination of amino acids, carbohydrates, or lipids and respond. This recognition is dependent on many factors, one of which is size. A molecule of several hundred daltons is required to initiate receptor recognition and immune response. This molecule is called the antigen. Many proteins are large enough to elicit an immune response and therefore are antigenic. Many other molecules, or small proteins, are not and must first be attached to a larger molecule. These small molecules can then be recognized by the immune system and are called haptens. When a foreign molecule is introduced to the body, it is recognized as such in conjunction with the particular human leukocyte antigen (HLA) receptors on macrophages. The macrophage digests the molecule and presents certain combinations of external groups of atoms called epitopes on the surface of the cell. The epitope is then brought into contact with helper T-cell lymphocytes, which help present the epitope to B-cell lymphocytes. The B cells synthesize immunoglobulin protein

chains with the ability to bind specifically with the epitope. The cell makes a light chain that is 30 daltons in size and of either the lambda or the kappa type, which is based on molecular structure. Two light chains combine with two heavy chains, measuring 60 daltons in size, of the mu, gamma, delta, alpha, or epsilon types of molecular structure to form the immunoglobulin molecule. In response to antigenic stimulus, this molecule is called an antibody. The antibody site reacting specifically with an epitope is called the idiotype. In response to stimulus, the B cell transforms to a plasma cell and secretes large quantities of the antibody into circulation. When these molecules are harvested, they can be concentrated to react specifically with the antigen causing the response, or immunogen. Each antigenic substance has many epitopic sites, also called determinants, capable of combination with antibody. In vivo, the response to an antigen will be broad, and the resulting set of antibodies reactive with the determinants on the antigen is called a polyclonal antiserum. Monoclonal antibodies are produced by fusing one particular line of B cells exhibiting a unique idiotype with an immortal transformed plasma cell that consistently produces a certain immunoglobulin. The result is an antibody with a specific idiotype that is constantly produced from an immortal transformed hybrid cell.

Whether monoclonal or polyclonal antibodies are employed, the presence of the specific chemical antigen in tissue can be determined by allowing the antibodies to bind and then locating them with a detection system designed to identify the particular species of immunoglobulin antibody. The techniques for doing so are numerous, but the overall technology is essentially the same. An antibody to the substance to be examined is used, followed by a detection system identifying the immunoglobulin of the species from which the antibody was obtained, and then allowing the reporter molecule of the detection system (enzyme, fluorochrome, gold particle, etc.) to react with its respective visually detectable substrate. These identifiable substrates are permanent enough to offer a documentation of localization that can be seen through the microscope at the cellular level. In this way, individual cells or groups of cells can be monitored for the presence of the specific analyte, directly.

Using antibodies to identify specific substances in human tissues has emerged as a powerful new tool in pathology. Whereas, in the past, anatomic pathologists observing diseased tissue have had to rely on morphological characteristics highlighted through the use of special dyes, immunohistochemical technology makes it possible to visualize a specific substance and identify its location. The histogenesis of tumors can be more easily determined through the identification of tissue- or organ-specific molecules, and the recent introduction of several proliferation-associated markers has allowed for prognostic information to be derived as well.

This chapter will deal with the technique and also the technology of this assay system as it is applied at the Armed Forces Institute of Pathology (AFIP). The details of methodology, the interpretation of the results, problem-solving ideas, and the requirements of a large-volume laboratory will be addressed.

AVIDIN-BIOTIN COMPLEX METHOD FOR ANTIGEN DETECTION

PRINCIPLE:

Immunohistochemical assays identify specific substances in tissue using derived antibodies and a detection system that allows visual identification. The identification of specific compounds enables the physician to determine more accurately the histogenesis of particular lesions and possibly provides prognostic information about them as well.

Antibodies are raised against antigenic determinants present on the compound of interest. These antibodies are allowed to specifically combine with the compound in tissue and are detected by the use of other antibodies that are designed to recognize the immunoglobulin from the species exposed to the original compound. These detection antibodies (anti-antibodies from other species) are tagged with some reporter molecule such as fluorescein or an enzyme that can catalyze a further reaction towards a visible product.

At the AFIP, the avidin-biotin complex (ABC) detection system is used on specimens of paraffin-embedded tissue, 6 microns thick. The specific antibody to the compound of interest is allowed to combine with the antigen and is detected with an anti-antibody produced in another species that recognizes the first antibody as an antigen. This "secondary" antibody has biotin molecules attached to it, enabling further detection with the protein avidin. Biotin, a vitamin, is relatively small, and attachment to the chains of the immunoglobulin does not interfere with its ability to recognize the "primary" antibody. Avidin, a protein, has such a high affinity for the biotin molecule that the combination is virtually irreversible. Each avidin molecule has four combination sites for biotin, and each biotin molecule has two combination sites for avidin. The horseradish peroxidase (HRP) molecule is the reporter molecule in use at the AFIP. This enzyme is bound by biotin and incorporated in an avidin-biotin complex in such a way that, when it is brought in proximity to the secondary biotinylated anti-antibody, the complex binds through a remaining biotin-binding site on one of the avidin molecules. This essentially provides enzyme at the site of the original primary antibody-antigen interaction. The enzyme then, acting on hydrogen peroxide, promotes the transfer of electrons from a chromogenic compound that precipitates as an insoluble pigment. At the AFIP, the chromogen in use is 3,3'-diaminobenzidine tetrahydrochloride (DAB). This molecule is essentially colorless in solution at dilute concentrations and precipitates dark brown during the oxidation. The precipitate is insoluble in alcohol, allowing the sections to be counterstained with hematoxylin, dehydrated with ethanol, cleared with xylene, and coverslipped.

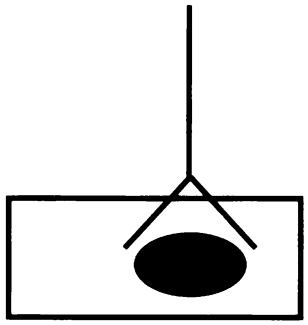
For example, suppose a liver biopsy specimen was suspected of harboring the hepatitis B virus. A rabbit immunized with hepatitis B virus would produce rabbit anti-hepatitis B virus antibodies. Incubation of this antiserum (primary antibody) with the liver biopsy specimen at proper dilution and pH would result in the antibodies binding with the virus if it is present in the specimen. The second stage incubation occurs with a specific secondary antiserum. In this case, commercially prepared goat anti-rabbit immunoglobulins (secondary antibody) are used, being derived from the immunization of goats with rabbit immunoglobulin molecules. These goat antibodies have been prepared with attached biotin molecules that are used in the detection process. Incubation with the biopsy specimen will result in these antibodies binding to the primary antiserum immunoglobulins wherever they are located in the specimen. The third stage in the process involves the ABC, which is prepared from commercially available avidin, and biotin attached to the HRP enzyme. In combination, the complex builds to form many avidin molecules attached to molecules of biotin linked to enzyme. This ABC, when applied to the biopsy specimen, attaches to the biotin molecules present on the secondary antibodies. Thus, the HRP enzyme is essentially linked to the location of the hepatitis B virus wherever it exists in the biopsy specimen. In order to determine virus localization, the enzyme is allowed to catalyze the oxidation reaction with hydrogen peroxide and DAB. This results in the DAB precipitating brown in close proximity to the peroxidase enzyme.

$$HRP + H_2O_2 = HRPH_2O_2 + DAB = DABpolymer + H_2O + HRP$$

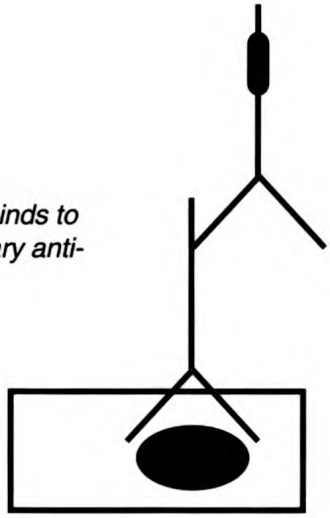
This brown precipitate is insoluble and permanent. The specimen may be counterstained to determine morphological parameters of interest and permanently coverslipped in order to view the DAB precipitate with the use of the light microscope. The location of the virus, if present, will be identified by the observation of DAB precipitate.

To better visualize these essential three steps in the procedure, a diagrammatic representation of antigen identification follows.

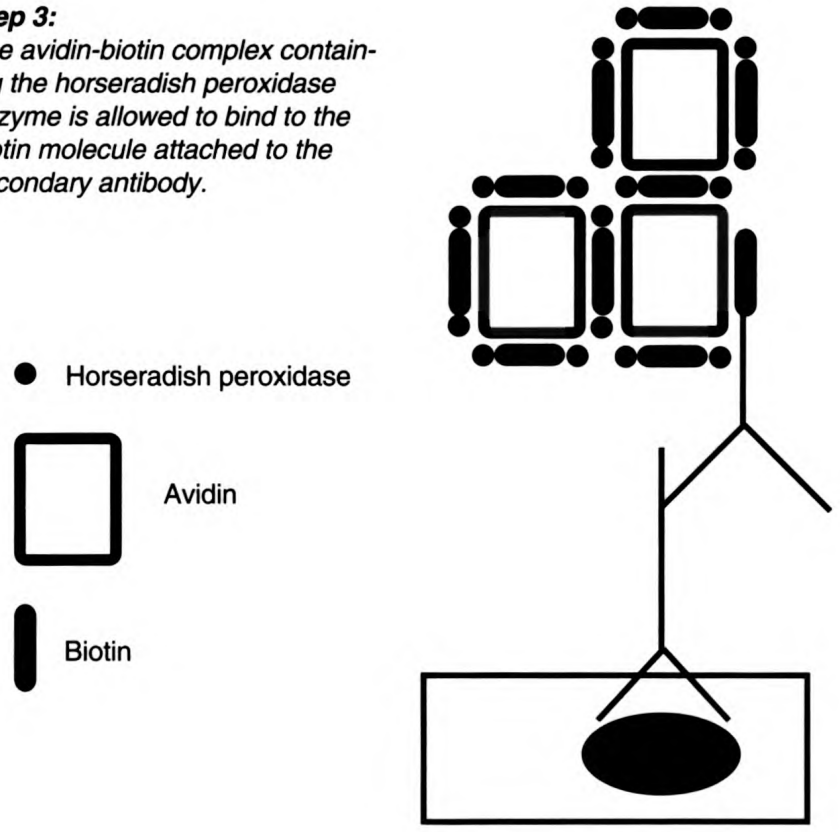
Step 1:
The primary antibody binds to the corresponding antigen in the tissue section.



Step 2:
The secondary antibody binds to determinants on the primary antibody.



Step 3:
The avidin-biotin complex containing the horseradish peroxidase enzyme is allowed to bind to the biotin molecule attached to the secondary antibody.



MATERIALS AND SOLUTIONS:

Water bath
Oven at 60°C
Magnetic stir plates and stirring bars to agitate solutions
Humidity chambers with level staining trays
Microliter pipettes
45-liter carboy
Gloves
Sodium hydroxide (NaOH) 10N
Hydrochloric acid (HCl) Conc.
Ethanol: 100%
Xylene
Permount (Fisher Scientific)

0.01M phosphate buffered saline (PBS), pH 7.40 +/- 0.05

Sodium chloride (NaCl) 0.89% ..... 400.5 g
Sodium phosphate (NaH_2PO_4) ..... 54.0 g

Place in a 4-liter flask. Fill flask with deionized water to 3 liters. Mix on a stirring plate until completely dissolved. Using NaOH, adjust to a pH of 6.80. Pour the solution into a 45-liter carboy and bring to 45 liters with deionized water. Mix thoroughly; adjust pH with HCl or NaOH to 7.40 +/- 0.05.

0.1M phosphate buffer, pH 7.8

Sodium phosphate ($NaH_2PO_4.H_2O$) ..... 52.4 g
Deionized water to a final volume of ..... 3.8 L

Place in a 4-liter flask. Mix on a stirring plate until completely dissolved. Adjust pH to 7.80 using NaOH or HCl.

Proteolytic digestion solution

0.1M phosphate buffer ..... 500 ml
Protease VIII (Sigma, St. Louis) ..... 0.25 g

Mix well and place in a 37°C water bath. Solution is ready to use when it reaches 37°C.

Endogenous oxidation blocking solution

30% hydrogen peroxide (H_2O_2) ..... 52.6 ml
Methanol ..... 1-pint bottle (approximately 473 ml)

This solution should be used as soon as possible.

Caution: 30% hydrogen peroxide is a very strong oxidant and will burn. It is advisable to wear gloves when handling and avoid skin contact.

10% Normal serum

Deactivated serum .....50 ml
PBS .....450 ml

Deactivate the serum by placing it in a 56°C water bath for 30 minutes. The serum is obtained from the same species as the biotinylated secondary antibody. EXAMPLE: If the secondary antibody is horse anti-mouse IgG, then 10% horse serum should be used.

Primary antibody

The primary antibody is chosen specifically for the detection of a desired antigen. This product is usually purchased commercially and diluted with PBS. Titrations should be performed to determine the proper dilution of concentrated antibodies.

Secondary antibody

The secondary antibody, conjugated with biotin, should be directed against the animal that produced the primary antibody. EXAMPLE: If the primary antibody was produced by a mouse, then a biotinylated horse anti-mouse IgG can be used. Titrations should again be done to determine the proper dilution, with PBS used as the diluent.

Avidin-biotin complex (ABC) reagent

The reagent is purchased commercially (Vector Laboratories, Burlingame, CA) and prepared according to the manufacturer's specification.

0.016% Diaminobenzidine tetrahydrochloride (DAB) chromogen solution

DAB (Sigma, St. Louis) .....80 mg
PBS .....500 ml
30% hydrogen peroxide (H_2O_2) .....400 µl

Fill the staining dish with PBS, add the DAB, and mix until DAB is dissolved. Then add 400 µl of 30% H_2O_2 to this solution. The solution should be made immediately before use and have only a slight tint when compared to normal PBS. When the solution becomes light brown to reddish brown it should be discarded. **Caution: DAB is a suspected carcinogen and should be considered a hazardous material. DAB should be used under a fume hood. The technician should wear gloves, safety glasses, and a protective covering. As much as possible, limit exposure to the powder form.** This laboratory stores

2% DAB in PBS in 4-ml aliquots at -70°C. One frozen vial is used for 500 ml of chromogen solution.

Gill's hematoxylin

Commercially prepared Gill's hematoxylin (Fisher Scientific) is used mostly for convenience and its long shelf life. Other hematoxylins or stains can be substituted for Gill's provided they produce a light counter-stain.

Ammonia water

Conc. ammonia ..... 10 ml
Deionized water ..... 500 ml

Specimen

The specimen of choice is formalin-fixed, paraffin-embedded tissue sectioned at 6 microns and mounted on positively-charged glass slides or slides coated with poly-L-lysine, silane (see pages 44 and 60), or 15% Elmer's glue diluted in water. While other specimens are acceptable, i.e., frozen sections or specimens fixed in an alternate fixative, slight changes will have to be made to the procedure to achieve desired results. Some specimens may not be suitable for a given analyte, but only by testing can this determination be made. It is imperative to always perform a negative control with each specimen and to understand that a negative result only indicates an inability to *detect* the substance analyzed.

PROCEDURE:

1. Position the slides in a staining rack and heat in an oven at 60°C for 30 minutes in order to properly anneal the section to the glass slide.
2. Place the rack of slides in the first staining dish of xylene immediately after taking them from the oven (see Note 1). Continue to deparaffinize with 4 changes of fresh xylene, allowing the slides to remain in the xylene dishes for 5 minutes each.
3. Place in 100% ethanol for 4 changes, 3 minutes each. For sections that do not require digestion go directly to Step 7.
4. Rinse sections requiring digestion in deionized water for 3 minutes.
5. Incubate sections in proteolytic digestion solution at 37°C for 1 to 30 minutes. The standard time for digestion is 3 minutes.
6. Rinse sections in 2 changes of deionized water for 2 minutes each, then 3 changes of 100% ethanol for 3 minutes each.

7. Place all sections in endogenous oxidation blocking solution for 30 minutes.
8. Rinse sections 3 times with deionized water for a total time of 10 minutes.
9. Place sections in 10% normal serum solution at 4°C overnight to reduce nonimmunologic binding of antibody. Under rare circumstances, it may be necessary to decrease the serum blocking time. This is accomplished by incubating sections in 10% normal serum at room temperature for 45 minutes.
10. Place slides horizontally on leveled bars in a humidity chamber (Fig. 1-1). Distribute slides according to the primary antibody that is to be applied. Care should be taken so that the sections do not touch during incubation. Separation of slides into groups will help preclude any chance of contaminating a section with the wrong antibody. Gently shake off excess normal serum and cover sections with 3 to 4 drops of primary antibody (Fig. 1-2). Incubate for 30 minutes at room temperature, making sure the lid to the humidity chamber is on to prevent air-drying.
11. Wash sections with PBS 3 times and gently shake off excess (Fig. 1-3).
12. Flood sections twice with 10% normal serum for 10 minutes each.
13. Wash sections with PBS 3 times and gently shake off excess.
14. Cover sections with 3 to 4 drops of biotinylated secondary antibody for 30 minutes at room temperature. Secure the lid to the humidity chamber to maintain a moist environment.
15. Wash sections 3 times with PBS and gently shake off excess.
16. Cover sections with 3 to 4 drops of ABC reagent for 30 minutes at room temperature. Make sure the lid to the humidity chamber is on to prevent air-drying of sections.
17. Wash sections 3 times with PBS; place sections once again in staining racks for a final 10-minute soak in PBS.
18. Develop in 0.016% DAB chromogen solution for 10 to 25 minutes at room temperature. The usual time for optimal development of DAB is 15 minutes, but this may vary with different lots of DAB (Fig. 1-4).
    Special precautions should be taken when handling DAB because of its possible carcinogenic properties. The solution should be kept under a safety hood. The technician should wear gloves and protective coverings when handling the DAB solution.
19. Wash sections 3 times with deionized water for a total time of 5 minutes.
20. Counterstain with Gill's hematoxylin for 3 to 30 seconds, depending on the concentration of the solution.
21. "Blue" sections with ammonia water for 30 seconds.

22. Wash in deionized water for 5 minutes.
23. Dehydrate using 4 changes of 100% ethanol, 3 minutes each.
24. Clear with 4 changes of xylene, 3 minutes each.
25. Coverslip sections with Permount.

Positive antibody/antigen reaction is dark brown, with the background a light blue.

NOTES:

1. Care should be taken when transferring the slides from the oven to xylene. The sections should still be warm but cool enough that the tissue won't float off the slide when placed into the xylene. When rehydrating numerous slides, allow extra incubation time in xylene. This is especially true for the first dish because the majority of the paraffin will be dissolved in this step. It is important to effectively remove all of the paraffin because paraffin can mask epitopes from the primary antibody, creating a muddy appearance or a false-negative reaction.
2. It is important to insure that the humidity chamber staining rack is level before placing the slides on it for primary antibody incubation. If there is a problem with the level of the rack, the immunoglobulins may not have enough time to effectively bind the target.
3. There are several factors that were considered when preparing the endogenous oxidation blocking solution. Methanol was chosen as a base solution because it appears to be stable for a longer period of time, when compared to water or PBS. ✋ **Caution should be used when handling 30% hydrogen peroxide. The 30% hydrogen peroxide reagent is a caustic compound and can cause severe burns. Wear appropriate protective clothing, including gloves, and bring spills to the attention of the supervisor.**
4. Throughout the procedure, keep drying of the sections to a minimum in order to prevent destroying epitopes on the cell membrane. Drying will also cause nonspecific background staining.
5. **Special precautions should be taken when handling DAB because of its possible carcinogenic properties: gloves, organic ventilators, and protective clothing should be worn. All procedures using DAB should be performed under a safety hood.**
6. This procedure can be adapted for just a few slides. Make 50 ml of digestion and blocking solutions in small Coplin jars. Additionally, only small quantities of antibody reagents and ABC have to be prepared.

ADDITIONAL SAFETY CONCERNS:

1. Treat all biological materials, including lyophilized materials, as potentially hazardous.
2. All hazardous materials contained in glass bottles should be carried in a polypropylene bottle carrier.
3. Hands should be washed with a bacteriostatic soap as soon as practical following contact with human unfixed tissue, serum, urine, or other biological fluids.
4. Spills should be promptly flooded with a disinfectant and cleaned up.
5. All biological hazards and anything that comes in contact with them should be disposed of in biohazard bags.
6. Biohazard bags should be sealed with special autoclavable seals, labeled with autoclave tape, and autoclaved at 20 pounds pressure at 120°C for not less than 1 hour before final disposal.
7. Controlled substances should be kept under lock and key.
8. All flammables are to be kept in a closed fire cabinet.
9. Hazardous materials should be handled as previously described.
10. To avoid contact with DAB, the following procedure can be used.
    a. Wearing gloves, goggles, protective covering, and an organic filtered mask, dissolve DAB powder (5-g vial) in manufacturer's glass container with PBS in the chemical safety hood.
    b. Pour solution into a 250-ml volumetric flask and bring to 250 ml with PBS.
    c. Immerse a magnetic stir bar and stopper the flask. Stir solution until completely dissolved.
    d. Aliquot 4 ml into 1-dram glass vials and freeze at -70°C.
    e. Extreme care should be used when making any solutions of DAB. Stock solution can vary from dark brown to purple.

PEROXIDASE-ANTIPEROXIDASE METHOD FOR ANTIGEN DETECTION

PRINCIPLE:

The avidin-biotin system can at times lead to erroneous results. This often occurs when dealing with poorly fixed samples or frozen sections with viable endogenous biotin. It is better in these instances to change the method and abandon the need for biotin. One of the most successful is the completely immunologic peroxidase-antiperoxidase (PAP) technique. Of course, the enzyme of choice is subject to preference. In this technique, a linking or bridge antibody is used to bind both the primary antibody and a complex made up of antibodies directed against horseradish peroxidase derived from the same species as that responsible for primary antibody production, and the HRP enzyme, in a soluble immune complex.

PROCEDURE:

Steps 1 to 13, follow the ABC procedure (see pages 8 and 9), then proceed as below.

14. Apply swine anti-rabbit immunoglobulins to the sections (in the same manner described in Step 14 of the ABC procedure) for 30 minutes.
15. Rinse the sections in PBS thoroughly, allowing buffer to drain off the slide. Repeat three times in rapid succession. It may be beneficial to allow the excess buffer to remain on the section for a few minutes to completely allow the excess immunoglobulin to diffuse off the section before draining.
16. Decant excess buffer and add the horseradish peroxidase-rabbit anti-horseradish peroxidase complex. This immune complex is soluble and obtained already prepared. Work quickly to avoid drying of the section. Allow to incubate for 30 minutes.
17. Continue with the ABC procedure, Step 17, from this point on.

NOTE:

The swine anti-rabbit immunoglobulin will bind both the primary rabbit antibody and the rabbit antibody bound to the peroxidase enzyme (with the remaining available site), but only if it is in molar excess. Otherwise, there will not be enough antigen-binding region available to the PAP antibody complex. It should be noted that the serum used in this example to achieve blockage of non-specific binding would have to be normal swine serum, 10% in PBS. Also, pH plays a larger role in assays of this type, due to the complete immunologic nature of the binding. Diligence is essential to assure that all buffers are physiologic, including that used for the preparation of the DAB.

ALTERNATIVES

AEC CHROMOGEN

There may be instances when the use of another procedure is necessary to bring about the desired effect. For instance, if there is an excess of pigment, such as melanin or hemosiderin, in the tissue section, the use of a different chromogenic compound other than DAB is often beneficial. There are several chromogens that can be used that precipitate as a color other than brown upon exposure to peroxidase and hydrogen peroxide. One such chromogen is 3-amino, 9-ethyl carbazole (AEC), which precipitates red in color. This chromogen is soluble in alcohol, so specimens cannot be dehydrated in the usual fashion but must instead be air-dried and covered with Crystal/Mount (Biomeda Corp., Foster City, CA). Crystal/Mount dries to a solid plasticlike coating over the section and removes the need for alcohol dehydration and xylene clearing.

AEC Preparation

(Prepared according to manufacturer's instructions [Biomeda Corp.])

1. Place 5 ml of deionized H_2O in a 15-ml test tube.
2. Add 2 drops of Solution A (concentrated buffer) to test tube and mix well.
3. Add 1 drop of Solution B (AEC concentrate) to test tube and mix well.
4. Add 1 drop of Solution C (H_2O_2) to test tube and vortex.

PROCEDURE:

Follow the ABC procedure through Step 17 (see pages 8 and 9).

18. Place slides in a humidity chamber that has been heated to approximately 50°C.
19. Drop the AEC solution on the sections and incubate for 7 minutes.
20. Drain off heated AEC from sections. Reapply fresh AEC solution to sections and incubate for an additional 7 minutes.
21. Wash sections with deionized water and counterstain with hematoxylin for 3 to 30 seconds.
22. Rinse with deionized water, tap off excess, and dry around tissue section with gauze.
23. Apply Crystal/Mount and bake flat in a 60°C oven until Crystal/Mount is set and all water has evaporated.

24. Sections can be reviewed at this time and/or coverslipped with permount.

Positive precipitation appears bright red in color, with the nuclei staining blue.

NOTE:

This solution should be used as soon as possible to prevent precipitation of the AEC. The red precipitate is soluble in alcohol. The sections cannot be dehydrated organically. Permount is the only permanent mounting medium that should be used, since others can dissolve the precipitate. Permount can only be applied, however, after the section has been protected by the use of Crystal/Mount. Although no further preparation is needed following Crystal/Mount baking, it should be noted that Permount and a coverslip will prevent scratches or alterations to the surface of the dried Crystal/Mount which can lead to problems with section interpretation.

ADDITIONAL ALTERNATIVES

Other alterations to the color utilized with these reporter system applications can be achieved if desired. DAB can precipitate black in color by the addition of metal ions to the developing solution. DAB should be prepared as above with the addition of 1 ml of 1% $CoCl_2$ to the 500 ml of solution. The resultant precipitate will be black not brown. This is sometimes needed to increase contrast. Some of the nuclear location analyses are more easily studied using the black form of the chromogen. To increase the contrast further, 1% methyl green in water can be used as a counterstain for 5 minutes in place of hematoxylin. DAB, whether black or brown, is insoluble in alcohol, so these sections can be dehydrated, cleared with xylene, and coverslipped.

There may also be instances in which entirely new facets of the technology may be necessary. While all are similar in principle, the use of different marker molecules allows for more flexibility in assay design. The most common enzyme system in use other than peroxidase is alkaline phosphatase. This enzyme acts on many substrates to provide a variety of colored product combinations.

The stipulations for whichever enzyme is used are that the appropriate enzyme substrate must be available to allow visualization and the substrate must be insoluble as a precipitate to remain localized in the section. Also, if the enzyme is normally present in the section, it must first be blocked to avoid interference with its use as a marker. At the AFIP, the peroxidase enzyme is used because of the adequate elimination of endogenous activity achieved with the use of a relatively simple blocking procedure.

Other marker molecules such as fluorescent compounds or gold particles may be utilized with subtle changes in technique and means of detection. These techniques are not routinely performed, and for general use, a standard assay that is reliable is preferred.

There are other technique changes that can be employed if desired, such as indirect techniques having the enzyme or marker attached directly to a detecting secondary antibody or direct techniques with the reporter molecule attached directly to the primary antibody. Both of these variations are useful in certain situations, but the enzymatic assays are more sensitive and less fraught with background when used in sandwich assays as described above. Conjugation of a large enzymatic protein to an immunoglobulin, as required in direct and indirect procedures, can lead to inactivation of the immunoglobulin due to steric interferences. In the event that a fluorescent approach is desirable, however, fluorescein-labeled secondary antibodies are ideal for discreet UV visualization. There are also valid reasons, such as desired resolution or the need to examine ultrastructure, for the utilization of a heavy metal such as gold or gold enhanced with silver deposits.

Notes on Sample Treatments

In the pathology laboratory the fixative of choice is 10% neutral buffered formalin. Most of the assays in use at the AFIP are designed to detect substances fixed in this manner. Occasionally though, a specimen is fixed in another fixative, or frozen, and certain modifications need to be made. For most fixatives the standard protocol should suffice, bearing in mind that glutaraldehyde fixation may require more extensive enzyme digestion and ethanol fixation may not require any.

Sometimes antigenicity may be enhanced with the use of longer proteolytic enzyme digestion pretreatments. At other times antigenicity may be improved by the use of an antigen-retrieval method. These methods use dilute metal solutions and a microwave oven to boil the tissue substrate. The following protocol (Biogenex Corporation, San Ramon, CA) is often used.

1. Deparaffinize and hydrate slides to deionized water.
2. Place sections in a plastic Coplin jar and fill three-fourths full with dilute antigen-retrieval solution (1:4 with deionized water).
3. Fill a large surface area, one-pint vessel, with water.
4. Place Coplin jar in water vessel and place vessel in a 700-watt microwave oven.
5. Microwave on high for 3 to 5 minutes.
6. Check the level of retrieval solution and if necessary fill to three-fourths with deionized water. Reposition the water vessel with Coplin jar in a different location in the microwave.
7. Microwave on high for 3 to 5 minutes.
8. Place Coplin jar on countertop for 15 minutes at room temperature.
9. Rinse in deionized water for 3 minutes. Incubate sections requiring

digestion in proteolytic digestion solution at 37°C for 3 minutes.

10. Rinse in deionized water for 3 minutes. Place in PBS modified endogenous oxidation blocking solution (450 ml PBS, 50 ml 30% hydrogen peroxide) for 30 minutes.
11. Continue with ABC procedure, Step 7 (see page 9).

At times it may be necessary to assay a previously stained section. This can be accomplished, in most instances, using a previously assayed negative control or a hematoxylin and eosin-stained slide. If only the nuclei in the section are stained and the compound of interest exists in the cytoplasm, the slide need not be destained. When restaining a negative-control slide, it helps to know whether a section was proteolytically digested and what was used as the negative sera. In fact, destaining in acid/alcohol may inhibit the detection of the remaining material. When destaining is necessary though, the following method is used.

1. Remove coverslip and place in xylene for 3 minutes to remove mounting media.
2. Place in two more changes of xylene for 3 minutes each.
3. Place in 100% ethanol for 3 changes, 2 minutes each.
4. Place in 1% acid alcohol (conc. HCl in 70% ethanol) until slides appear color or stain free to the eye.
5. Rinse in deionized water for 3 minutes.
6. Proceed to digestion Step 5 (see page 8), if required, or rinse in 3 changes of 100% ethanol for 3 minutes each.
7. Proceed to endogenous oxidation blocking, Step 7 (see page 9).

It should be noted that destaining in 1% acid alcohol may inhibit the reactivity of the section. The quality of immunostaining will not be as good as that achieved using an unstained section.

Tissues that have first been decalcified can also be problematic due to treatments involving nitric acid. Many antigens will not survive the concentrated acidity required by some protocols. This is not true for all, however, and each system should be tested on an individual basis. It is recommended though, when contemplating immunohistochemical analysis of a specimen that must first be decalcified, that the reagent used to decalcify be a milder one, such as EDTA. Even though the results may not be ideal for other work on the specimen and the process will take longer, a milder treatment will help to preserve the greatest number of antigens for testing.

INTERPRETATION OF RESULTS

Expected Results

The results of these assays should show the location of the desired colored pigment with a consistency that denotes specificity. The results are interpreted and reported by the pathologist. In dealing with DAB-stained slides, there is a definite quality to the specific reaction product. The intense brown color is easy to detect in a typical tissue section (Fig. 1–5). The hardest interpretive process involves discerning specific from nonspecific reactivity. Specific reactions are usually more granular, although that is not always the case (Fig. 1–6). Precise localization is also an indicator of reliable reactivity. Note the numerous discreet cell types and the strength of the reaction product versus surrounding tissue in this smooth muscle cell identification of desmin (Fig. 1–7). Nonspecific reactions often have a very homogeneous dull color or appear a rather blushy reddish brown, but are always without the qualities unique to specific deposition. Melanin, lipofuchsin, hemosiderin, or some other crystallin substance present in the tissue may cause conflict in interpretation. Endogenous pigments can be discerned with practice and usually appear more refractile in sections, with the exception, sometimes, of melanin in the skin. Location in the basal layer of the epidermis should be a factor in making the determination of this interfering pigment.

Sometimes, changing the color of the reaction product is essential to avoid misinterpretation. The bright-red color product of AEC as the chromogen is seen in this assay for the antigen recognized by monoclonal antibody HMB 45 (Fig. 1–8A), in comparison with the DAB reaction product (Fig. 1–8B). Enhancement can also be obtained through the use of alternate color precipitation of DAB. Shown here, the assay for S100 protein in the Schwann cells surrounding nerves is made more visible by using DAB in the presence of $CoCl_2$ as the chromogen with a methyl green counterstain (Fig. 1–9).

Artifacts

As a rule, artifacts occur through the random deposition of precipitate, not necessarily in conjunction with primary antibody location. Artifactual false-positive results have many causes and many appearances and can be confusing when examining immunostained sections. The nonspecific binding associated with a nonspecific antiserum or a sticky tissue surface can be very difficult to distinguish from valid results.

A lack of sufficient peroxidase quenching, for example, will result in DAB being deposited at the site of endogenous enzyme. This is, however, easily recognized in control sections, the deposits being primarily associated with areas of heavy red blood cell accumulation (due to their peroxidase-like activity) or widespread

inflammatory regions containing numerous macrophages and polymorphonuclear leukocytes (Fig. 1–10). An insufficient serum protein incubation with resultant charged sites left on the tissue could also present some problems in antibody binding and aberrant DAB deposition. A dull brownish hue is usually seen associated with the areas of connective tissue in the section and may be seen in especially sticky cells such as plasma cells or mast cells having a large amount of glycoprotein binding antibody non-specifically. This too can be easily observed on control sections and is the most common and sometimes unavoidable example of nonspecific binding, as in this assay for the Her 2 protein on the surface of breast carcinoma cells (Fig. 1–11A). Note the homogeneous appearance of the DAB and the connective tissue location in relation to the specific membrane reactivity. The same assay with appropriately reduced background reactivity is seen in Figure 1–11B.

Edge artifact due to drying or enzyme entrapment is a common finding, especially in a large laboratory that uses proteolytic digestion to enhance reactivity. When performing the procedure on numerous sections at once, it is important to work quickly to prevent the sections from drying. Drying will result in enzyme complex binding to denatured areas of the section and cause inappropriate DAB deposition. Proteolytic enzyme digestion is necessary in many instances, but it also abrades the tissue enough to sometimes leave the edges loose. Enzyme complexes can be trapped in these areas and result in DAB deposition (Fig. 1–12). Another common problem that occurs with the use of proteolytic enzyme digestion is the overdigestion of some tissues. This is unavoidable when dealing with a large volume of slides, since an average digestion time is performed to ensure the highest number of successful assays. This practice can result in an occasional sample being prepared in such a way that the average digestion time is too extensive. The DAB will be deposited throughout the section, and often nuclear decoration will be seen along with a washed-out cellular appearance.

One artifact that continues to be a problem is the pooling of reagent in sections that are not cut exactly even. This may be due to dull or loose knives, or it may be a factor of the tissue itself. "Laking" of the reagent in depressed regions of the section causes the tissue to appear as though parts of it were not tested, leaving the impression of an incomplete reaction (Fig. 1–13). This can also occur when using an assay chamber that is not level because antibody molecules can migrate by gravity flow (Fig. 1–14).

Poor tissue preservation and necrosis can also lead to false-positive results. These need to be recognized as tissue-derived causes of inappropriate reactivity, showing the standard signs of nonspecificity. Some, in fact, are due to the adherence of DAB itself, especially when using an older solution. Others seem to be associated with the inappropriate binding of the ABC usually seen with fresh or frozen samples (Fig. 1–15). In these cases, a PAP technique will help to provide an interpretable result. It is imperative to review the negative controls to rule out the possibility of a false-positive result caused by the detection system. Some tissues have intrinsic binding affinity for ABC detection systems. Nevertheless, these systems are preferred because of their sensitivity. This is not,

incidentally, exclusively limited to frozen specimens and points out the need to use and examine negative controls (Figs. 1–16 through 1–21).

In addition, there are also problems with various antibody preparations. Some are intrinsically nonspecific, harboring reactivities to many elements not expected by the user. These are not true artifacts but must be considered when reviewing immunostained specimens at least during the initial phase of antibody selection. Thus, establishing a routine program for specificity testing is all the more important, since such a program provides experience from which to judge the true utility of an antiserum. There is a fine distinction between true unwanted clonal reactivity and that which may occur through cross-reactivity. Cross-reactions with polyclonal antisera may be removed through absorption, but in the case of a monoclonal reagent it is usually wiser to use a different clone.

PROBLEM SOLVING

When confronted with a problem result, it is desirable to examine the process in reverse. Determine whether the encountered problem has anything to do with the chromogen. If not, move on to the detection system, to the primary antibody, through to the tissue itself to identify the cause. Once the cause is identified, decide how that step may be altered to alleviate the problem. Finally, incorporate the changes into the daily routine to prevent the problem from recurring.

Problems with the results of immunohistochemical assays fall into two very broad categories. Either there is too much reactivity, excess background, and seemingly nonspecific staining of areas that are not thought to contain the antigen in question, or the slides are too weakly reactive to be useful, if not completely negative.

False-Negative Results

An examination of the cause of negative (or weakly reactive) results depends on the prior knowledge of the antibody system employed. If just starting out with a new technique or a new primary antibody, it is not unusual for the first experiments to be less than satisfactory. In this instance it is important to make sure that the tissue being tested contains the antigen being assayed. Don't assume the carcinoma contains cytokeratin, but rather, use a skin section that by definition is keratinized. Be absolutely certain that the antigen is present before testing. Assuming antigen exists, begin with the chromogen and make sure that it does in fact precipitate in the presence of the detecting enzyme. Try to achieve positive results by using concentrated forms of the antisera. The background this will cause can be dealt with after a positive signal has been obtained. Sometimes, even after exhausting experimentation, antigen just cannot be identified. This is either the fault of the tissue fixation, which cannot be altered practically, or the fault of the primary antibody. A new antibody should be sought in this case. It is easier and surprisingly cheaper to screen several antibodies than to waste time performing countless futile experiments to track down a problem.

If, on the other hand, the negative result occurs with a known reliable system, the individual test run is suspect. This is the reason for performing positive controls. Usually, a negative result on an occasional basis is related to individual technique. However, antibodies do denature in the refrigerator with time, and freezing aliquots at -70°C to prevent this is recommended. A schedule for replacing antibodies in use should be developed to lessen the chance of a reagent failure. Some of the more common causes of negative results are failing to add H_2O_2 to the chromogen solution, forgetting a step in antibody addition, working on a surface that is not level, or accidental contamination with a cleaning agent, such as bleach. The first step in troubleshooting these cases is to repeat the assay. If the results are still negative or poorly reactive, alternate control material could be tried. Sometimes, it seems, control material that is precut on slides may with time

lose some effectiveness. Some control slides are kept at 4°C for this reason. Additionally, antibodies do gradually lose strength in the freezer and may suddenly appear to be nonreactive when entering the awareness threshold of the reviewer. Periodic retitrations can help to prevent this problem.

False-Positive Results

The more difficult immunostaining problems are those involving nonspecific reactivity or excess background. A technology as sophisticated as this should not yield results that are subjective. There are steps that can and should be taken to identify and correct the causes of invalid reactivity. All sections tested should remain completely negative in the absence of primary antibody. If PBS is substituted for the primary antibody and positive immunostaining results, the problem exists with the detection system, the tissue preparation, or both.

The detection system should be examined, beginning with the chromogen solution. Endogenous enzyme activity may still be present in the tissue, confusing the marker enzyme location. The chromogen solution itself may be too concentrated, too old, or contain too much hydrogen peroxide. Also, incubation times may need to be adjusted. If the chromogen alone does not cause reactivity, the detecting enzyme complex may be the cause of the problem. Sometimes, depending on the sample or pretreatment, certain enzyme complexes may be unsuitable for use. Endogenous biotin or available immunoglobulin Fc receptors on cell surfaces can cause inappropriate background problems in frozen sections. Also, the pH and ionic strength of the PBS play a role in immunoglobulin binding, and adjusting these can have an effect on the result.

Most often, problems are due to either the primary or secondary antibody reagents. Usually, success in the use of secondary antibodies almost always involves proper titration. If problems still exist, again, there are too many good reagents available to devote much time to a poor one.

Should problems occur with the primary antibody, a few alterations can be tried to counter them. Difficulties with antibody specificity emanate from unwanted clonal reactivities, cross-reactivities, or nonspecific binding due to immunoglobulin Fc receptors, denatured antibodies, or fragments.

The tissue can be better prepared for the problem reagent by increasing the strength, the time, or the temperature of treatment with the blocking solution for nonimmunologic binding. In addition, a protein such as bovine serum albumin can be added to the antibody dilutions to prevent further nonspecific attachment by dissolving 2 grams into 100 milliliters of the PBS diluent. Also, the use of detergents can prevent any additional binding due to charges. A 0.1% solution of Triton X-100 in PBS allows less material to stick by decreasing the surface tension. This also can enhance reactivity by increasing the fluidity of the reagent. It may, however, decrease the reactivity of an antiserum with low avidity.

If true unwanted clones exist, they can be absorbed from the solution by introducing antigen in vitro. If reactivity is seen intravascularly, 0.5% human serum can be

added directly to the antiserum to remove the reactivity. This is only valid for those instances in which the desired antigen to be identified has little or no blood stream concentration. If antigen can be obtained in large enough concentrations, affinity columns can be prepared that contain the antigen in the column matrix. Antiserum is passed through the column, and the clones reactive with the desired antigen remain attached to the column. These clones are then eluted from the column by low pH or high salt concentrations and dialyzed back into PBS for use on tissue. If the reactivity problem is the result of cross-reactivity, there is little that can be done, especially if it is present in a monoclonal reagent. As stated before, in these situations it is prudent to examine other sources of antibodies.

Once the cause of an erroneous result is recognized and corrective actions are taken, prevention of repeated failures is necessary to ensure confidence in future assays. Special diligence to subtleties in the laboratory that may indicate a potential for error is essential. By being well informed on the nature of the technology and the reasons for performing these assays, the technical staff become better prepared to identify and prevent most of the problems that can undermine the work.

LABORATORY ORGANIZATION

When shifting from an occasional assay to production-line volumes, it is helpful to start with a basic idea of how the various parts of the laboratory should be organized. First, the essential equipment should be decided upon (e. g. freezers, refrigerators, etc.) and space allocated accordingly. Next, the segments of the workload need to be addressed to determine where the case material should be stored, received, assayed, assembled, and logged out. A workload schedule should be drawn up that includes the duties of the personnel and determines work stations to be divided and rotated from week to week. Finally, a system of reagent documentation and quality control should be instituted in order to function as a clinical laboratory.

Antibody Selection

The most important part of any immunohistology laboratory is the person doing the test, and the second most important is the antibody used. Antibodies can be obtained from many sources. Some of the suppliers are also referenced through catalogues. Purchased individual reagents go farther and are often better characterized than preparations that are prediluted for histology. Purchasing a primary antibody reagent is the first step in starting an immunohistochemistry lab. One should know, or have access to knowledge about, the antigen in question and its location in the tissue. After settling on a few antibodies to try, a detection system is needed. The most universal of these is the ABC system, since there are numerous biotinylated antibodies available to act as secondary antisera against numerous species' immunoglobulin.

When all of the supplies are in place, the testing phase can begin. Initially, a two-stage titration needs to be performed. A known antigen-rich tissue is selected, and dilutions of primary and secondary antibody solutions are made. After a titer has been determined for a secondary antibody reagent, further primaries may be tested in a one-dimensional titration, keeping the secondary titer constant. Usually, a concentration of around 10 to 20 μg/ml of immunoglobulin will provide adequate reactivity. If the concentration is unknown, a two-step titration should be performed. All new assays are routinely performed with and without the standard 3-minute proteolytic enzymatic digestion. Thus, for each dilution of antibody, two slides are examined. The antibody is diluted serially, 1:10, with resulting dilutions of 1:10, 1:100, 1:1000, and 1:10,000. Generally, one of these reactions will demonstrate some sort of reactivity on either the digested or undigested section. If, for example, the 1:100 undigested section looked the best, the next step would be to retiter with dilutions of 1:25, 1:50, 1:100, 1:200, 1:400, and 1:800, using the best digestion protocol. A precise titration can in this way be determined, and the digestion protocol for this antibody/antigen system established.

Once the reagent has been reliably titered and the results are seemingly valid, the

specificity and performance testing phases can begin. Every reagent antibody is tested on a series of normal tissue control slides representing various tissues, organs, and cells. This tissue screen is done to examine, in actual working situations, the extent of cross-reactivity or background not fully appreciated in the original titration. After specificity testing, the assay is put to use on many actual specimens while paying careful attention to the spectrum of reactivity. Only after considerable actual work experience is an assay considered of value in a consultative situation.

The primary antibody is stored in aliquots at -70°C, the volume of the aliquot depending on the titer and the total volume of concentrated reagent. Reagents are aliquotted into 4-ml glass vials with screwcap lids to prevent evaporation. The smallest volume recommended is 20 μl/vial, although for smaller volumes as little as 10 μl has been used. Ordinarily, the reagent will be thawed and diluted 1:10 with PBS. This concentrate or stock solution is kept at 4°C for one week. From this stock, daily working volumes are prepared. If the reagent titer is 1:500, a 1:50 dilution is prepared from its stock solution, in enough volume to be used that day. Reagents maintain their viability longer this way. Alternatively, a reagent may be frozen in aliquots so that dilution of the aliquot to working concentrations may be done directly. These reagents are usually used for not more than two days. It is important not to let the antibodies stay at room temperature for too long. It is also important not to freeze and thaw them too many times. Contamination and denaturation are hard to troubleshoot, and disposing of reagent is an expensive exercise. In a large-volume laboratory it is less cost-effective to use the prediluted kits that are available from many sources because of the sheer volume of reagent needed. Also, maintaining a freezer inventory is easier than keeping track of kit boxes in the refrigerator. Antibodies frozen in this manner remain effective for many years. In fact, there is less reason to suspect denaturation, since antibodies in high concentrations seem to be more stable. On the average, monoclonal antibodies seem to be less stable in the refrigerator than polyclonals, although that is strictly an observation and may not be true for all laboratories. The judicious use of controls helps to determine when a reagent is performing optimally, and self-derived titrations can be adjusted if changes occur. A kit kept at 4°C in a prediluted state and used over an extended period of time provides more chance for contamination, more chance for denaturation, and less available reagent for use in a large-volume setting. Also, the simplistic designs of some of the kits on the market lead to a false sense of security about the procedure. Only by becoming familiar with antibody test components through titration, specificity, and performance testing can one truly appreciate the intricacies involved in this most sensitive of technologies.

Case Management and Tracking Systems

All cases entering the laboratory consist of tissue sections on slides labeled with an accession number and should be accompanied by a form requesting the tests desired, the physician's name, the date, the number of sections submitted, and, if possible, information about the tissue preparation. The date should be stamped on each case when it arrives in the laboratory. The sections are then labeled as

to the requested assays and required digestion times with an indelible pen, and all information is entered into a computer or log book. The slides are separated into groups according to the assay requested, species of the antibody, and whether or not digestion is needed. Known positive-control slides are then added to each test group, and all of the slides are placed in staining racks. The slides requiring digestion for assays using antibodies made in the same species are placed together, as are the slides not requiring digestion for those assays. For example, slides assayed with monoclonal antibodies that require proteolytic digestion would be placed together in a rack, and the slides not requiring digestion would be placed in a different rack. The slides assayed with antibodies from other species would be similarly segregated. The slides are then processed according to the general ABC procedure. When the slides are completed, they are sorted by their accession number and labeled with the number, test, and date performed. Finally, the sections are placed with the accompanying forms and materials, and the entire case is logged out and returned to the requesting physician.

Quality Control

A proper attempt at providing an immunohistochemistry laboratory must include a sound quality control program. A responsible procedure manual should be developed—more extensive than the one rendered here—outlining every procedure, solution, piece of equipment, and quality control program, including laboratory safety guidelines. The antibody of course is the most important item requiring control, beginning with the storage of the specificity testing data for each lot of each reagent. With each assay, a negative control should be performed, using a section similar to the unknown and normal immunoglobulin in substitution for the primary antibody in the procedure. This identifies any possible problems with the specimen. With each assay, a positive control should also be performed, using an antigen-rich specimen treated the same as the unknown. This identifies any problems having to do with the test itself. The positive controls can be graded subjectively, for example, on a scale of 1 to 4+ reactivity, with only sections greater than 2+ considered acceptable. All assays demonstrating inferior reactivity on controls should be examined and repeated, if possible. In addition, survey participation is required. An occasional self survey with the cooperation of some other laboratory can be a good test of the ability to identify a given substance.

Documentation is an important facet in most laboratories today and should extend to the immunohistochemistry laboratory. The recommended documentation consists of the specificity sheets for each lot of reagent outlining the determined titer and the tissue screen results. In addition, the inventory control for each reagent should include the dates in use, for retrospective lot analysis. The daily positive-control results of all the reagents need to be recorded, along with any unusual findings or occurrences within the run, for each day that assays take place. The positive-control slides ought to be retained for examination by the pathologist, if desired.

The above descriptions and advice are offered as an introduction into the technology of immunohistochemical staining. There are many techniques available and

many antibodies on the market, with more being developed continuously. The power of the assay will insure its use in pathology for years to come, provided the results are accurate and unequivocal. It is important, therefore, to always question unexpected results and to never stop examining the intricacies of antibody-antigen interactions.

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APPENDIX 1

Appendix 1 is an abbreviation of the immunohistochemical ABC procedure and intended as a handy bench reference guide.

ANTIGEN DETECTION IN TISSUE BY ABC IMMUNOHISTOCHEMISTRY

PRINCIPLE:

The presence of a certain antigen is demonstrated using the avidin-biotin-peroxidase complex (ABC) method of immunohistochemistry.

Tissue samples are submitted on slides dipped in 15% Elmer's glue.

MATERIALS/SOLUTIONS:

Oven set at 60°C
Water bath set at 35-40°C
Magnetic stir plate, stir bars
Gloves
Pipettes
Xylene
100% ethanol
0.05% protease VIII (Sigma, St. Louis) in 0.1M sodium phosphate buffer, pH 7.8
100% methanol with 3% hydrogen peroxide (H_2O_2)
0.01M sodium phosphate, pH 7.40, 0.15M NaCl (PBS)
Normal serum (specific for secondary antibody species)
Primary antibody
Biotinylated secondary antibody
Avidin-Biotin Complex (ABC) reagent
0.016% diaminobenzidine tetrahydrochloride (DAB), 0.24% H_2O_2 in PBS
Gill's hematoxylin
2% conc. ammonia in deionized water
Permount (Fisher Scientific)

PROCEDURE:

1. Paraffin sections mounted on pretreated slides are heated in an oven for 30 minutes.
2. Deparaffinize sections using 4 changes of xylene for 5 minutes each, followed by 4 changes of 100% ethanol for 3 minutes each. For sections that do not require digestion, go directly to Step 6.
3. For sections requiring digestion, rinse in deionized water for 3 minutes.
4. Incubate sections in buffered protease VIII at 37°C for 1 to 30 minutes. The standard time for digestion is 3 minutes.
5. Rinse sections in 2 changes of deionized water for 2 minutes each, then 3 changes of 100% ethanol for 3 minutes each.
6. Block endogenous peroxidase activity with 3% H_2O_2 methanol for 30 minutes.
7. Rinse sections 3 times with deionized water.
8. Reduce nonimmunologic binding of antiserum by placing sections in 10% normal serum-PBS derived from the same species as the biotinylated (secondary) antibody (e.g., if biotinylated antibody is goat anti-rabbit antiserum, use normal goat serum) at 4°C overnight.
9. Shake off excess normal serum and place slides in leveled staining trays. Distribute the slides according to the primary antiserum that is to be applied. Care should be taken so that the sections do not touch during incubation with primary antisera. The need to separate the slides into groups in order to preclude any chance of contaminating a section with the wrong antibody cannot be over emphasized. Cover section with primary antiserum, e.g. rabbit antiserum specific for desired antigen, for 30 minutes at room temperature. Make sure the chamber lid is on to prevent air-drying during the incubation.
10. Wash sections with PBS 3 times.
11. Flood sections twice with 10% normal serum for 10 minutes each, and then rinse in PBS.
12. Shake off excess buffer and cover sections with biotinylated secondary antibody. Incubate for 30 minutes at room temperature, making sure the chamber lid is on to prevent air-drying of sections.
13. Wash sections 3 times with PBS.
14. Shake off excess buffer and cover sections with Vectastain ABC reagent, prepared according to the recommendations of the manufacturer, for 30 minutes at room temperature, making sure the chamber lid is on to prevent air-drying of the sections.

15. Wash sections 3 times with PBS. Place sections in staining racks for a final 10-minute soak in PBS.
16. Develop in 0.016% DAB and 0.24% H_2O_2 in PBS solution for 10 to 25 minutes at room temperature. The usual time for optimal development of DAB is 15 minutes, but this may vary with the lot of DAB being used.
17. Wash sections for 2 minutes 3 times with deionized water.
18. Counterstain with Gill's hematoxylin.
19. "Blue" sections with ammonia water.
20. Wash in deionized water for 5 minutes.
21. Dehydrate and mount sections with Permount.

RESULTS:

Positive antibody-antigen reaction is brown, with nuclei blue.

NOTES:

Throughout the procedure, keep drying of the sections to a minimum in order to prevent destroying epitopes on the cell membrane. Drying also causes nonspecific background staining.

In Step 2, it is important to complete deparaffinization because paraffin can mask epitopes from the primary antibody.

In Step 16, special precautions should be taken when handling DAB because of its possible carcinogenic properties.

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Hsu SM, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J Histochem Cytochem.* 1981;29:577-580.

APPENDIX 2

LIST OF REAGENT ANTIBODIES

Following is a list of reagent antibodies in use currently at the AFIP. This document is offered as a guide and in no way constitutes an endorsement of any individual product. The suppliers listed and the titers given are unique to the Immunopathology Division and should be considered as a reference. Each laboratory is responsible for developing its own individual procedures.

ANTIGEN	ANTIBODY ABBREVIATION	NEGATIVE CONTROL	TITER	COMPANY
ACTH	ACTH	NRS PO	400	DAKO
ACTIN-SMOOTH MUSCLE	S.M. ACTIN	NMS PO	4000	SIGMA
ACTIN-MUSCLE SPEC.	ACTIN	NMS PO	50	ENZO
ADENOVIRUS	ADV P3	NMS P3	400	CHEMICON
ALBUMIN	ALB P3	NRS P3	6400	DAKO
ALPHA-1-ANTICHYMOTRYPSIN	ACT P3	NRS P3	200	DAKO
ALPHA-1-ANTITRYPSIN	AAT P3	NRS P3	800	DAKO
ALPHA-AMYLASE	AMYL-A P3	NRS P3	1000	SIGMA
ALPHA-LACTALBUMIN	ALA P3	NRS P3	10	DAKO
BETA-AMYLOID	B-AMY	NMS P0	150	DAKO
BOMBESIN	BOMBESIN	NRS P0	150	INCSTAR
C-ERB-B2 ONCOGENE PROTEIN	C-ERB-B2 P3	NMS P3	80	BIOSCIENCES
CALCITONIN	CALCITONIN	NRS P0	200	DAKO
CD 15	LEU M1	NMS P0	50	BECTON DICKINSON
CD 20	L 26	NMS P0	200	DAKO
CD 30	KI-I P3	NMS P3	80	DAKO
CD 34	Q BEND	NMS P0	200	SEROTEC
CD 57	LEU 7	NMS P0	32	BECTON DICKINSON
CD 68	KP1 P3	NMS P3	40	DAKO
CD3	CD3 P10	NRS P10	25	DAKO
CD45RB	LCA	NMS P0	100	DAKO
CD45RO	UCHL-1	NMS P0	100	DAKO
CEA-NCA REACTIVE	P-CEA P3	NRS P3	400	DAKO
CEA-SPECIFIC	M-CEA P0	NMS P0	400	SAN BIO
CHOLECYSTOKININ	CCK	NRS P0	4000	INCSTAR
CHROMOGRANIN A VETERINARY USE	CHRM-VET	NMS P0	80	ENZO
CHROMOGRANIN A	CHROMOGRAN	NMS P0	1600	BOEHRINGER MANNHEIM
COLLAGEN TYPE IV	COLLAGEN IV	NMS P5	2000	CHEMICON

ANTIGEN	ANTIBODY ABBREVIATION	NEGATIVE CONTROL	TITER	COMPANY
CYTOKERATIN COCKTAIL (AE1/AE3&CK1)	KERMIX P3	NMS P3	200	DAKO/ HYBRITECH
CYTOMEGALOVIRUS	CMV P3	NMS P3	800	CHEMICON
DESMIN	M-DESMIN P3	NMS P3	40	BOEHRINGER MANNHEIM
EPITHELIAL MEMBRANE	BER EP4 P3	NMS P3	100	DAKO
EPITHELIAL MEMBRANE ANTIGEN	EMA P3	NMS P3	100	DAKO
FACTOR XIII A	F XIII A P3	NRS P3	2000	CALBIOCHEM
FIBRONECTIN	FIBRONECTIN	NRS P20	200	CHEMICON
FOLLICLE STIMULATING HORMONE	FSH	NRS P0	1600	DAKO
FIBRINOGEN	FIBRINO P3	NRS P3	200	DAKO
GASTRIN	GASTRIN	NRS P0	2000	INCSTAR
GLIAL FIBRILLARY ACIDIC PROTEIN	GFAP P3	NRS P3	500	DAKO
GLIAL FIBRILLARY ACIDIC PROTEIN(MONO)	M-GFAP P0	NMS P0	100	DAKO
GLUCAGON	GLUCAGON	NRS P0	800	DAKO
GROWTH HORMONE	GH	NRS P0	1600	DAKO
HEPATITIS B CORE ANTIGEN	HBCAG	NRS P0	1600	DAKO
HEPATITIS B SURFACE ANTIGEN	HBSAG	NRS P0	400	DAKO
HERPES SIMPLEX VIRUS 1&2	HSV	NRS P0	1600	DAKO
HUMAN CHORIONIC GONADOTROPIN	HCG	NRS P0	400	INCSTAR
HUMAN PANCREATIC POLYPEPTIDE	HPP	NRS P0	1000	DAKO
HUMAN PLACENTAL LACTOGEN	HPL	NRS P0	8000	DAKO
IMMUNOGLOBULIN-ALPHA	IG A P3	NRS P3	3200	DAKO
IMMUNOGLOGULIN-GAMMA	IG G P3	NRS P3	1600	DAKO
IMMUNOGLOGULIN-KAPPA	KAPPA P3	NRS P3	2000	DAKO
IMMUNOGLOBULIN-LAMBDA	LAMBDA P3	NRS P3	8000	DAKO
IMMUNOGLOBULIN-MU	IG M P3	NRS P3	1600	DAKO
INSULIN	INSULIN	NGP P0	2000	DAKO
LAMININ	LAMININ P20	NMS P20	8000	SIGMA
LEUCINE ENKEPHALIN	L-ENKEPHAL	NRS P0	2000	INCSTAR
LUTEININZING HORMONE	LH	NRS P0	3200	DAKO

ANTIGEN	ANTIBODY ABBREVIATION	NEGATIVE CONTROL	TITER	COMPANY
LYSOZYME	LYSO P3	NRS P3	400	DAKO
MAC 387	MAC 387	NMS P0	400	DAKO
MELANOCYTE-SPECIFIC	HMB 45	NMS P0	400	DAKO
MILK FAT GLOBULE PROTEIN	MFG P3	NMS P3	800	CHEMICON
MYOGLOBIN	MB	NRS P0	800	DAKO
NEUROFILAMENT-200MW	NFP P3	NMS P3	300	LABSYSTEMS
NEURON-SPECIFIC ENOLASE(MONO)	NSE-M	NMS P0	160	DAKO
NEURON-SPECIFIC ENOLASE(POLY)	NSE-P	NRS P0	1600	DAKO
PROLACTIN	PRL	NRS P0	800	DAKO
PROLIFERATING CELL NUCLEAR ANTIGEN	PCNA	NMS P0	40	DAKO
PROSTATE SPECIFIC ANTIGEN	PSA P0	NRS P0	800	DAKO
PROSTATIC ACID PHOSPHATASE	PAP P0	NMS P0	800	DAKO
S100 PROTEIN-MONOCLONAL	MS-100	NMS P0	1000	CHEMICON
S100 PROTEIN-POLYCLONAL	S100 PROT	NRS P0	800	DAKO
SEROTONIN	M-SEROTON	NMS P0	200	DAKO
SOMATOSTATIN	SOMATOSTAT	NRS P0	250	CHEMICON
SYNAPTOPHYSIN	SYNAPTO	NMS P0	40	BOEHRINGER MANNHEIM
T-CELL SUBSET ALPHA-BETA	B-F1 P3	NMS P3	100	T CELL SCIENCES
T-CELL SUBSET CD4 (?)	OPD4	NMS P0	50	DAKO
TAG-72	B72.3 P0	NMS P0	40	BIOMED TECH
THYROGLOBULIN	TG	NRS P0	800	DAKO
THYROID STIMULATING HORMONE	TSH	NRS P0	2000	DAKO
UBIQUITIN	UBIQ	NRS P0	300	DAKO
VIMENTIN	VIM	NMS P0	2000	BIOGENEX
VON WILLEBRAND FACTOR	F VIII RAG P3	NRS P3	20	DAKO

NMS = NORMAL MOUSE SERUM
NRS = NORMAL RABBIT SERUM
NGP = NORMAL GUINEA PIG SERUM
P# = MINUTES OF PROTEASE DIGESTION REQUIRED

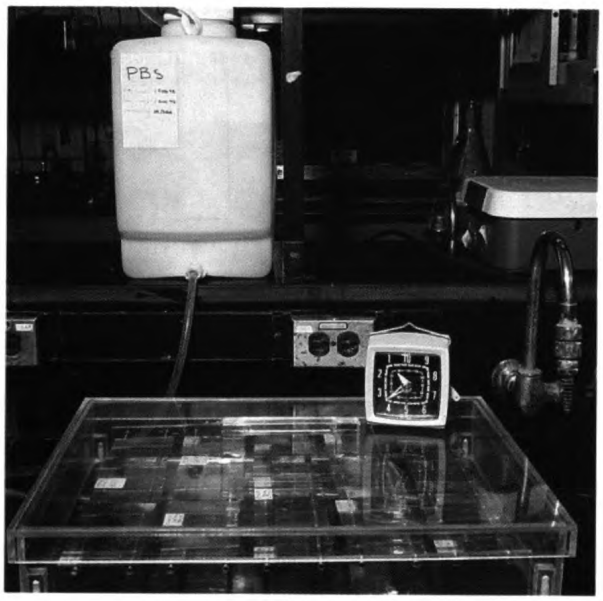


Fig. 1–1. Slides placed in humid chamber - note buffer siphon.

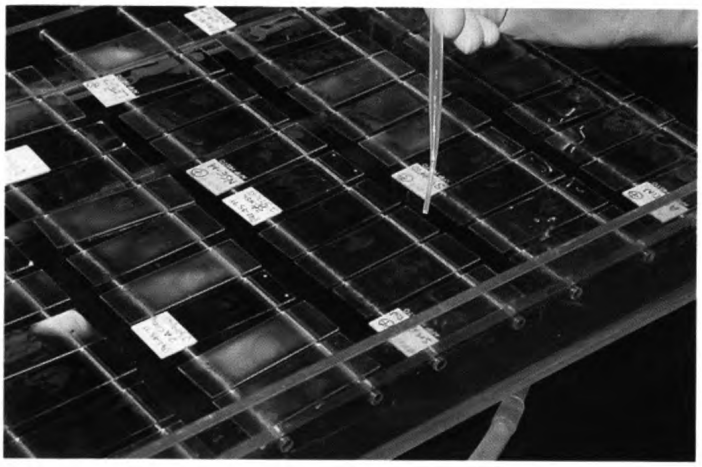


Fig. 1–2. Antibody application, enough to cover section.

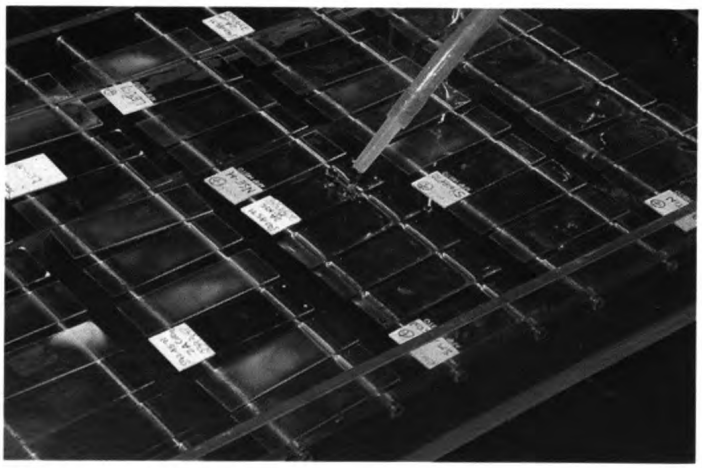


Fig. 1–3. Buffer rinse, using siphon, washing entire slide.

Fig. 1–4. Sections immersed in DAB chromogen for 15 minutes. Note brown tint on the sections. It is important that the DAB solution is nearly colorless when sections are immersed. Brown tinted solutions should not be used.

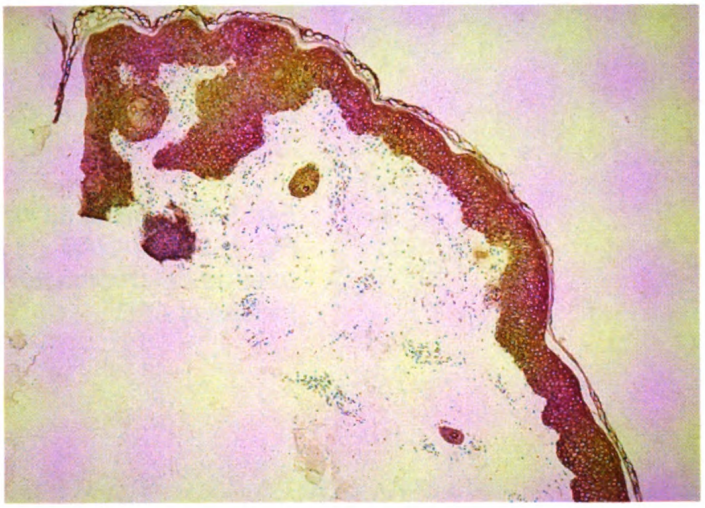


Fig. 1–5. Cytokeratin demonstrated in skin section, ABC method with DAB.

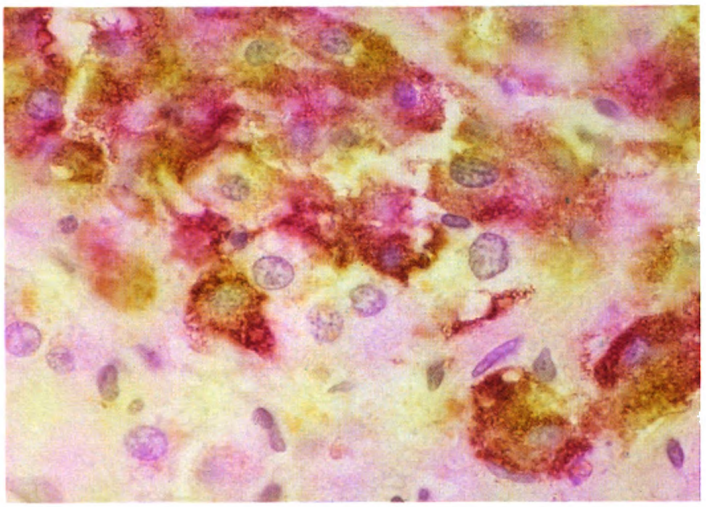


Fig. 1–6. Chromogranin granularity demonstrated in cells of the adrenal medulla, ABC method, DAB.

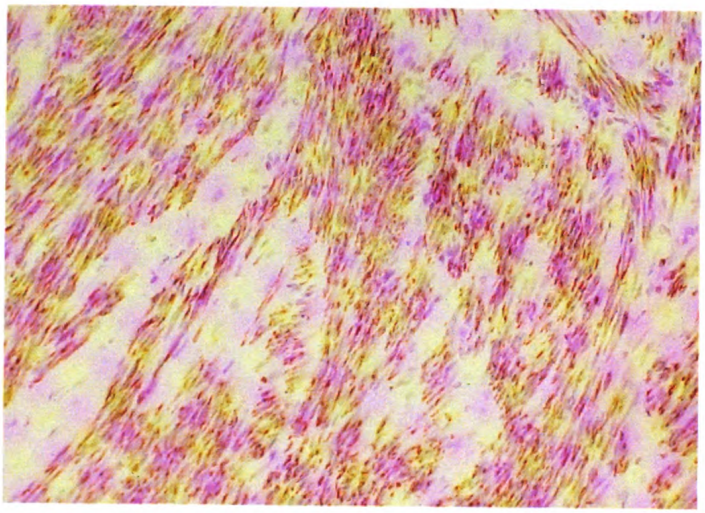
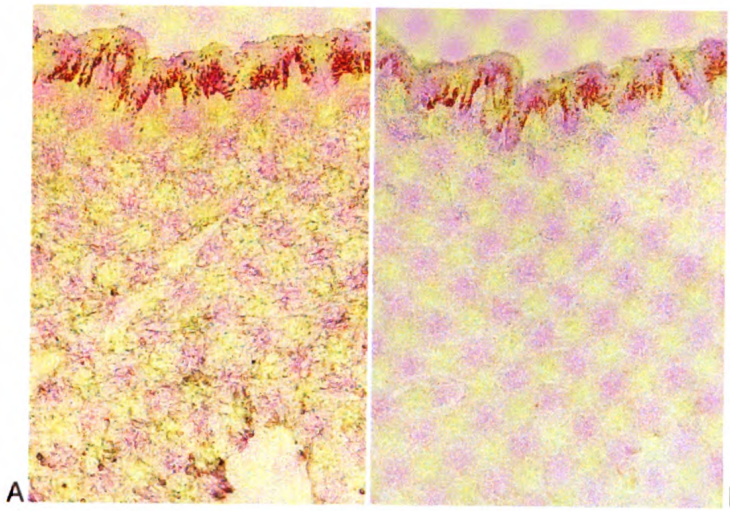


Fig. 1–7. Desmin localization in smooth muscle of the uterus, ABC method, DAB.



A B

Fig. 1–8A. HMB 45 antibody deposition in melanoma of the skin, ABC method, with AEC.

Fig. 1–8B. Same as in Fig. 1–8A with DAB used as the chromogen instead of AEC.

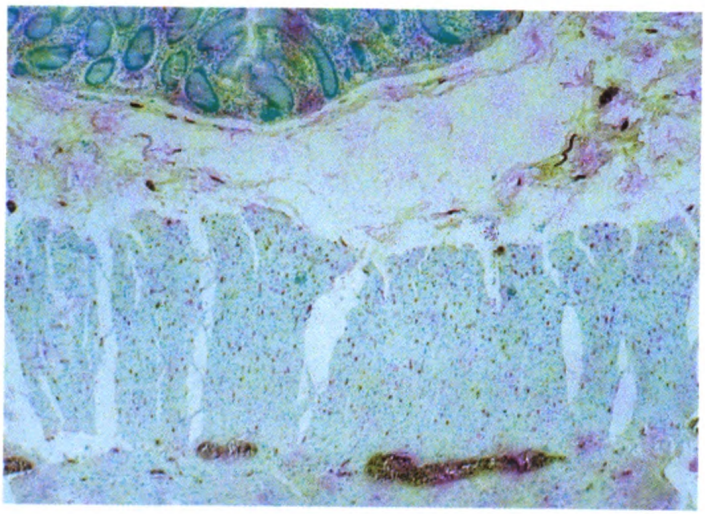


Fig. 1–9. S100 protein demonstrated by the ABC method with cobalt enhanced DAB in section of intestine.

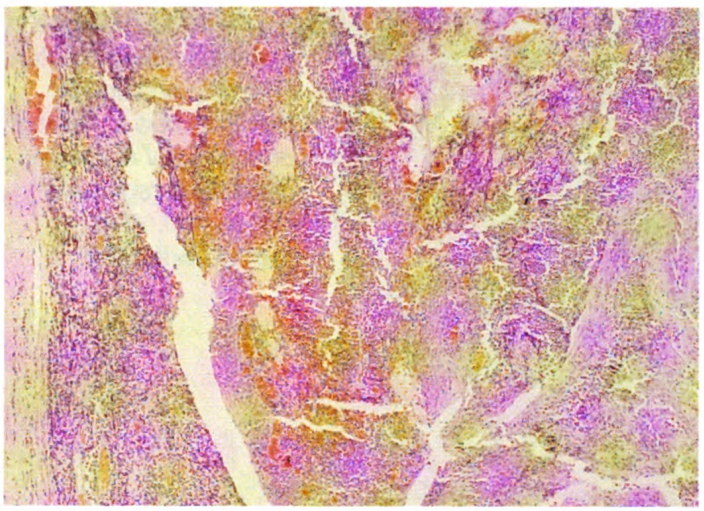
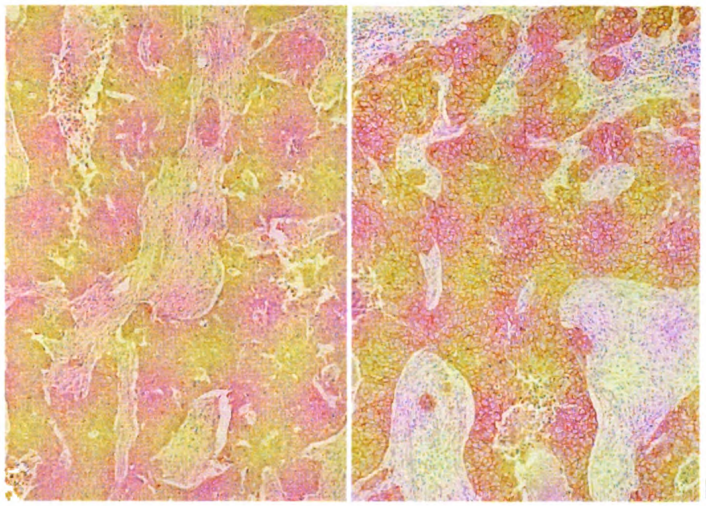


Fig. 1–10. Tonsil, DAB solution only, demonstrating endogenous peroxidase-like activity.



A B

Fig. 1–11A. Her 2 oncoprotein, non-specific antibody binding causing background reactivity in breast, ABC method with DAB.

Fig. 1–11B. Same as in Fig. 1–11A, background reactivity reduced.

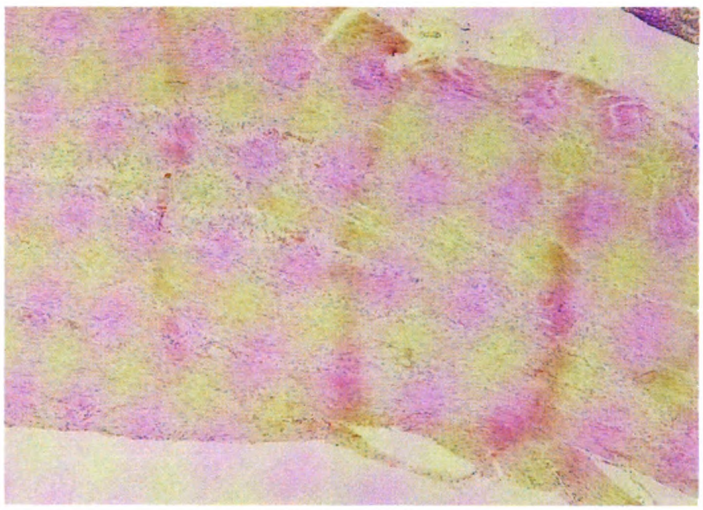


Fig. 1–12. Assay for Neuron-specific enolase (NSE), demonstrating ridges of background reactivity in section of intestine caused by horseradish peroxidase enzyme entrapment. ABC method with DAB.

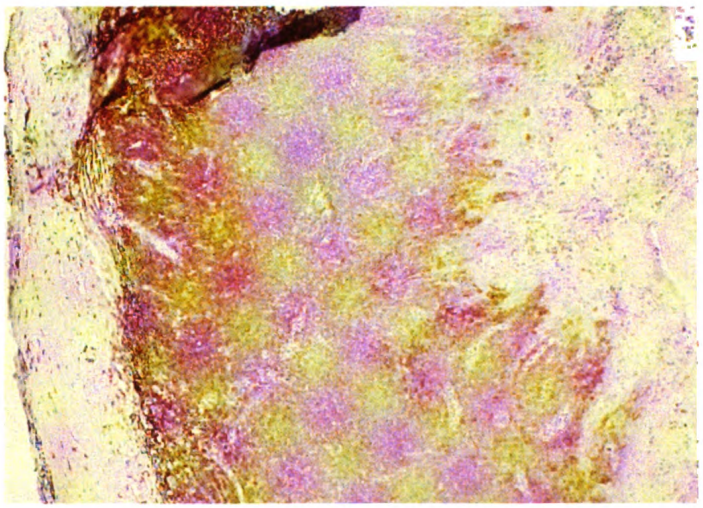


Fig. 1–13. Leukocyte common antigen (LCA) in tonsil, incomplete reactivity due to "laking" or pooling of reagent in depressed regions of the section. ABC method with DAB.

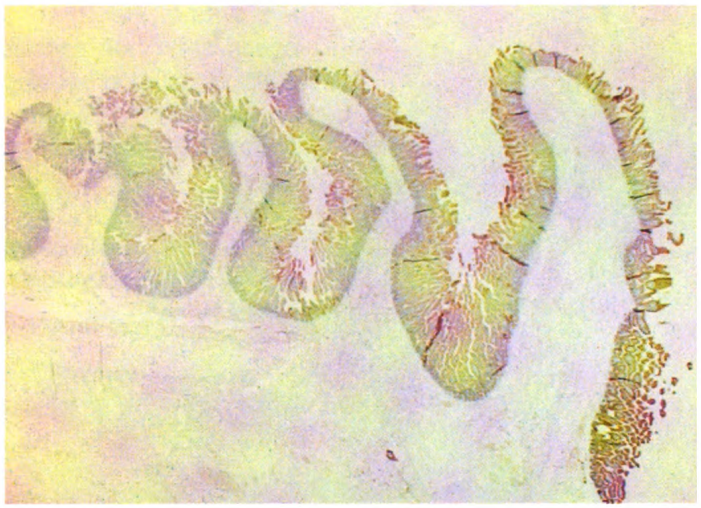


Fig. 1–14 Cytokeratin in the intestinal mucosa, incubation chamber not level, resulting in incomplete reaction pattern. ABC method with DAB.

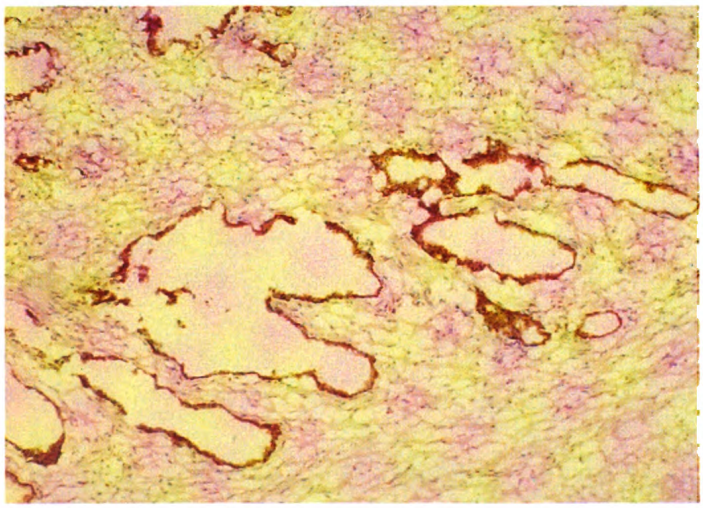


Fig. 1–15. Frozen section of prostate ABC reagent only, with DAB, demonstrating inappropriate binding of ABC reagent sometimes observed with fresh or frozen samples.

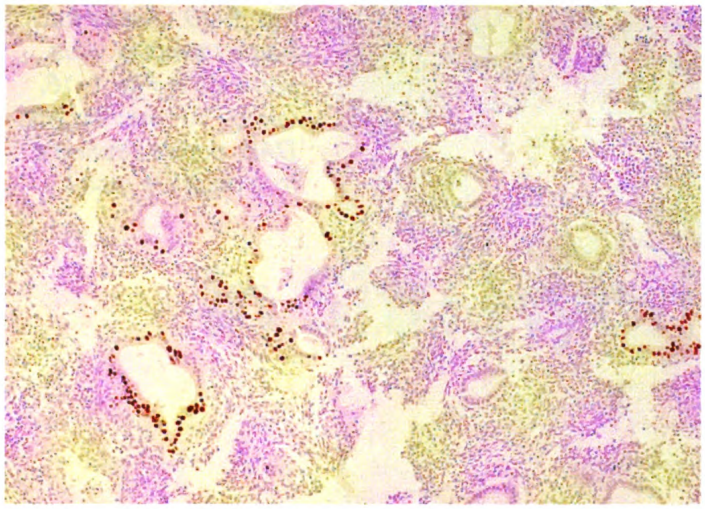


Fig. 1–16. Assay for herpes simplex virus using a polyclonal antiserum, ABC method with DAB, on formalin-fixed paraffin-embedded endometrial tissue, demonstrating a false-positive reaction in epithelial cells.

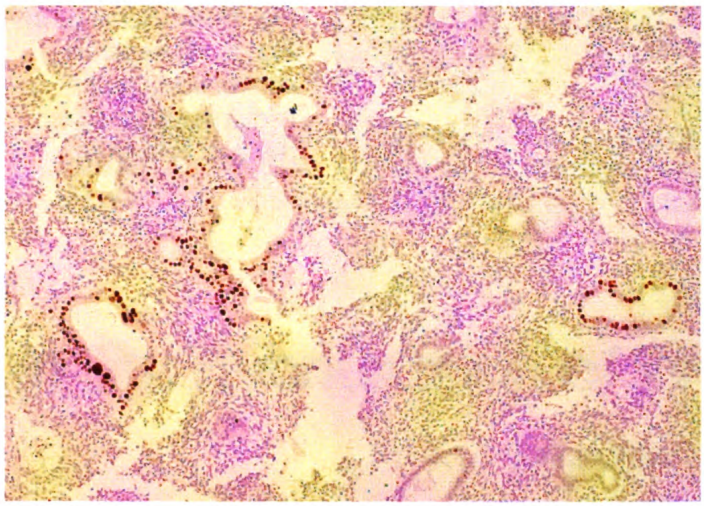


Fig. 1–17. Same as in Fig. 1–16, negative control using rabbit immunoglobulins in place of antiserum, confirming false-positive assay result.

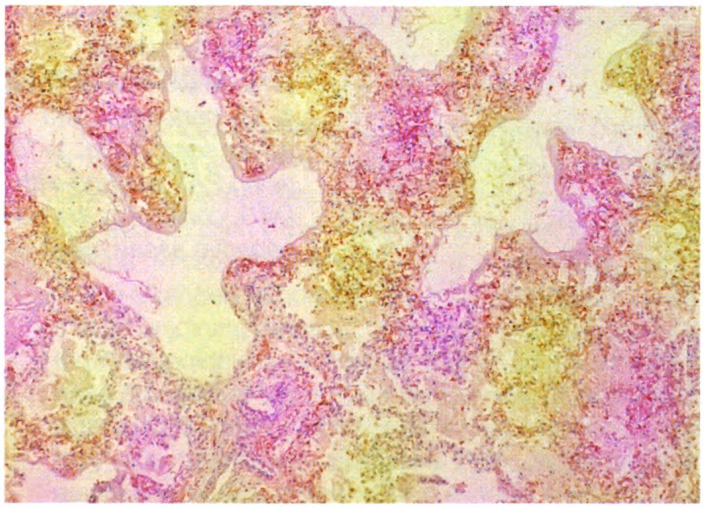


Fig. 1–18. Herpes simplex virus infected lung tissue. Positive control for herpes simplex virus performed with assay seen in Fig. 1–16, ABC method with DAB.

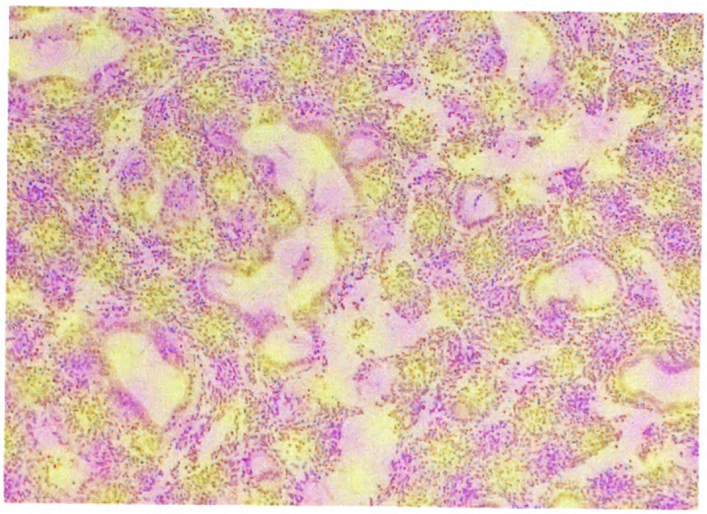


Fig. 1–19. Assay for herpes simplex virus same as in Fig. 1–16, using PAP method with DAB, demonstrating a true negative result.

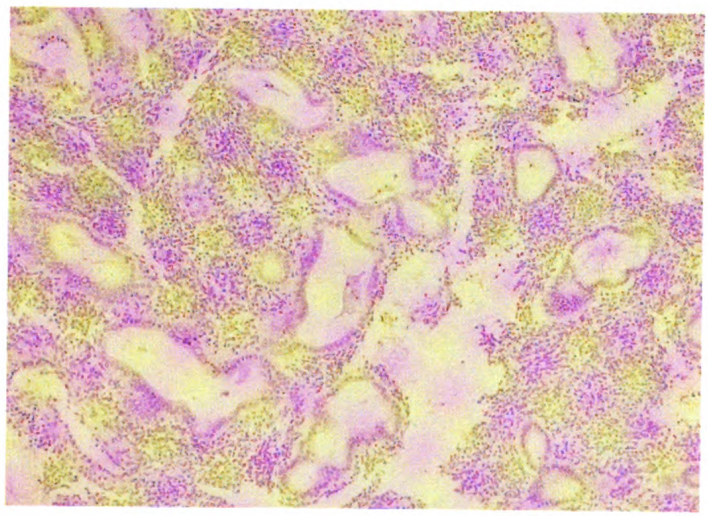


Fig. 1–20. Same as in Fig. 1–19, negative control using rabbit immunoglobulins in place of antiserum.

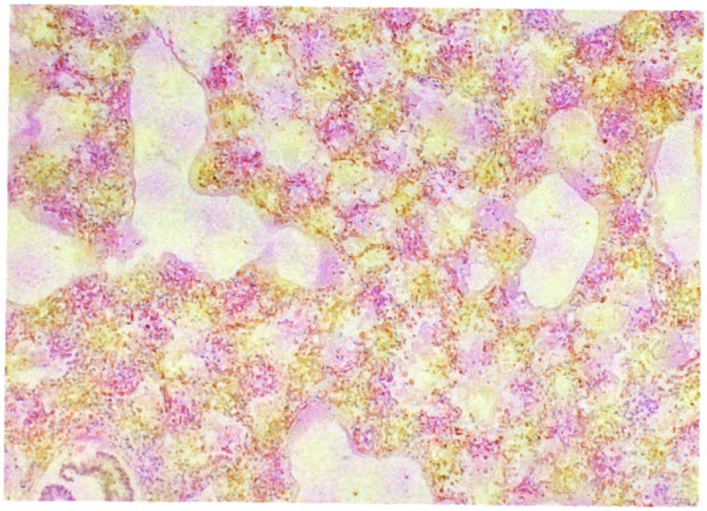


Fig. 1–21. Positive control for herpes simplex virus as in Fig. 1–18, performed with assay seen in Fig. 1–19 using PAP method with DAB, confirming the true negative assay result.

2

In Situ Hybridization

This chapter covers the in situ hybridization technique on paraffin-embedded material. The procedure can be varied in many different ways. We have chosen to present two versions, each discussed by a different department at the AFIP.

In Situ Hybridization of Viral Inclusions

Robert E. Cunningham

INTRODUCTION

The advances in immunology and molecular biology have revolutionized diagnostic medicine and have greatly contributed to biomedical research as a whole. One of the techniques that utilizes both immunology and molecular biology is in situ hybridization (ISH). In situ is Latin for "in its original place" and hybridization means "to cause the production of a hybrid, a cross"; in this case, a hybrid of single-stranded genomic DNA and single-stranded probe DNA. It accomplishes this by enjoining powerful methodologies from the two systems. The diversity of the immunologic detection capability and the exquisite sensitivity of specific DNA base recognition together provide a new powerful tool for diagnosis and research. Appropriate immunological stains enable recognition of phenotypic changes that may reflect genotypic alterations. ISH can reveal those genotypic changes as well as oncogene expression, chromosomal mapping, infectious disease detection,

and metabolic disorders.

ISH involves taking morphologically intact tissue, usually paraffin-embedded tissue or cells or chromosomes, through the hybridization process to demonstrate not only the presence of a particular piece of genetic information, but also its specific location within the tissue, cells, or chromosomes. DNA hybridization utilizes a labeled DNA probe to detect and identify specific DNA sequences. A DNA probe is a segment of DNA that is specific for and complementary to the target DNA sequence that is to be detected and identified.

The target nucleic acids are found intimately mixed with the proteins, other nucleic acids, and membranes that form the basis of the familiar staining pattern of hematoxylin and eosin. The goal is to make the target DNA available for hybridization while maintaining a recognizable staining environment so that the tissues, cells, and chromosomes can be identified by the landmarks of routine staining. ISH techniques represent a compromise between making the nucleic acid target available for hybridization and maintaining the morphologic integrity of the starting material.

Figure 2–1 illustrates a block diagram of the detection of a DNA probe in paraffin-embedded tissue sections. The first layer is the target DNA sequence, which is a section of paraffin-embedded tissue mounted on a glass microslide that has been treated with proteolytic enzymes and/or high salts or acids to not only remove histone proteins but also to begin the process of denaturation of the double-stranded DNA into single-stranded DNA. The second layer is a DNA probe with DNA sequence complementary to a portion of the target DNA segment. This probe is biotinylated. The third layer is a complex of streptavidin and biotinylated peroxidase, which react together in such proportion that some biotin-binding sites on the streptavidin molecule are not filled by biotinylated peroxidase but are free to react with the biotin in the second layer. The fourth layer is a reporter molecule, such as 3-amino-9-ethylcarbazole or 3,3'-diaminobenzidine, that reacts with the enzymatic substrate (peroxidase) and deposits an opaque-colored marker on the tissue.

PRINCIPLE:

The detection of specific nucleic acid sequences occurs within structurally intact cells or tissues. As such, the technique provides a unique opportunity to display nucleic acids superimposed in well-preserved cellular and subcellular structural details. DNA exists in vivo almost exclusively in the form of a double helix. Only during replication and transcription are regions of single-stranded DNA present. However, it is possible to separate (denature) the DNA strands, in vitro, by using high temperatures and/or solvents such as formamide. The helix will unwind; the hydrogen bonds will break and separate the base pairs into single-stranded DNA (ssDNA). Reversing the denaturation condition by slowly cooling the single strands can reform the original base pairs, and the duplex is restored. This process is called annealing. When this annealing reaction occurs between nucleic acid molecules from different sources with complementary base pair

sequences, a "hybrid" double-stranded molecule is formed. This reaction is called "hybridization" (Jones and Robertson 1970).

Tissues are prepared by standard histologic and immunologic techniques, with an emphasis on decreasing the amount of nucleic acid degradation by endogenous nucleases. The specimen is usually fixed, processed, and embedded in paraffin and then cut on a microtome and immobilized on the microslide. Alternatively, tissue culture cells may be grown directly on glass slides and used in that way. Chromosome preps are "squashed" onto the slide to open up the cell and spread the chromosomes. Sample preparation involves some method of affixing the starting material to a microscope slide, so that light-microscopic evaluation can be done. The slide becomes the solid support that carries the specimen through all the following steps of the hybridization assay. This step is not trivial because the conditions of the hybridization assay can be quite harsh and loss of sample through detachment during the assay is a major concern. Often specially treated microslides are used to offset this problem.

Before hybridization the sample is subjected to various enzymatic digestions to improve the accessibility of the nucleic acids to the probe. Commonly, a protease is used (e.g., Proteinase K). DNA probes are consensus sequences of DNA that recognize particular sequences of genomic DNA. When these DNA strains oppose each other, they can bind as two genomic strands of DNA naturally bind. In this state, there is no signal detection capability. Therefore, a signal-generating system must be employed. In this example, we use a deposit of a red or brown chromogen product to generate the signal.

In order to create this signal-generating system, the DNA probe must be modified. The system that we employ incorporates biotin into the DNA probe. Biotin-labeled DNA probes can be large (100 to 2,000 base pairs long); therefore, for the probe to reach its target and generate maximum signal, the fixed tissue requires enzymatic removal of DNA-associated histones and partial denaturation of the double-stranded DNA. This is the trickiest and most crucial step when using large probes. Streptavidin, a high-affinity (four sites), biotin-binding glycoprotein, is used to develop the chromogen in the presence of horseradish peroxidase. During this development, the soluble chromogen becomes insoluble and visible.

The detection complex is a biotin-binding protein linked to a signal-generating system. The signal-generating systems are either fluorescent or enzymatic. The enzymatic detection system is a soluble complex composed of streptavidin and a biotinylated enzyme. The streptavidin molecule, which has multiple biotin-binding sites, is complexed with a biotinylated enzyme, such as horseradish peroxidase. Because there are free binding sites on the streptavidin molecules in the complex, the entire complex can bind to biotinylated DNA.

Thus, the biotinylated enzyme is localized to the target site of the DNA probe. The enzyme then acts as a catalyst in an oxidation reaction with hydrogen peroxide and a chromogen such as 3-amino-9-ethyl carbazole (AEC) or 3,3'-diaminobenzidine (DAB). This results in the chromogen precipitating in close proximity to the peroxidase enzyme.

The complex is visualized after conversion of the substrate and chromogen (hydrogen peroxide and AEC or DAB) into a localized precipitate. The entire process is illustrated by Figures 2–2A through 2–2F.

Following counterstaining with Mayer's hematoxylin, the tissue is observed under a light microscope. The appearance of the red or brown precipitate within the nucleus of the cells indicates a degree of positivity (Brigati et al. 1983).

In this example, we are going to examine tissue sections with a biotinylated JC virus probe that is commercially produced by Enzo Diagnostics, New York, NY. The JC polyomavirus is the etiological agent of progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease of the central nervous system (Padget et al. 1976). The detection method will be a horseradish peroxidase-streptavidin utilizing AEC as the chromogen. There are many different versions of this procedure concerning the probe, the detection complex, and the chromogen. Further, the tissue preparation, prehybridization, hybridization, and posthybridization conditions can also be varied.

MATERIALS AND SOLUTIONS:

Micropipetters
Freezer -70°C
Refrigerator 4°C
Glass beads, tissue culture quality
Incubator 37°C
Heating block 90° to 95°C
Glass beaker
Glass slides
Coverslips
Crystal/Mount (Biomeda, Foster City, CA)
Staining dish
Glass Pasteur pipets
Poly-L-lysine-coated glass slides

Make a 50 µg/ml solution of poly-L-lysine in autoclaved, deionized water. (This solution may be kept for 1 month at 4°C.) On each glass slide, place approximately 1 ml of the poly-L-lysine solution and distribute evenly over each surface. Let the slides stand for 30 minutes at room temperature. Pour off the excess poly-L-lysine solution and rinse slides 3 times with deionized water. Let slides air-dry. (Dried slides may be stored for 1 week at room temperature.) As an alternative, "Probe-On Slides" (Fisher Scientific) can be used.

200mM EDTA, pH 8.0

Ethylene diamine tetraacetic acid (EDTA),
Disodium, dihydrate (Sigma, St. Louis) ..... 37.22 g
Distilled/deionized water ..... 400 ml

Adjust pH to 8.0 with 1N NaOH and add enough water to make a final volume of 500 ml. Filter through a 0.2-μm filter.

10% SDS

Sodium dodecyl sulfate (SDS) (Bio-Rad) ..... 10 g
Distilled/deionized water ..... 100 ml

Phosphate buffered saline (PBS) 10X, pH 7.4

Dulbecco's Phosphate Buffered Saline (DPBS) 10X, without calcium chloride and magnesium chloride,
0.07M dibasic sodium phosphate (Na_2HPO_4)
0.03M monobasic sodium phosphate (NaH_2PO_4)
1.5M sodium chloride (NaCl)
(Gibco BRL, Gaithersburg, MD)

PBSE (1X), pH 7.2

10X DPBS ..... 50.0 ml
200mM EDTA ..... 12.5 ml
Distilled/deionized water ..... 437.5 ml

Adjust pH to 7.2 and filter.

20X saline-sodium citrate (SSC) (Sigma, St. Louis)

0.3M citric acid trisodium
3.0M sodium chloride

2X SSC

20X SSC ..... 10 ml
Distilled/deionized water ..... 90 ml

Adjust pH to 7.0 and filter.

0.2X SSC

20X SSC ..... 1 ml
Distilled/deionized water ..... 99 ml

Adjust pH to 7.0 and filter.

2X SSC with 0.1% SDS

2X SSC ..... 99 ml
10% SDS ..... 1 ml

Adjust pH to 7.0 and filter

Tris buffer, 0.05M, pH 7.6

Trishydroxymethyl aminomethane ..... 6.1 g
Distilled/deionized water ..... 50.0 ml
(Dissolve Tris in the water and add)
1N hydrochloric acid ..... 37.0 ml

Distilled/deionized water to a final volume of 1,000 ml

Proteinase K solution

Proteinase K (Sigma, St. Louis) ..... 0.125 mg
PBSE ..... 1 ml

The solution is warmed to 37° C until use.
This enzyme is used to make the DNA accessible to the probe.

3% hydrogen peroxide solution

30% hydrogen peroxide (Fisher Scientific) ..... 1 ml
PBSE ..... 9 ml

This dilute peroxide solution removes endogenous peroxidase reactivity that would add to the overall staining of the tissue.

50% dextran sulfate solution

Dextran sulfate ..... 50 g
Distilled/deionized water ..... 100 ml

Mix. Sonication can be used to drive it into solution. This solution is very heavy, with a "syrupy" texture, and is light brown in color.

Formamide solution, 50%

Formamide (Sigma, St. Louis) ..... 50 g
Distilled/deionized water ..... 100 ml

Concentrations of formamide from 40% to 50% are used in ISH. Each 1 percent of formamide lowers the melting temperature of DNA (TM) by 0.7 percent. Commercial preparations of this chemical usually contain salt impurities as well as hydrolysis products, ammonium formate, NH_3, and formic acid. The formamide can be cleaned by stirring 1 liter of formamide with 10 grams of Norite A (Fisher Scientific) and 50 grams of mixed bed resin (AG501- X8(d), 20-50 mesh, Bio-Rad) at 4°C for 2 hours. Filter twice through Whatman #1 filter paper. Store aliquots at -70°C.

Carrier DNA

Salmon sperm is supplied with each DNA probe in a 1.5-ml tube at 10 mg/ml (Enzo Diagnostics) and is used in ISH as a blocking agent. Remove tube from freezer and boil for 10 minutes, place tube on ice, and add to DNA probe mixture at 100 μg/ml of hybridization solution.

DNA probe (negative control) (Enzo Diagnostics)

Concentration: .....25.0 μg/ml
Quantity: .....2.5 μg/slide
Fragment size:..... 200 to 2,000 base pairs (estimated)

pBR322 is a plasmid grown in *E. coli* HB101, extracted by standard molecular procedures and purified by isopyknic banding in CsCl gradient.

DNA probe (positive control) (Enzo Diagnostics)

Concentration: .....20.0 μg/ml
Quantity: .....1.6 μg/slide
Fragment size: .....200 to 2,000 base pairs (estimated)

A 4kB fragment of JC virus DNA is cloned into the Eco R1 site of pBR322.

Hybridization mixture

Deionized formamide, pH 6.8 to 7.2 .....50 μl
50% dextran sulfate .....20 μl
20X SSC .....10 μl
JC virus probe or pBR322 probe .....20 μl
Carrier DNA ..... 4 μl

This mixture is stable for 12+ months at 4°C. The amount is enough for 5 tissue samples measuring 20 mm x 20 mm.
Note: Many variations exist on the exact makeup of hybridization probe mixtures. Tissue type, fixative, DNA probe length, and DNA probe content of particular nucleo-tides are some of the conditions that may dictate an alteration in the hybridization mixture.

Glass microcarrier beads, 90 to 150 μm (Sigma, St. Louis)

The addition of glass beads decreases the adherence of the coverslip to the glass slide during the hybridization step. This is especially useful when a long hybridization time is necessary. The glass beads are added immediately before the coverslip. The beads can be diluted in the probe mixture, or they can be placed between the slide and coverslip dry.

Posthybridization wash 1 - 2X SSC

20X SSC ..... 50 ml
10% Triton X-100 ..... 5 ml
Distilled/deionized water ..... 445 ml

Posthybridization wash 2 - 0.2X SSC

20X SSC ..... 5 ml
10% Triton X-100 ..... 5 ml
Distilled/deionized water ..... 490 ml

Detection complex (HRP-SA)

Horseradish peroxidase-streptavidin (Amersham) ..... 0.025 ml
PBSE ..... 9.975 ml

Streptavidin is a high-affinity, biotin-binding protein, and when coupled with horseradish peroxidase, it is used to develop the chromogens, 3-amino-9-ethylcarbazole or 3,3'-diaminobenzidine in the presence of hydrogen peroxide. During this development, the soluble chromogens become insoluble and visible. Staining time is 15 minutes.

Chromogen AEC

AEC can be purchased as a peroxidase chromogen kit (20 mg/ml in . dimethylformamide) from Biomeda Corporation.

Working reagent:

Distilled/deionized water ..... 5 ml
Chromogen buffer (bottle 3A) ..... 2 drops
Concentrated chromogen (bottle 3B) ..... 1 drop

(Mix quickly, then add)

2% hydrogen peroxide (bottle 3C) ..... 1 drop

Store away from direct sunlight until used.
The chromogen used in this assay is 3-amino-9-ethylcarbazole (AEC). It has the ability to turn red in the presence of horseradish peroxidase, which is deposited in the detection complex. Chromogenic substrates, when oxidized, form colored compounds. AEC is soluble in alcohol, and AEC-stained slides are mounted from water in Crystal/Mount.

Chromogen DAB

If AEC is not the preferred chromogen, the logical alternative is 3,3'-diaminobenzidine tetrahydrochloride (DAB). DAB is probably the most utilized enzymatic stain in the detection of immunologic probes. DAB

produces a brown color that is insoluble in alcohol; therefore, the slides can be dehydrated through alcohols, cleared in xylene, and coverslipped with conventional mounting media.

DAB	6.0 mg
0.05M Tris	10.0 ml
3% hydrogen peroxide	0.1 ml

NOTE: AEC and DAB are potential carcinogenic substances and must be labeled as such and stored away from normal contact. Gloves should be worn when handling carcinogens, and these substances should be treated with extreme care.

Mayer's hematoxylin:

Distilled/deionized water	1,000 ml
Ammonium or potassium alum	50.0 g
Hematoxylin crystals	1.0 g
Sodium iodate	0.2 g
Citric acid	1.0 g
Chloral hydrate	50.0 g

Dissolve alum in deionized water, without heat; add and dissolve the hematoxylin crystals. Then add, in sequence, the sodium iodate, citric acid, and the chloral hydrate; stir until all components are in solution. It is important that each chemical is completely dissolved before adding the next chemical. The final color of the stain is deep reddish violet.

NOTE: Mayer's hematoxylin is used to highlight the topography of the tissue stroma by having a contrasting color (blue) to the color (red) of the chromogen. Other counterstains can be used.

PROCEDURE:

1. Deparaffinize and hydrate sections.

Xylene	x 2	for 2 minutes each
100% EtOH	x 2	for 2 minutes each
95% EtOH	x 2	for 2 minutes each
50% EtOH	x 2	for 2 minutes each
PBSE	x 2	for 5 minutes each

2. Wash in deionized water and air-dry sections at 37°C.
3. Digest sections in Proteinase K (0.125 mg/ml in PBSE) at 37°C for 10 minutes.

4. Block endogenous peroxidase by immersing sections in 3% hydrogen peroxide in PBSE at 37°C for 10 minutes.
5. Wash in PBSE x 3, 2 minutes each.
6. Wash in deionized water, 2 minutes.
7. Dehydrate sections through graded alcohols 50%, 95%, and 100% for 2 minutes each.
8. Add probe at 20 µl per 20 x 20-mm area of tissue.
9. Add a small amount (pipet dipped in vial) of glass beads to the probe and place a coverslip on top, one that is large enough to cover the tissue.
10. Denature both the probe and genomic DNA by heating the slides from 90° to 95°C on a hot plate for 10 minutes.
11. Remove slides from hot plate and place in a covered, moistened, humid chamber to hybridize at 37°C for 1 hour.
12. Remove coverslip by immersing each slide in 2X SSC.
13. Wash slides in 2X SSC with 0.1% SDS for 5 minutes.
14. Wash slides in 0.2X SSC with 0.1% SDS for 5 minutes.
15. Wash slides in PBSE for 5 minutes.
16. Add approximately 0.5 ml of detection complex (HRP-SA) to each slide and incubate at 37°C for 15 minutes.
17. Wash slides in PBSE x 3, for 2 minutes each.
18. Wash slides in deionized water.
19. Add approximately 5 drops of chromogen complex (AEC) to each slide and incubate at 37°C for 15 minutes. (For DAB staining, see page 51.)
20. Wash in deionized water x 3, for 2 minutes each.
21. Counterstain with Mayer's hematoxylin for 15 to 30 seconds.
22. Wash in tap water for 20 minutes.
23. Lightly dry slides and add Crystal/Mount to cover the sections.
24. Dry slides first at 25°C for 10 minutes. Then completely dry the Crystal/ Mount by heating the slides at 80°C for 10 minutes.
25. Option: Add 3 drops of Permount (Fisher Scientific) and coverslip. Other resinous mounting media may cause diminished red color of the AEC.

ALTERNATIVE PROCEDURE:

Follow the previous procedure, Steps 1–18, then proceed as follows:

19. Add approximately 5 drops of chromogen complex DAB to each slide and incubate at 37°C for 15 minutes.
20. Wash slides in PBSE x 3, 2 minutes each.
21. Counterstain with Mayer's hematoxylin for 15 to 30 seconds.
22. Wash in tap water for 20 minutes.
23. Dehydrate in 50% EtOH x 2, 95% EtOH x 2, and 100% EtOH x 2, for 2 minutes each.
24. Clear in xylene x 2 for 2 minutes each.
25. Mount in resinous mounting medium.

RESULTS:

(See Fig. 2–3)

1. Nuclear chromatin - blue.
2. Positive DNA Probe - deep rose red (DAB would show brown.)
3. Background - pink to rose (DAB would show tan to light brown.)

REQUIRED SLIDES FOR EACH "RUN":

Known positive-control slide — Run with JC (+) probe to control for known positive results.

Known positive-control slide — Run with pBR322 (-) probe to control for known negative control. This probe is deficient only in the JC virus complementary sequence.

Submitted case slide 1 — Run with JC (+) probe to evaluate the consult case.

Submitted case slide 2 — Run with pBR322 (-) probe to further control for false positivity.

If any positive case used as a positive control is negative or if any of the negative-control slides appear positive, a total review of all procedures must be done, followed by a repeat assay.

HOW TO AVOID PROBLEMS AND AIDS FOR TROUBLESHOOTING

- **Problem — no staining**

  Possible causes:

  1. Improper fixation and/or tissue processing of the specimens.
  2. Denaturation incomplete — not enough single-stranded DNA available to react with probe.
  3. Probe length (too long) creating a steric problem between the probe and target DNA sequence.
  4. Use of alcohol-based counterstain or alcohol-based mountant.
  5. Reagents used in incorrect order.
  6. Reagents not made correctly by omission of some component.
  7. Sodium azide in reagent diluent or buffers.
  8. Incompatible buffer for HRP complex or chromogen.
  9. Improper concentration of hydrogen peroxide in the HRP complex.

- **Problem — weak specific staining of positive control**

  Possible causes:

  1. Too much liquid (usually wash buffer) retained on sections when detection and chromogen dilutions are applied.
  2. Probe mixture incorrect.
  3. Proteolytic digestion omitted.
  4. One or more reagents defective.
  5. Insufficient hybridization time.
  6. Insufficient incubation time for HRP complex or chromogen.
  7. Denaturation temperature not high enough to make DNA accessible to probe.
  8. Old HRP complex and/or chromogen solutions.
  9. Improper storage of reagents.

- **Problem — weak specific staining of specimen (when assumed to be positive)**

  Possible causes:

  1. Specimen incorrectly fixed and processed.
     a. Overfixation
     b. Fixative degraded or too weak
  2. Specimen not uniformly fixed.
  3. Specimen not completely deparaffinized.

- **Problem — excessive background staining**

  Possible causes:

  1. Nonspecific binding of probe or detection HRP complex.
  2. Endogenous peroxidase activity not removed.
  3. Overdevelopment of HRP complex.
     a. Increased incubation temperature
     b. Excessive incubation time with HRP complex and/or chromogen
     c. Incorrect preparation of HRP complex and/or chromogen HRP (e.g. complex too concentrated)
  4. Slides inadequately washed.
  5. Probe incorrectly prepared or insufficiently diluted.
  6. Excessive adhesives used on slides.
  7. Adhesive contaminated by bacterial growth.
  8. Tissue sections too thick.
  9. Delay in initial fixation of the tissue leading to general autolysis.
  10. Wash procedures not adequate or not properly completed.

- **Problem — irrelevant staining**

  Possible causes:

  1. Degradation of target DNA.
  2. Probe DNA binds to target DNA not at specific sequence due to a slight mismatch in DNA pairing.

- **Problem — high background**

  Possible causes:

  1. Excessive incubation with substrate-chromogen reagents.
  2. Slides inadequately washed.
  3. Substrate-chromogen reagents mixed incorrectly due to substrate being too concentrated.
  4. Endogenous peroxidase activity not removed.
  5. Improper fixation.
  6. Paraffin improperly removed.
  7. Tissue adhesive used in excess amount.

- **Problem — stain covers areas not exposed to detection HRP complex**

  Possible causes:

  1. Excessive adhesives used on slides.
  2. Adhesive contaminated by bacterial growth.

- **Problem — staining appears blurry and/or streaky**

  Possible causes:

  1. Excessive proteolytic digestion.
  2. Necrosis of tissue.
  3. Incomplete deparaffinization.

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Jones KW, Robertson FW. Localization of reiterated nucleotide sequences in Drosophila and mouse by in situ hybridization of complementary RNA. *Chromosoma.* 1970;31:331-345.

Padget BL, Walker DL, Zu Rhein GM, Hodach AE, Chow SM. JC papovavirus in progressive multifocal leukoencephalopathy. *J Infect Dis.* 1976;133:686-690.

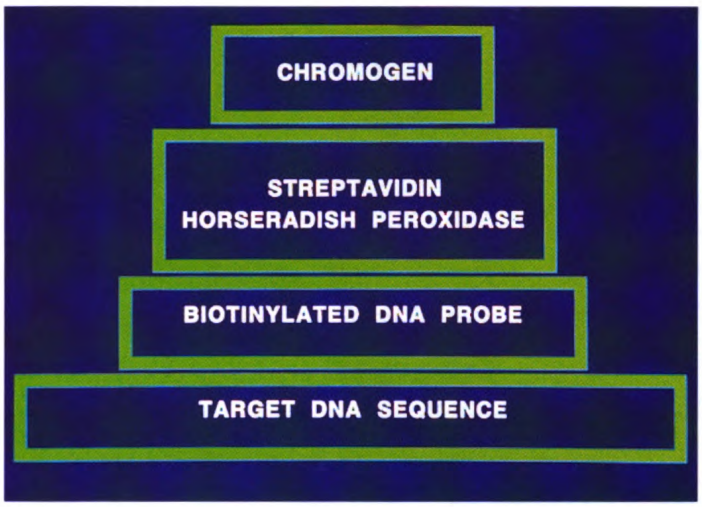
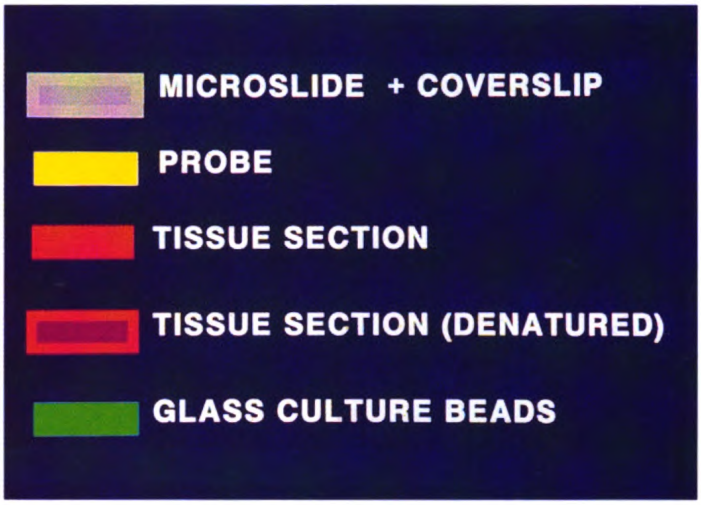


Fig. 2–1. Illustration of how the different layers bind together in the in situ hybridization technique.



Figs. 2–2A — 2–2F. Schematic representation of the procedure.

Fig. 2–2A. Color representation of the various steps.

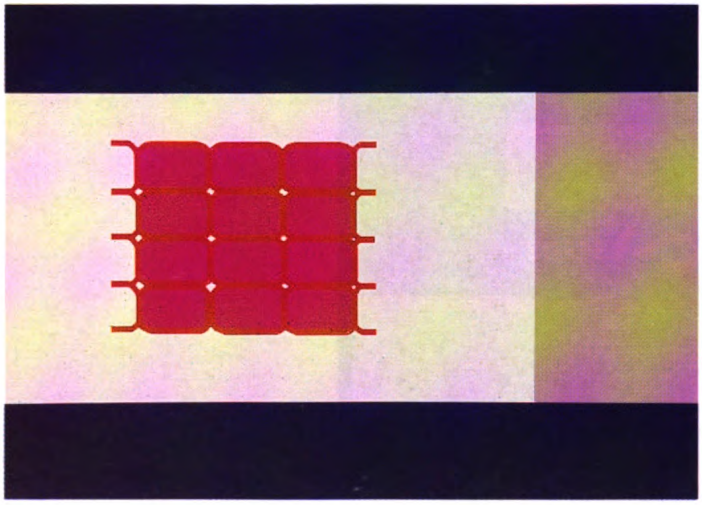


Fig. 2–2B. Tissue section, mounted on a glass slide.

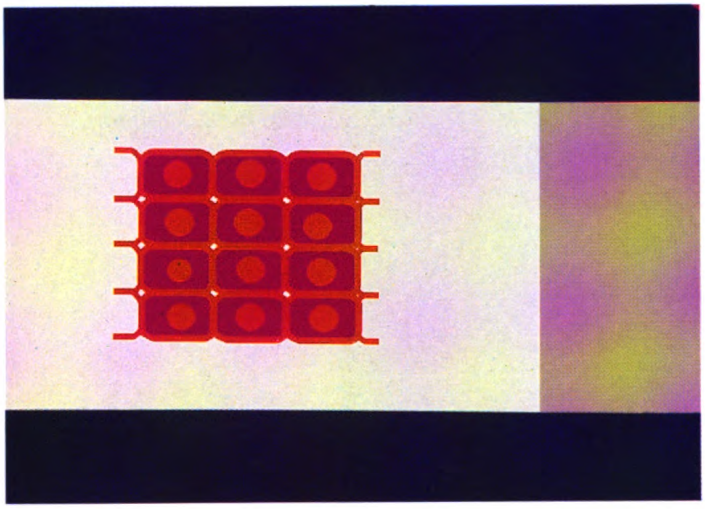


Fig. 2–2C. Section is denatured to make the DNA accessible.

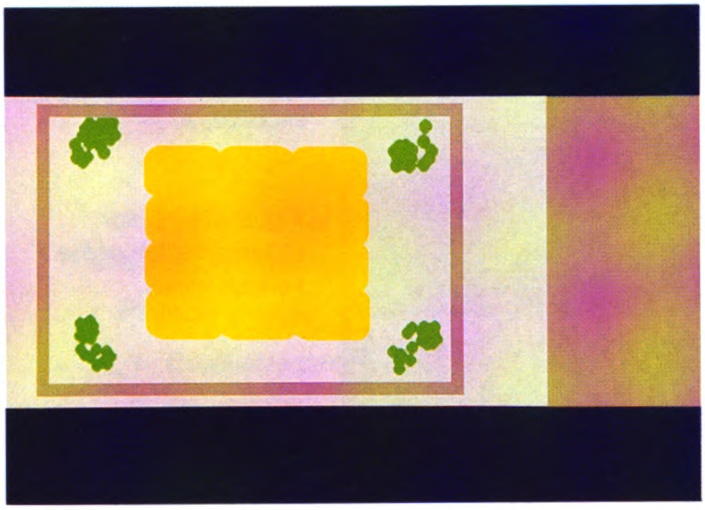


Fig. 2–2D. DNA probe, glass beads, and coverslip are added for the hybridization step.

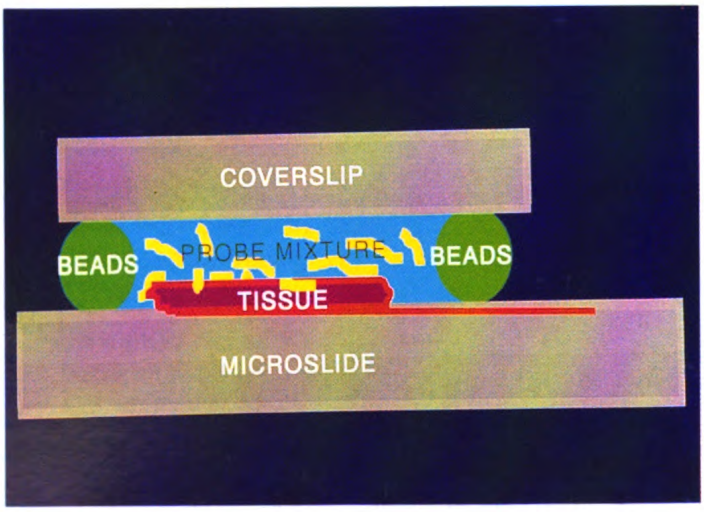


Fig. 2–2E Side view of Fig. 2–2D.

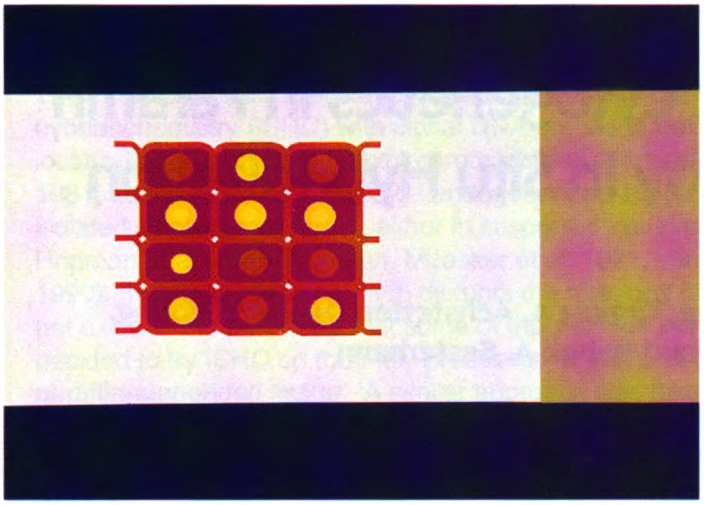


Fig. 2–2F. The detected DNA sequences are revealed by the chromogen.

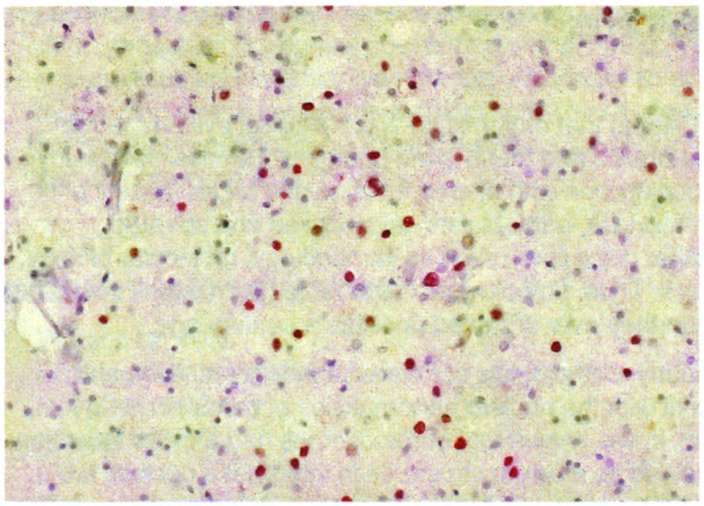


Fig. 2–3. Positive reaction with AEC for Jakob-Creutzfeldt virus in brain tissue.

Interphase Cytogenetics in Paraffin Sections by In Situ Hybridization

Frank A. Avallone, Virginia A. Achstetter, Denise Y. Young, and Isabell A. Sesterhenn

INTRODUCTION AND PRINCIPLES

In 1914, Ludwig Aschoff stated that morphology is a biological system, rather than a dead or inanimate object, and that it is the pathologist's mission to study structural alterations in conjunction with altered function and foster functional analysis. To accomplish this task, pathologists have utilized and adopted methods developed in other disciplines, such as immunology, molecular biology, and, recently, cytogenetics. Thus, pathologists have been able to study diseased tissues in more detail than they ever dreamed possible a century ago.

At the end of the last century, pathologists correlated abnormal mitoses and variable chromatin content with the nuclear abnormalities of cancer cells (Hansemann 1890). Also, it was known that chromosomes had a certain position in metaphases and remained as discrete structures in interphase nuclei (Rabl 1885). Somewhat later, Boveri postulated that chromosomal abnormalities cause malignant transformation and proliferation of cells.

Although DNA and RNA were discovered in the last century and the connection between abnormal chromosomes and tumors was made at the turn of the century, it has been within the last 50 years that modern techniques have enabled scientists to confirm the earlier theories. In 1944, Avery linked genes to DNA; in 1953, Watson and Crick described the double helix of DNA; and in 1986, Nelson and Coffey described the complex structure of chromosomes. Furthermore, although plant cytogeneticists have gathered extensive information since 1920, it was not until 1956 that Tjio and Levan detected the correct chromosome number of human cells. In 1960, Nowell and Hungerford described the first specific chromosome change in a tumor, and by 1987, cytogenetic information was available on 8,000 tumors. Eighty-seven percent of those were hematologic malignancies. In only 13% of solid tumors, some data about karyotypic abnormalities were available (Heim and Mitelman 1981). This discrepancy can be explained by the fact that most solid tumors grow only poorly in vitro. Also, it may

be difficult to determine if the metaphases are actually tumor derived or of stromal origin.

The introduction of molecular cytogenetics, particularly nonisotopic in situ hybridochemistry (ISHC) with clonal DNA probes targeting specific DNA, enables localization of individual human chromosomes in interphase nuclei (Bauman et al. 1981, Langer-Safer et al. 1982, Landegent et al. 1985). This can easily be done on isolated whole tumor nuclei, either in suspension or in smears (Cremer et al. 1986, Hopman et al. 1988, Hopman, Moesker et al. 1991, van Dekken et al. 1988, 1990). However, this approach disrupts the histology of the tumor and still does not exclude the possibility that some of the cells are nontumorous. Therefore, we decided to try ISHC on routinely processed sections of formalin-fixed, paraffin-embedded tissue. A similar approach was taken by Emmerich et al. (1989) and Walt et al. (1989) to evaluate interphase cytogenetics in testicular germ cell tumors and by Hopman, van Hooren et al. (1991) in bladder tumors.

We have applied the method described in this chapter to sections of bladder carcinomas (Sesterhenn, Mostofi, Davis, van Dekken, McCarthy, unpublished data), prostatic carcinomas (Sesterhenn et al. 1992), lymph nodes with follicular hyperplasias and follicular lymphomas (Abbondanzo et al. 1992), gestational moles, and streak gonads. It should also be mentioned that interphase cytogenetics is not only helpful in neoplastic diseases but also in clinical diseases such as sex chromosome abnormalities.

MATERIALS AND SOLUTIONS:

Water bath 70°C
Shaking water bath 40°C
Hot plate 98°C
Incubator 37°C
pH meter
Magnetic stirring hot plate
Microwave oven (Sigma, Emerson model, AT736A)
Micropipetters from 1 to 1,000 microliters
Square Bio-Assay culture dishes, 245 x 245 x 20 mm, (NUNC, Inc., [A.H. Thomas])
Vortex Geni, (A.H. Thomas)
Silane-coated glass slides or "Super Frost Plus" slides (Fisher Scientific)
Biotinylated DNA probes for chromosome centromeres (e.g. Oncor, Gaithersburg, MD; Imagenetics, Inc., Naperville, IL)
Mouse monoclonal antibody to biotin (Dako, Carpinteria, CA)
Biotinylated antimouse IgG (Vector, Burlingame, CA)
ABC complex (Vector, Burlingame, CA)
Carrier DNA - sonicated herring sperm (Digene, Silver Spring, MD)
Proteinase K (Tritirachium album) (Boehringer Mannheim, Indianapolis, IN)
3,3'-diaminobenzidine tetrahydrochloride - DAB (Sigma, St. Louis)
Copper sulfate (Fisher Scientific)

Imidazole
3-Aminopropyltriethoxysilane, Silane (Sigma, St. Louis)
Dextran sulfate (Sigma, St. Louis)

Silane solution for coating glass slides

3-Aminopropyltriethoxysilane ..... 12 ml
Acetone ..... 588 ml

Place precleaned slides in racks and soak in silane solution for 2 minutes with gentle agitation. Drain slides and rinse in 2 changes of deionized water. Change water every 2 to 3 racks. Dry slides in 60° C oven overnight. Cool and store slides in their original boxes. Do not touch slide surfaces. Fingerprints will become permanently attached and cannot be removed. This solution is usable for 8 hours. Do not allow water contamination of silane stock or silane/acetone solution. This renders the solutions ineffective.

Blocking solution for endogenous peroxidase (0.6%)

Methanol ..... 250 ml
30% hydrogen peroxide (H_2O_2) ..... 5 ml

This solution should be made prior to use and is used to prevent staining of endogenous peroxidase activity in the tissue, which could interfere with specific immune staining.

CAUTION: Hydrogen peroxide is a very strong oxidant and will burn. Wear gloves.

0.1N HCl with 0.05% Triton X-100

0.1N HCl (Fisher Scientific) ..... 1,000 ml
Triton X-100 (Sigma, St. Louis) ..... 0.5 ml

0.1M Tris buffer, pH 7.5

Tris HCl ..... 12.7 g
Tris base ..... 2.36 g
dH_2O to a final volume of ..... 1,000 ml

Adjust pH to 7.5 with 1N HCl or 1N NaOH as required.

Phosphate buffered saline (PBS) (Dulbecco)

Sodium chloride (NaCl)	32.0 g
Sodium phosphate dibasic (Na_2HPO_4)	4.6 g
Potassium phosphate monobasic (KH_2PO_4)	0.8 g
Potassium chloride (KCl)	0.8 g
dH_2O to a final volume of	4,000 ml

Adjust pH to 7.4 with 1N HCl or 1N NaOH as required.

5% nonfat dry milk (NFDM)

NFDM	0.5 g
PBS	10 ml

This solution should be prepared fresh each time. However, if an antibacterial agent like 0.1% Thimerosal (Sigma, St. Louis) is added, the solution can be kept for a week or more, if stored at 0° to 4°C. Discard if bacteria appear on slides.

20X Saline sodium citrate - 20X SSC (Digene, Silver Spring, MD)

2X Saline sodium citrate - 2X SSC

20X SSC	100 ml
dH_2O	900 ml

Adjust pH to 7.0 with 1N HCl or 1N NaOH, as required.

Formamide solution, ultrapure (Boehringer Mannheim, Indianapolis, IN)

If formamide is not ultrapure, commercial preparations may contain significant amounts of the hydrolytic product, ammonium formate, which interferes with the procedure. Deionization is then necessary and is accomplished by mixing 50 grams of AG 501-X8 D Resin (Bio Rad, Hercules, CA) in 1 liter of formamide. Mix with a magnetic stirrer at 4°C for 1 hour. Filter into a clean, dry, amber bottle for storage. Smell the just-opened bottle before use to assure that there is no acrid odor of ammonia. High amounts of ammonium formate render formamide unusable for this protocol. Purchase high-grade formamide with an expiration date of at least 8 to 12 months after purchase and store unopened bottles (Boehringer Mannheim) at room temperature. Purchase formamide in small quantities (100- to 500-ml bottles) to avoid excessive waste. Usage should determine quantity size. Store protected from light.

DNA carrier

Sonicated herring sperm ..... 950 µl
dH_2O ..... 50 µl

Vortex solution for 5 seconds. This solution is kept at 0° to 4°C and is stable for several months. Keep 1-ml stock aliquots at -20°C in a standard freezer, not a frost-free one.

Hybridization mixture

	60%	70%
Formamide*	12 ml	14 ml
Dextran sulfate	2.5 g	2.5 g
Tween 20	2.0 µl	2.0 µl
20X SSC	2.0 ml	2.0 ml
dH_2O	6.0 ml	4.0 ml
Total volume	20.002 ml	20.002 ml

Adjust pH to 7.0 with 0.1N HCl. Dispense in 1-ml aliquots and store in a standard household freezer at -20°C. **Do not use a frost-free freezer.**
*See page 61.

Probe mixture per slide

Probe ..... 1.5 µl
DNA carrier ..... 1.5 µl
Hybridization mixture ..... 12.0 µl
Total volume ..... 15.0 µl

This amount (15 µl) should be enough to cover an area under a 22 x 22-mm coverglass. For larger sections, prepare additional mixture in the same proportions.

Denaturation solution

70% formamide in 2X SSC

Deionized formamide ..... 70 ml
20X SSC ..... 10 ml
dH_2O ..... 20 ml

Total volume ..... 100 ml

Adjust pH to 7.0 with 1N HCl.

Posthybridization wash solution

Formamide	70 ml
20X SSC	10 ml
dH_2O	20 ml

This solution contains 70% formamide. Percentage of formamide depends upon the chromosome-specific probe to be hybridized. Some probes are better washed out with a solution that contains 50% or 60% formamide. Do not alter 20X SSC volume when making different percent solutions.

1M Imidazole enhancer

Imidazole	68.08 g
dH_2O to final volume of	1,000 ml

(Final concentration of imidazole in PBS/DAB solution is 0.01M.)

DAB stock solution

3,3'-diaminobenzidine tetrahydrochloride	4.5 g
dH_2O	100 ml

Using a magnetic hot plate, dissolve DAB powder in warm dH_2O water (40° to 47°C) with agitation. When completely dissolved, dispense into 1-ml aliquots and freeze. **CAUTION: Wear gloves, work in a safety hood, and treat as hazardous material. DAB is a suspected carcinogen.**

DAB working solution (0.015%)

Stock solution 1 vial	1 ml
PBSD	296 ml
Imidazole (enhancer)	3 ml
Total volume	300 ml

Add 0.4 ml 30% hydrogen peroxide just prior to use and mix well.

Copper sulfate - 0.5% in 0.9% NaCl

$CuSO_4 5H_2O$	7.8 g
NaCl	9.0 g
dH_2O	1,000 ml

Mayer's hematoxylin

Ethanol: 70%, 95%, 100%

Xylene

Mounting medium - Permount or other synthetic mounting media

HYBRIDIZATION PROCEDURE:

1. Cut paraffin sections at 4 to 6 microns and pick up on silane-coated slides. (Dry slides in 60°C oven for 30 minutes.)
2. Deparaffinize slides in 3 changes of xylene, 2 minutes each change, with gentle agitation.
3. 100% ethyl alcohol, 2 changes, 3 minutes each.
4. Place slides in blocking solution (methanol + 0.6% hydrogen peroxide) for 20 minutes. This prevents excessive background.
5. Wash in 2 changes of dH_2O, 2 minutes each.
6. Microwave slides in dH_2O for 10 minutes. (Use a plastic Coplin jar immersed in a plastic container containing tap water.) Note: In order to obtain consistent uniform results, strict adherence to protocol must be observed. Deviations will produce erratic results. (See Figs. 2–4, 2–5).
    a. Microwave ovens that possess a carousel tray are the instruments of choice. All other types of microwave ovens have hot spots within their chambers and as a result give erratic results.
    b. The same microwave treatment must always be used. Each microwave oven emits different amounts of energy per cubic inch. Always use full-power setting and time according to protocol. We have found that the Emerson Model AT736A, carried by Sigma, meets all our requirements.
    c. All containers must be microwavable plastic. **Do not use glass containers.**
    d. Use the same number of slides in each Coplin jar; use blank slides if necessary. Fill Coplin jar with the dH_2O to the very top and loosely cover with lid. **Do not tighten lid at any time. Do not put in microwave without lid in place.**
    e. Set Coplin jar in another plastic container containing plain water. The water level in the second container must be as high as possible without making the Coplin jar buoyant. Normally this is at least three-fourths of the way up the side of the Coplin jar. When this level has been determined, measure the water volume. Always use the same container and water volume each time the procedure is performed.
    f. Use extreme caution when handling containers after microwaving. The solutions will be extremely hot.
    g. Microwave for 5 minutes; check to be sure that there is no solution evaporation from the Coplin jar. If any evaporation is noted, fill Coplin jar back to original volume with tepid distilled water and microwave for an additional 5 minutes. The total time

of 10 minutes is critical.

h. All solutions must be at room temperature when starting either single or multiple runs.

7. Treat sections in 0.1N HCl with 0.05% Triton X-100 for 15 minutes.
8. Wash with dH_2O, 2 changes, 2 minutes each.
9. Incubate slides in 70°C, preheated, 2X SSC, pH 7.0, and leave in 70°C water bath for 30 minutes. (Fig. 2–6)
10. Wash in several changes of 0.1M Tris buffer, pH 7.5. This may also be a brief holding point.
11. Digest tissues by placing slides, leveled, in a moist environment and flooding them with prewarmed 37°C Proteinase K, diluted in Tris buffer. Cover the dish and incubate in a 37°C incubator. The concentration of the enzyme Proteinase K and the time of incubation has to be determined for each tissue type. Dilutions can vary from 1:100 to 1:5000 and incubation times from 2 to 3 minutes to 15 minutes or more. Enzyme concentrations and incubation times must be determined for each tissue type.

    Remove sections in same order as Proteinase K was applied. This will ensure constant digestion time on each slide.
12. Wash in several changes of dH_2O to stop digestion. This washing step is critical.

    Prepare probe mixture at this time and vortex probe mixture just prior to use for 5 seconds. If using Wet Method continue on page 66.

ISH DRY METHOD

13. Dehydrate slides through graded alcohols 70%, 95%, and 100%, 2 changes each, for 1 minute each.
14. Air-dry slides quickly by fanning them or by using a hair dryer on the cold setting.
15. Place probe mixture on top of sections; carefully place a coverslip on top of each section; express all air bubbles (Figs. 2–7, 2–8). Denature DNA probe and tissue DNA by placing slides on a 90° to 100°C hot plate for 10 minutes (Fig. 2–9). Timing is critical, as is temperature. We denature on an aluminum block placed in an oven (Fig. 2–10).
16. Hybridize slides in an environmental chamber (Fig. 2–11). Incubate at 37°C for 2 hours to overnight. The norm seems to be 24 hours. **Do not exceed 48 hours. Do not allow moist chamber to become dry at any time.** Continue on to Step 17.

ISH WET METHOD

13. Denature slides in denaturing solution, 70% formamide, prewarmed in a 70°C water bath. The probe mixture is denatured in the same water bath at the same time. Slides are denatured for 2 minutes and probe mixture (placed in a small vial and partially immersed in the water) for 5 minutes. To assure proper denaturation time, slides must be removed in the same order in which they were placed in the denaturing solution. (Fig. 2–6)
14. Dehydrate slides immediately through cold (0° to 4°C) graded alcohols: (70%, 95%, and 100%), 2 changes each for 1 minute each.
15. Air-dry slides quickly by fanning them.
16. Place probe mixture on top of sections as described under dry method, (Figs. 2–7, 2–8) and hybridize slides in a moist environment in the incubator at 37°C for 2 hours to overnight (Figs. 2–11, 2–12).

SECOND DAY

17. Wash slides in prewarmed posthybridization washing solution, (50% to 70% formamide, depending on probe), 2 changes, in a shaking water bath (not exceed 19 to 21 cycles per second) at 40°C, for a total time of 15 minutes. Use 2 changes: first change 1-2 minutes, second change 13 to 14 minutes.
18. Wash in prewarmed (40°C) 2X SSC, 2 changes, in a shaking water bath for a total of time of 15 minutes.
19. Place slides, leveled, in a moist environment and cover sections with 2 to 3 drops (depends on size of tissue) of mouse monoclonal antibody to biotin (Biotin, Dako) diluted 1:100 in 5% NFDM. Place coverslip on top of the sections and incubate them at 37°C for 30 minutes. It is imperative that all air bubbles be dispersed (Fig. 2–12).
20. Rinse in PBS, several changes, a total of 20 minutes.
21. Incubate sections with biotinylated antimouse IgG, diluted 1:400 in 5% NFDM, 37° to 40°C, for 30 minutes. (NFDM, as in Step 19, is used to reduce background staining of endogenous avidin.)
22. Rinse in PBS, several changes, a total of 20 minutes.
23. Cover sections with 1 to 2 drops ABC complex (made in PBS only) and incubate sections as in Steps 19 and 21, (37° to 40°C) for 30 minutes.
24. Rinse in PBS, several changes, a total of 20 minutes.
25. Transfer slides to a Coplin jar and stain in DAB with imidazole as an enhancer for 8 minutes.
26. Rinse slides in several changes of distilled water.
27. Enhance DAB coloration with 0.5% copper sulfate in sodium chloride for 5 minutes.

28. Wash in several changes of tepid H_2O.
29. Counterstain in Mayer's hematoxylin for 1 to 5 seconds. Discretion of user.
30. Wash in tepid H_2O, or alkaline H_2O, to blue nuclei.
31. Dehydrate slides through graded alcohols, 70%, 95%, and 100%, 2 changes, for 3 minutes each.
32. Clear in xylene, 2 changes, 5 minutes each.
33. Mount in resinous mounting medium.

RESULTS:

Reaction products appear as dark-brown to black dots in the nucleus. In normal cells, autosomes should be visible as 2 spots per nucleus. Sex chromosomes in females: 2 spots for X chromosome; 0 spots for Y chromosome. In males: 1 spot for X and Y each. See Figures 2–13 through 2–20B.

TROUBLESHOOTING:

- Minor binding sites
    1. Deterioration of formamide.
    2. Excessive heating during denaturation.
    3. Too-low concentration of formamide in hybridization mixture and/or posthybridization wash.

- Other factors that give poor or no results:
    1. Poorly fixed tissue through overfixation or underfixation, or old acidified formalin.
    2. Overdigestion or underdigestion of tissue.
    3. Too-high concentration of formamide in hybridization mixture or posthybridization wash.
    4. Improper immunohistochemical procedure.
    5. Allowing slides to dry after Step 19.

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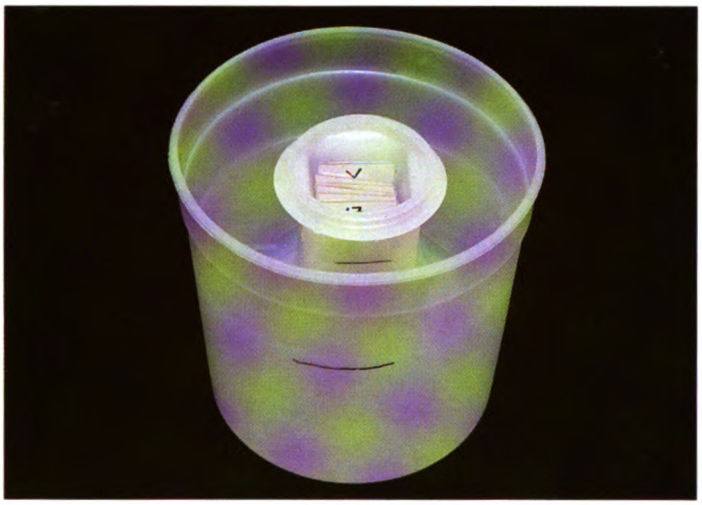


Fig. 2–4. Preparing slides in plastic Coplin jar for microwaving. Step 6, Wet and Dry Methods.

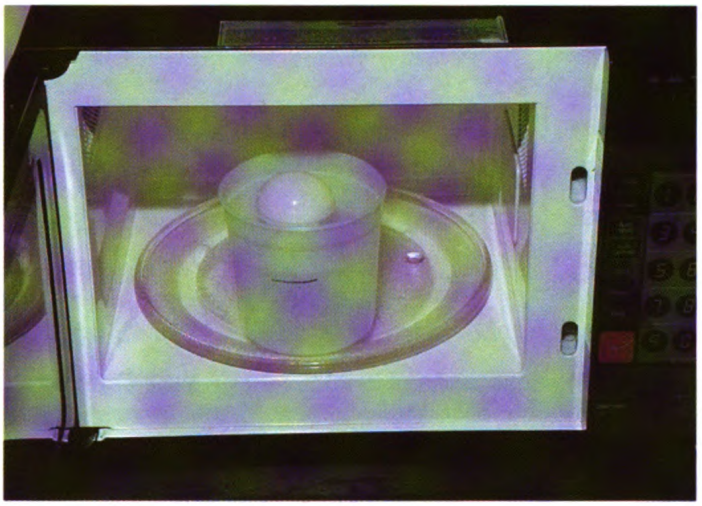


Fig. 2–5. Placement of container on microwave carousel tray prior to microwaving. Step 6e, Wet and Dry Methods.

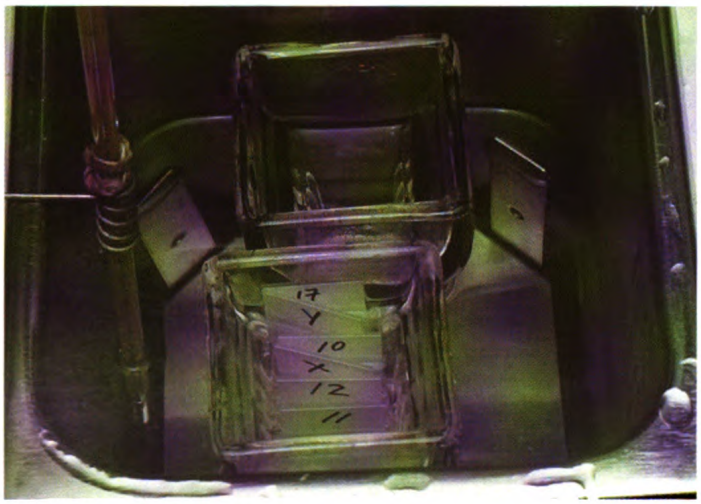


Fig. 2–6. Slides in Coplin jar placed in 70°C waterbath in pre-warmed 2X SSC. Additional container of pre-warmed formamide denaturing solution.***

** Step 9, Wet and Dry Method.*

*** Step 13, Wet Method Only.*

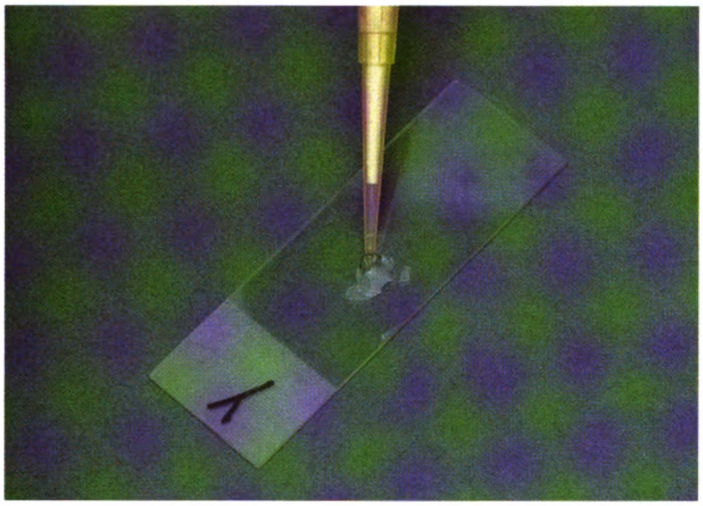


Fig. 2–7. Applying probe to section. Step 15, Dry Method. Step 16, Wet Method.

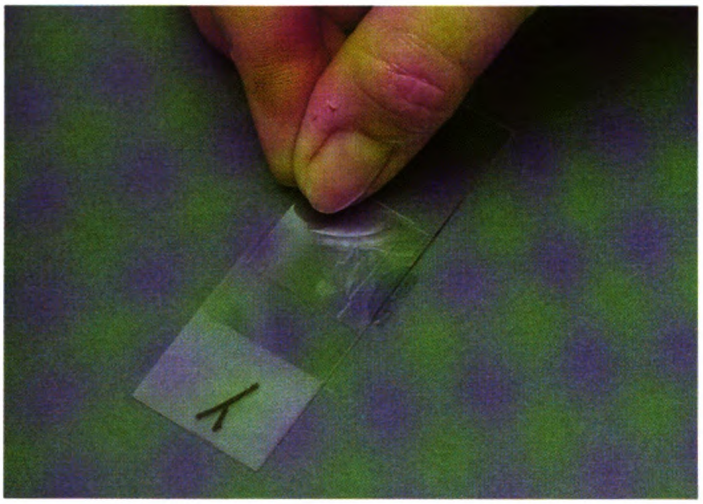


Fig. 2–8. Applying cover slip over section after probe has been dispensed. Step 15, Wet and Dry Method.

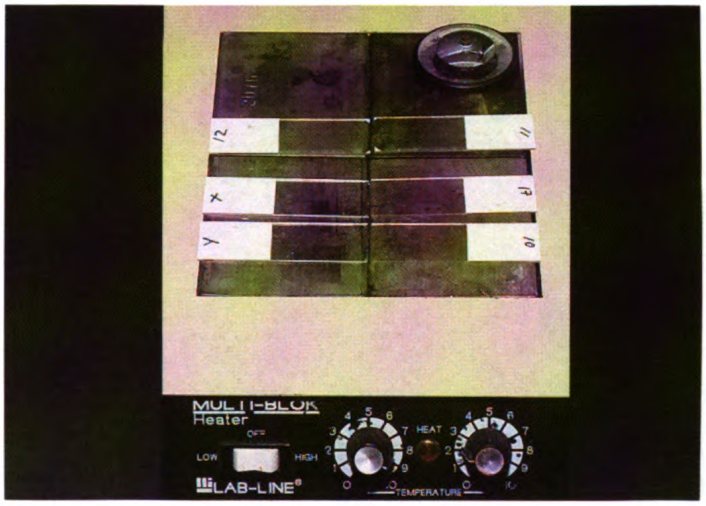


Fig. 2–9. Denaturing using dry heat block 98° to 100°C.

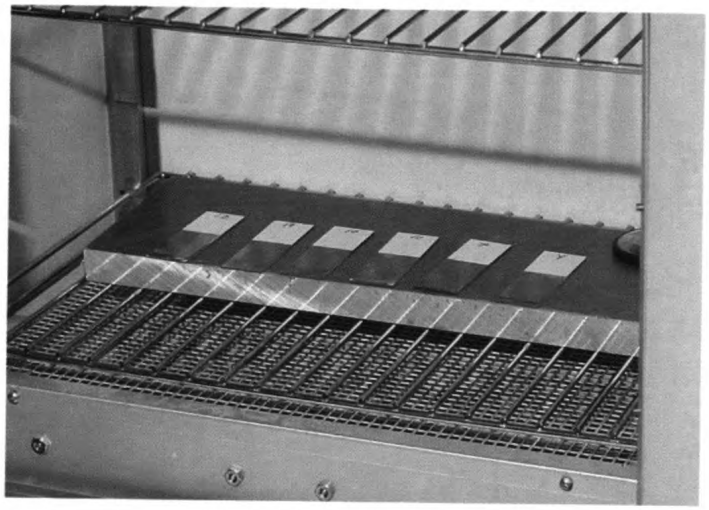


Fig. 2–10. Denaturing using dry heat plate in oven. Step 15, Dry Method. Note: Surface thermometer must be used to adjust temperature of block. Ambient temperature of oven will not give correct temperature of block due to its location, lowest point in oven. Once determined, temperature will remain very constant.

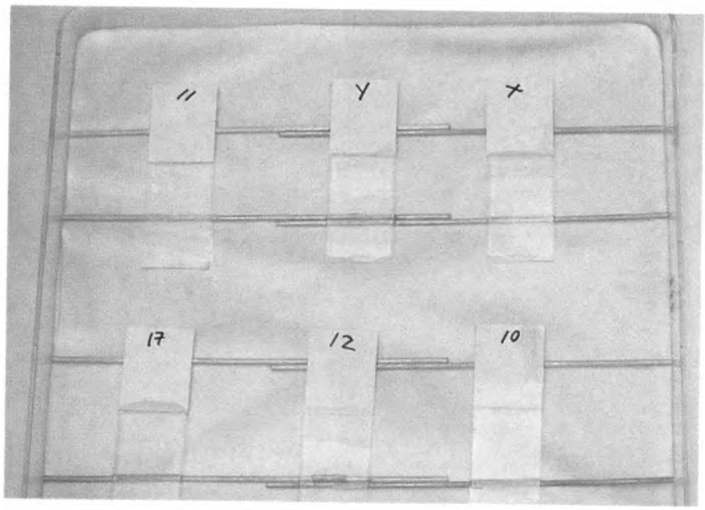


Fig. 2–11. Preparing slides for hybridization at 37°C using a square culture dish 245 x 245 x 20 mm.
Step 16, Wet and Dry Methods. This same container is used in Steps 11, 19, 21, and 23, Wet and Dry Methods.

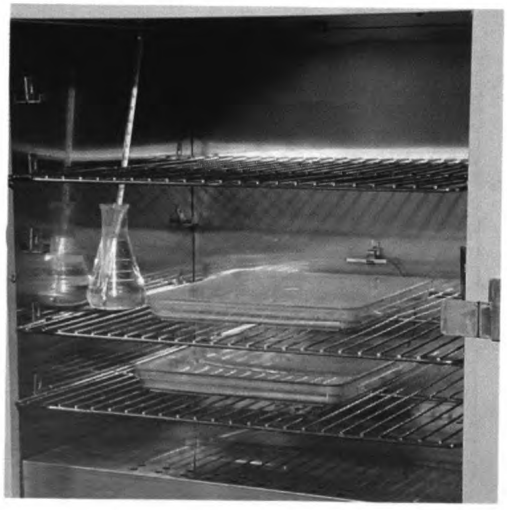


Fig. 2–12. Culture dishes with slides in 37°C incubator. Used in all steps indicated in protocol.

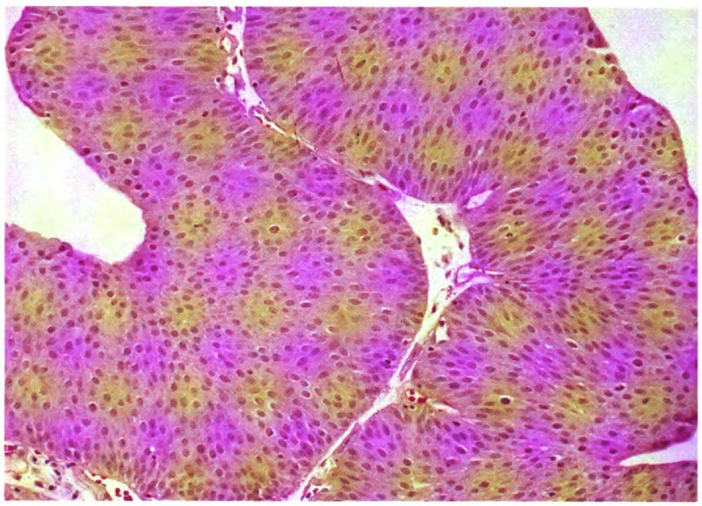


Fig. 2-13. Bladder biopsy. Transitional cell carcinoma, Grade 1. H&E stain.

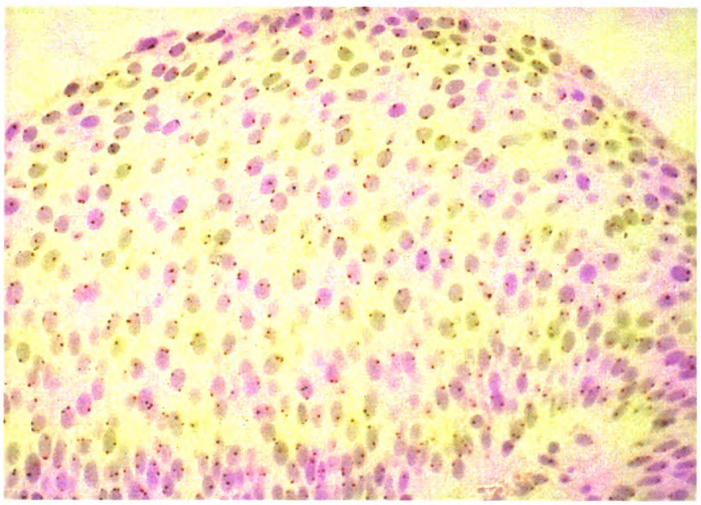


Fig. 2–14. ISHC for Chromosome 1 centromere. Same case as Fig. 2–13. Mostly 2 dots per nucleus. Diploid tumor.

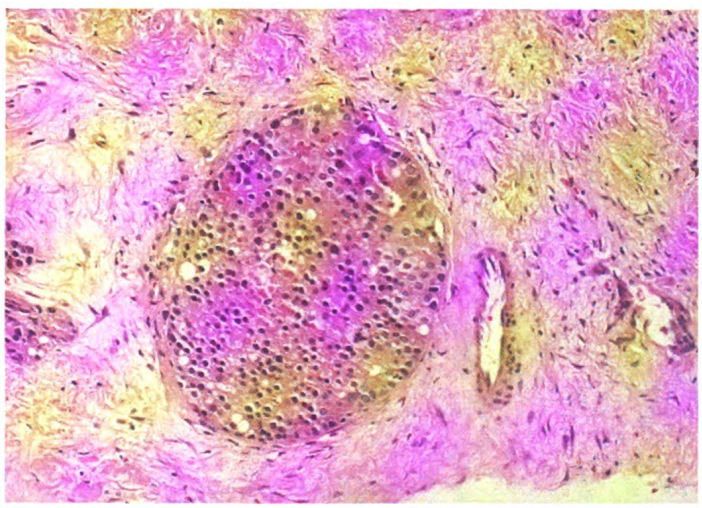


Fig. 2–15. Prostate biopsy. Poorly differentiated carcinoma. H&E stain.

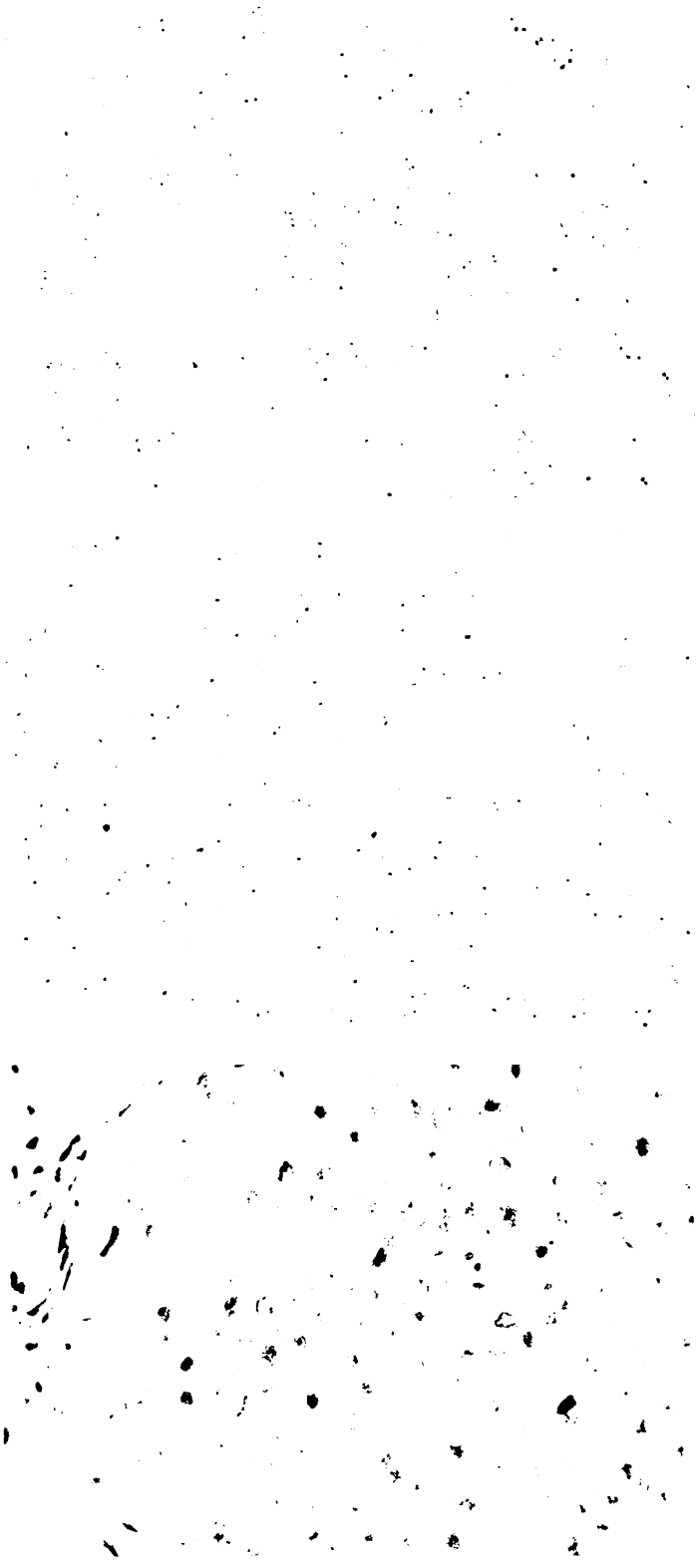


Fig. 2–16. ISHC for chromosome 17 centromere. Same case as Fig. 2–15. Note there are up to 4 dots per nucleus.

Fig. 2–17. ISHC for chromosome X. Same field as Figs. 2–15 and 2–16. A number of nuclei show 2 dots instead of 1 dot.

Fig. 2–18. ISHC for chromosome Y. Same fields as Figs. 2–15, 2–16, and 2–17. One dot in each nucleus.

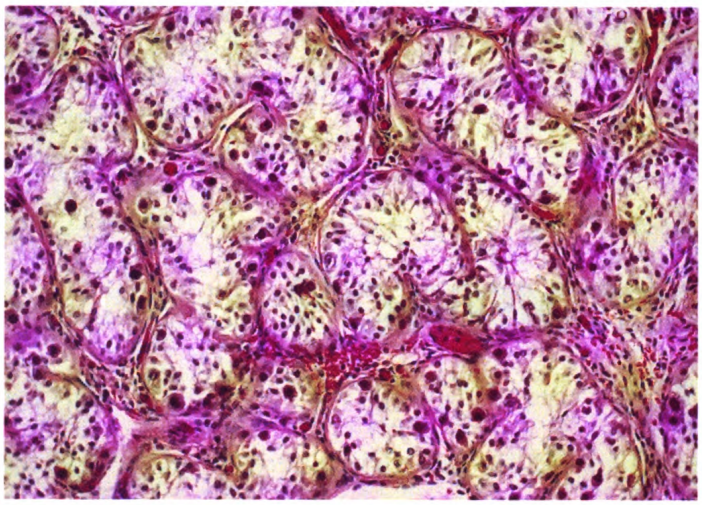


Fig. 2–19. Intratubular malignant germ cells. H&E stain.

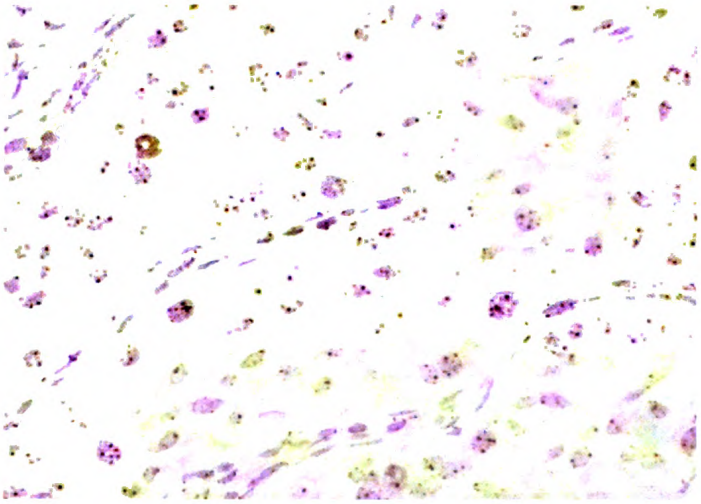


Fig. 2–20A. ISHC for chromosome 12 centromere with poor result. Same case as Fig. 2–19. Minor binding sites.

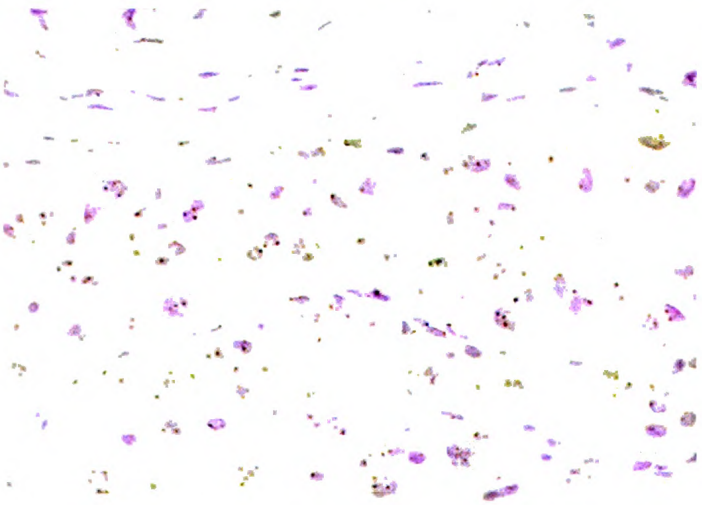


Fig. 2–20B. ISHC for chromosome 12 centromere. Same case as Figs. 2–19 and 2–20A. The result is good after more stringent post-hybridization wash.

3

Polymerase Chain Reaction

Ann H. Reid

The polymerase chain reaction (PCR) is used to make large numbers of copies of a specific DNA sequence. Normal human genes are present at the level of two copies per cell, and viral or bacterial pathogens may be present at even lower concentrations. Standard laboratory techniques are unable to detect specific DNA sequences at such low levels. PCR allows the DNA to be copied over and over again (a process called amplification) until it is present in sufficient quantity to be easily detected. PCR can be used to demonstrate the presence or absence of a gene; to detect mutation, amplification, or rearrangement of a gene; and to detect viral or bacterial DNA.

The DNA double helix is made up of two strands, each of which consists of a chain of deoxyribonucleotides. The backbone of the helix is formed by a series of phosphodiester bonds in which the 5' carbon of one sugar is bound to the 3' carbon of the next sugar by a phosphate. Thus, a DNA strand is said to have directionality. In a double helix, one strand runs in the 5' to 3' direction, while its complement runs in the 3' to 5' direction. The nitrogenous bases stack one on top of the other within the helix. There are four nucleotides that make up DNA: two purines, adenine and guanine, and two pyrimidines, cytosine and thymine. Each of these is bound by a hydrogen bond across the double helix with only one partner; thus, adenine is always found paired to thymine, and guanine is always found paired to cytosine. The exclusivity of this binding means that the sequence of one strand of DNA can always be deduced from the sequence of the other. (For more information on DNA structure, see reference 3.) The polymerase chain

reaction is based on the ability of DNA polymerase to make a strand of DNA using the complementary strand as a template.

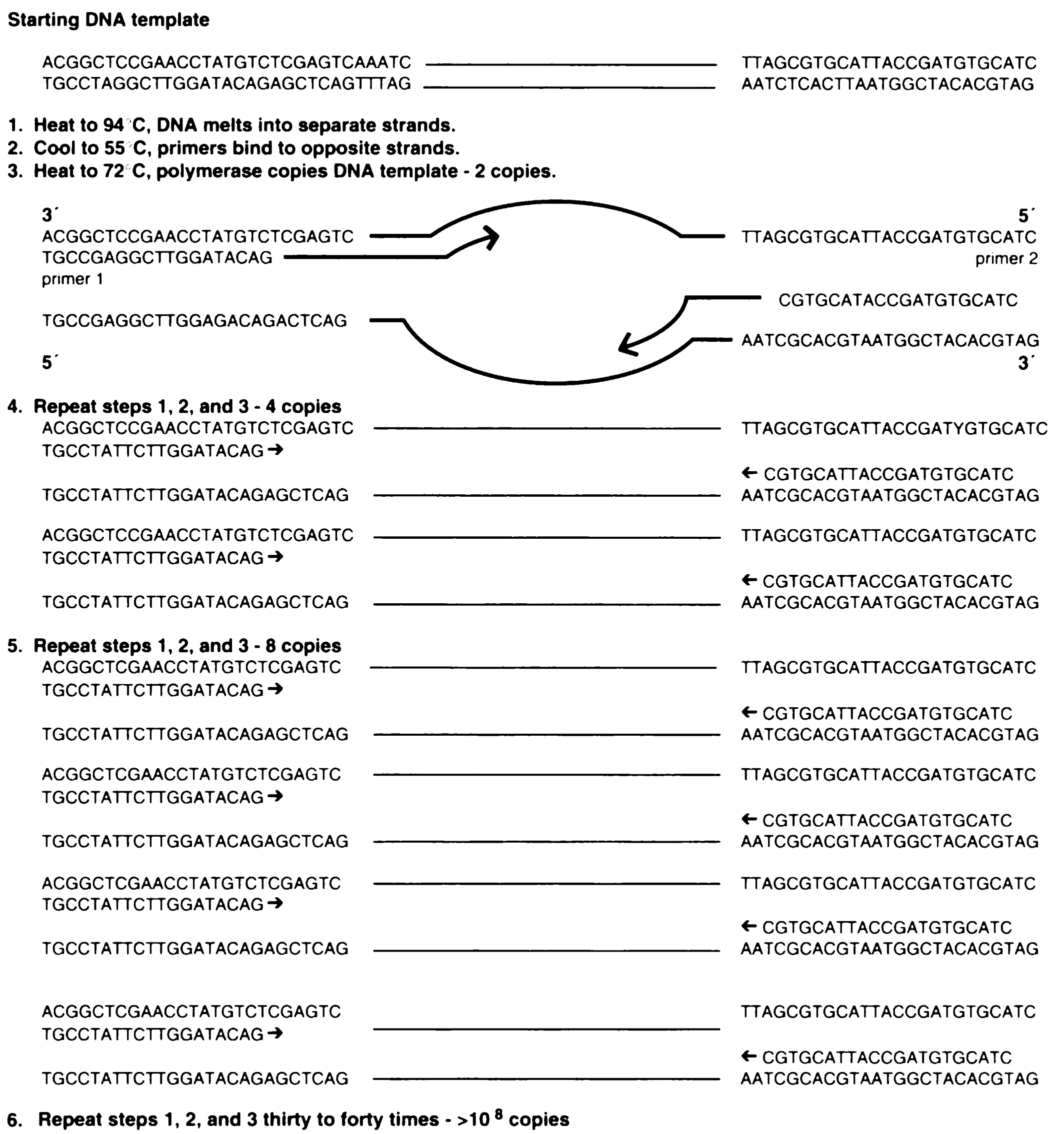
Automated PCR, which will be described in this chapter, depends on Taq DNA polymerase, which is isolated from thermophilic bacteria and is capable of withstanding very high temperatures. Two short lengths of single-stranded DNA, known as primers, are designed to bind to the ends of the DNA to be copied. The target DNA, combined with a large molar excess of the two primers, is heated to 94°C, which "melts" the DNA into single strands. The reaction is then cooled to between 37°C and 60°C, allowing one primer to bind to the sense strand and one to the antisense strand. The temperature is then raised to 72°C, the optimum temperature for the DNA polymerase, which adds nucleotides to the ends of the primers, producing one copy of each strand of the target DNA. This process is then repeated 30 to 40 times, with the number of copies of the target DNA doubling with each repetition (see Fig. 3–1). Theoretically, 40 cycles of PCR would result in over two trillion copies of the target sequence. In practice, 40 cycles of amplification yields a detectable amount of DNA in about 90% of cases, using paraffin-embedded, formalin-fixed tissue.

The PCR procedure is very simple. A master mix is made, containing 10X polymerase buffer, magnesium chloride, nucleotides, and primers. This mix is aliquotted into reaction tubes along with a small bead of specially formulated wax. These components are heated and then allowed to cool; the wax then forms a barrier over the reaction components. The DNA template and polymerase are then layered over the wax, and the tubes are placed in a thermal cycler, which performs the temperature changes needed to repeat the priming/synthesis/melting cycles.

When the amplification is complete, the next step is to determine which of the samples contained the target DNA. The product of the amplification reaction is loaded onto an agarose gel containing ethidium bromide, a fluorescent dye that binds to DNA. An electric current is then applied, and DNA migrates toward the positive pole at a rate inversely proportional to its size. When the gel is viewed under UV light, the DNA appears as thin bands parallel to the loading wells. The use of agarose gel electrophoresis allows a rapid assessment of the size and amount of DNA produced.

For many PCR applications, especially on formalin-fixed, paraffin-embedded tissues, 40 cycles of PCR do not produce enough product to be visible by ethidium bromide staining. Furthermore, ethidium bromide stains any nonspecific DNA produced in the PCR reaction as well as the target DNA. Therefore, it is often necessary to transfer the DNA from the gel to a nylon membrane and probe it with a radioactively labelled probe. The probe is a 30 to 40 base length of DNA complementary to a section of the expected PCR product between the two primers and which has been labelled at its 5' end with ^{32}P. After the probe is allowed to hybridize to the nylon membrane, the membrane is washed and placed on X-ray film. Wherever the probe has bound to DNA on the membrane, the X-ray film will be exposed. Positive samples will show a thin black line on the X-ray film at the appropriate distance from the loading wells. Probing with

Fig. 3–1. **The polymerase chain reaction**: each cycle doubles the number of copies of the target sequence.



radioactive probes improves sensitivity at least a hundredfold over ethidium bromide staining and also demonstrates that the specific target DNA has been amplified. (See Figs. 3–10A and 3–10B)

While the polymerase chain reaction is a straightforward technique, it is subject to some difficulties. First, the primers must be carefully designed so that they bind only to the intended DNA target and neither fold upon themselves nor bind to

each other. Second, the reaction conditions, such as priming temperature and magnesium concentration, must be optimized for each primer pair. Finally, scrupulous care must be taken to ensure that there is no contamination of the starting material.

QUALITY ASSURANCE

PCR is so sensitive that contamination of a sample with as little as one molecule of target DNA or previously amplified DNA will lead to a spurious positive signal. As a result, rigorous negative controls and careful observance of procedures for extracting DNA are crucial.

The most common source of contamination is from previously amplified product. Therefore, the following rules must be followed at all times.

1. Manipulation of samples prior to the PCR must be carried out in a different room from the analysis of amplified product. It is crucial to have two entirely separate sets of equipment—pipets, centrifuges, microcentrifuge tubes, and racks—for use with unamplified versus amplified DNA. **Under no circumstances should amplified DNA be brought into the PCR room. This includes storage of product, blotting of product-containing gels, centrifugation of product, or incubation of product-enzyme mixtures in water baths.**
2. Gloves should be changed whenever entering the PCR room.
3. When making up buffers and adding them to samples, use either disposable pipets or pipetters clearly labeled "NO DNA." A separate set of pipetters must be used to add sample lysate to PCR mix. If there is any suspicion that the pipetters have been contaminated, they should be carefully dismantled and the barrel and plunger should be washed in detergent, rinsed with alcohol, and allowed to dry. The handgrip should be wiped off with alcohol. Be careful not to lose the O-rings—note how they are arranged when you dismantle the pipetter. Aerosol-resistant pipet tips, which have a plug between the tip and the pipet, may help prevent the contamination of the pipetter with aerosolized reagents.

Because the opportunities for contamination are so numerous, appropriate negative controls must be included with every set of samples. These controls will be described at the appropriate points in the procedures to follow.

EXTRACTION OF DNA FROM FORMALIN-FIXED, PARAFFIN-EMBEDDED TISSUE

PRINCIPLE:

In the polymerase chain reaction technique, DNA polymerase makes repeated copies of a specific sequence of DNA. In order for the DNA contained in a formalin-fixed, paraffin-embedded (FFPE) tissue sample to be made available in a PCR reaction, the tissue must be deparaffinized and digested with protease. Once the DNA has been released into the lysis buffer, cellular debris can be centrifuged out and the lysate is suitable for use in a PCR reaction to amplify any DNA target sequence.

MATERIALS AND SOLUTIONS:

Gloves
2 small beakers
1.5-ml microcentrifuge tubes
Microtome
Extra microtome blades
Micropipetter
1- or 2-ml pipets
Microcentrifuge
Sterile cotton swabs or 5-µl microcapillary pipets
Water bath set at 55°C
Water bath or heat block set at 95°C
Xylene
Absolute ethanol

Proteinase K (2.5 mg/ml in water, store at -20°C)

1M KCl (100 ml):

KCl ..... 7.46 g
H_2O to final volume of ..... 100 ml

0.5M Tris buffer, pH 8.3 (100 ml):

Tris-base ..... 3.52 g
Tris-HCl ..... 3.31 g
H_2O to final volume of ..... 100 ml

1M $MgCl_2$ (100 ml):

$MgCl_2$	9.52 g
H_2O to final volume of	100 ml

Extraction buffer (store in 1-ml aliquots at -20°C):

	stock concentration	final concentration	amount to add
KCl	1.0M	50.0mM	0.5 ml
Tris, pH 8.3	0.5M	10.0mM	0.2 ml
$MgCl_2$	1.0M	2.5mM	25.0 µl
Tween 20	100%	0.45%	45.0 µl
Nonidet P-40	100%	0.45%	45.0 µl
H_2O			9.2 ml

PROCEDURE:

1. Read the quality assurance section of this chapter before beginning.
2. Wear gloves.
3. Change the microtome blade between blocks, if possible. If this is impossible, the blade must be wiped with xylene and ethanol between samples. The microtome itself and any tools that may have touched the specimen must also be wiped off with xylene.
4. Place two 6-micron sections into each of two sterile 1.5-ml microcentrifuge tubes.
5. Cut an additional section to be mounted and stained with hematoxylin and eosin. This will be used to verify the presence of the feature of pathological interest.
6. Pour approximately 1 ml/sample of xylene into one beaker and approximately 2 ml/sample of ethanol into another beaker. Do not pipet directly from stock bottles.
7. Thaw 1 ml extraction buffer for every nine samples, Add 25 µl of 2.5 mg/ml Proteinase K. Keep on ice until ready to use.
8. Include an empty tube with each set of samples. Label this tube "paraffin negative control." Treat this empty tube exactly the same as a sample tube.
9. Add 800 µl of xylene to each sample and vortex at full speed for 5 seconds.
10. Add 400 µl of ethanol, vortex at full speed for 5 seconds, and centrifuge at full speed for 5 minutes.

11. CAREFULLY decant the liquid. Resuspend the tissue in 800 µl of 100% ethanol, vortex at full speed for 5 seconds, and centrifuge at full speed for 5 minutes.
12. Remove the ethanol by decanting or pipetting and remove residual ethanol with a microcapillary pipet or cotton swab.
13. Resuspend the pellet in 100 µl extraction buffer + Proteinase K. Incubate in a water bath at 55°C for 1 hour.
14. Place in a water bath or heat block at 95°C for 10 minutes to inactivate the Proteinase K.
15. Centrifuge at full speed for 5 minutes to pellet debris. The supernatant now contains the DNA from the lysed cells and is called the lysate. The sample can be stored with the pelleted debris in place. Label the tube and store at -20°C.

NOTES:

1. The specimen may consist of any paraffin-embedded tissue fixed in formalin or non-cross-linking fixatives, such as ethanol. Specimens that have been fixed in B-5 or other mercury-based fixatives do not perform well in PCR reactions.
2. The most common problem in this protocol is loss of sample because the pellet does not stick to the microcentrifuge tube after Step 4.
   (a) If you experience difficulty because none of your samples stick to the tube after centrifugation, try different brands of microcentrifuge tubes until you find one to which most samples stick.
   (b) If an occasional sample floats off after centrifugation, either try pipetting out most of the xylene-ethanol mixture before adding pure ethanol or, if the problem is severe, add more ethanol to the xylene-ethanol mixture and recentrifuge.
3. If after PCR the paraffin negative control gives a positive signal, discard all reagents used for that set of samples and repeat the extraction using decontaminated micropipetters and disposable plasticware.
4. Removal of ethanol after Step 12 is important. If too much ethanol is left in the tube, the PCR reaction may be inhibited. The goal should be to leave no more than one or two microliters in the tube. If a large proportion of samples fail to amplify the positive control gene (more than 20%), it would be appropriate to make sure that virtually all the alcohol is being removed at this step.

RESULTS:

Lysate is suitable for use as template DNA in a standard PCR reaction. Each set of cases has an internal negative control— the paraffin negative control—to demonstrate that no contamination was introduced during the extraction process.

OPTIMIZING PCR FOR A SPECIFIC APPLICATION

PRIMER DESIGN:

PCR can be used to detect any human, bacterial, viral, or other pathogenic DNA sequence. As long as enough is known about the sequence to design primers flanking the region of interest, PCR can be attempted. For many applications, such as detecting common pathogens or frequently mutated alleles, primer pairs are commercially available from a variety of suppliers. Published applications will always include the primer sequences, which can then be ordered from a custom synthesis supplier. For new applications, it will be necessary to design the primers. Briefly, the following general rules should be used when designing primers for use with formalin-fixed, paraffin-embedded samples:

1. Locate the primers between 150 and 250 base pairs apart.
2. Make each primer approximately 20 bases long.
3. The forward primer binds to the strand running in the 3' to 5' direction. Its sequence will be identical to the 5' to 3' strand. The reverse primer binds to the other strand. Its sequence will be identical to the 3' to 5' strand. By convention, published sequences are reported in the 5' to 3' direction. Therefore, the forward primer should be identical to the published sequence for the gene of interest. The reverse primer would bind to the published sequence and therefore must be its reverse complement. When the two primers are bound to the target DNA, their 3' ends should be pointing towards each other (see Fig. 3-1).
4. About half of the bases in each primer should be G's and C's. Try to make the final 3' base a G or C.
5. Once two sequences are tentatively identified, compare them carefully to each other. Make sure they cannot bind to each other for stretches of more than two or three bases. Check each primer for internal complementarity that may lead to folding.
6. Look at the last five 3' bases of each primer. Check the entire target sequence and ensure that there is only one sequence that exactly matches those five bases. Since the polymerase adds on to the 3' end of the primer, the greatest potential for mispriming is at the 3' end. Mismatches can be tolerated at the 5' end.

CONTROLS:

It is also necessary to include the appropriate controls for any PCR application.

Because PCR is so sensitive, it is crucial to include controls that can help rule out the possibility of false-negative and false-positive results. Three control reactions must be included with every set of samples:

1. **Paraffin negative control**—one empty tube should be included when cases are being deparaffinized and lysed. The empty tube should go through the same steps (xylene, alcohol, Proteinase K digestion) as the sample cases. If the lysate from the empty tube gives a positive signal after PCR, it means that contamination was introduced during the extraction process. If this is the case, all of the cases in that batch must be reextracted (starting from new paraffin slices) using entirely new reagents.
2. **PCR negative control**—one empty tube should be included when setting up the PCR reactions themselves. Distilled water should be added instead of sample lysate. If this sample gives a positive signal after PCR, contamination has been introduced during the PCR process itself. If this is the case, all of the cases in that batch must be reamplified. The same lysates may be used, as the contamination may have been introduced after the extraction process. If upon reamplification the PCR negative control is negative and the paraffin control is positive, the entire process must be repeated, starting from fresh paraffin slices.
3. **Positive control**—every set of reactions also requires a positive control to demonstrate that the PCR is working properly. The positive control DNA should be used at a dilution that gives a consistently clear positive signal but not an overwhelming amount of product. Since previously amplified product is the most common source of contamination, it is desirable to reduce to a minimum the amount of product generated unnecessarily.

Finally, if the purpose of the PCR is to verify the presence or absence of a rare DNA sequence (such as a pathogen or a translocation), it is necessary to demonstrate that the sample contains enough DNA of sufficient quality to allow successful PCR. A significant proportion of formalin-fixed, paraffin-embedded cases (up to 10%) are of such poor quality that the DNA is too degraded to be amplified. In such cases, failure to see an amplified product does not prove that the sequence of interest is absent. Therefore, when PCR is used to look for the presence or absence of a sequence, it is important to amplify a control gene from each case. The control gene can be any DNA sequence that is known to be contained in every cell. Primers for many single-copy genes can be purchased from a variety of suppliers, and any of these can be used. The control primers should be chosen so that they amplify a sequence of approximately the same length as the target sequence primers. If a case gives a positive signal for the control gene and a negative signal for the target gene, one can conclude that the target sequence, if present at all, is present at a level less than two copies per cell. If a positive signal cannot be obtained for the control gene, the case must be considered unsuitable for analysis by PCR.

OPTIMIZATION

Since each sample is different and each set of PCR primers has slightly different characteristics, it is important to optimize the PCR process for each specific

application. The protocol given in the next section is typical—the conditions described work for a wide variety of samples and primers and should be considered a good starting point. It is usually worthwhile to optimize a particular application by adjusting several of the parameters, namely: priming temperature, primer concentration, magnesium concentration, and sample concentration.

1. **Priming temperature**—The optimal priming temperature is the temperature at which the primers are able to bind to the target DNA but are unable to bind to DNA that does not match the target DNA exactly. In practice, it is unnecessary to determine this temperature exactly. If the primers match the target sequence perfectly and have a G/C content of approximately 50%, the priming temperature should be between 50°C and 60°C. If the target sequence is variable or contains known mismatches, use a priming temperature between 37°C and 50°C, at least for the first 10 cycles.

2. **Primer concentration**—Primers are included at a high concentration so that when denatured DNA begins to cool, it is more likely to bind to a primer than to reanneal to itself. However, if the primer concentration is too high, there can be problems with the primers binding to each other or to non-target DNA, resulting in nonspecific DNA products. A final primer concentration of 200nM (nM = 10^{-9}moles/liter) is usually successful; 100nM is often sufficient, but higher concentrations of up to one micromolar can be attempted if necessary.

3. **Magnesium concentration**—Taq polymerase requires magnesium ion, so magnesium chloride is included in the reaction mixture. Deoxynucleotide triphosphates bind free magnesium, as can a variety of substances that may be present in the sample lysate (such as EDTA). Therefore, it is important to titrate the optimal magnesium concentration for each project. The range of magnesium chloride concentrations to assay is 1mM to 5mM (final). Generally, the best results are in the two to three millimolar range.

4. **Sample concentration**—Paraffin-embedded tissues samples vary greatly in their size, cellularity, quality of fixation, DNA integrity, and presence of potential PCR inhibitors. In general, digestion of two 6-micron sections in 100 μl of lysis buffer will give enough lysate for 50 to 100 PCR reactions, using one or two microliters per reaction. Occasionally, it is necessary to use up to 5 μl of the lysate to obtain a signal. Sometimes it is necessary to dilute the lysate and use the equivalent of 0.2 μl to 0.5 μl of lysate in order to get a positive signal. Usually, 2 μl of lysate will give a positive signal in 70% to 80% of samples. Retesting the other samples with both more and less lysate will generally yield positive signals in another 10% to 20% of cases.

The design of primers and optimization of PCR reactions is discussed in greater detail in references 1 and 2.

PROGRAMMING THE THERMAL CYCLER

The PCR is carried out in a thermal cycler instrument, which automatically executes the cycles of temperature changes needed to make copies of the template DNA. There are many different manufacturers of thermal cyclers, and each cycler has different capabilities. Therefore, it is not possible to give precise programming instructions here. Instead, some general principles of designing a successful cycling program will be presented. The specific programming instructions accompanying the machine should be used to enter these parameters.

A PCR program generally has three parts: an initial denaturation step; a cycling step that includes priming, synthesis, and denaturation and is repeated 25 to 40 times; and a final extension step.

1. **Denaturation**—The initial denaturation is carried out at 94°C and should last for 3 to 5 minutes for a standard 100-µl reaction. It is not helpful to denature for more than 5 minutes, as prolonged high temperatures are more likely to damage the polymerase than to further denature the DNA.
2. **Cycling**—Each cycle consists of three steps: a priming step at 37°C to 60°C (see page 86), a synthesis step at 72°C, and a denaturation step at 94°C. In a standard thermal cycler, with 100-µl reactions layered with 50 ul of mineral oil, each of these steps should be 1 to 2 minutes long. If the target is very long (>300 base pairs), the synthesis step should be increased to 2 to 3 minutes.
3. **Extension**—After the cycling is complete, the reactions are held at 72°C to allow the polymerase to complete any unfinished syntheses. Five to seven minutes is more than enough for this purpose.

A typical PCR program looks like this:

	5 minutes at 94°C;
40 cycles of:	1 minute at 94°C
	1 minute at 55°C
	1 minute at 72°C;
	7 minutes at 72°C

Most thermal cyclers offer the possibility of holding the samples at 4°C indefinitely at the end of the program. This is useful for storing samples after a program has run overnight.

Some thermal cyclers have radically different capabilities and therefore require modification of the above standard procedure. The product literature accompanying these machines will explain how to adapt the above cycle parameters.

THE POLYMERASE CHAIN REACTION

PRINCIPLE:

Polymerase chain reaction allows the amplification of a specific DNA sequence. Sample DNA is mixed with polymerase and primers specific to the target DNA sequence. The reaction mixture is cycled through a series of temperature changes that cause repeated separation of the DNA strands, binding of the primers, and synthesis of new DNA. The process allows the rapid detection of specific DNA sequences that would not be detectable by other means.

MATERIALS AND SOLUTIONS:

Thermal cycler—programmed appropriately (see page 87)
PCR tubes (microcentrifuge tubes that fit thermal cycler)
Microcentrifuge
NO DNA pipetters—a set of three micropipetters (1-20 µl, 20-200 µl, 200-1,000 µl) reserved for non-DNA work
DNA pipetters—a set of two micropipetters (1-20 µl, 20-200 µl) reserved for adding sample DNA to PCR reactions
Pipet tips, preferably aerosol-resistant type
Taq polymerase (available under a variety of brand names from many suppliers—some may provide 10 X buffer with the enzyme)
Mineral oil or Perkin-Elmer Ampliwax beads
Sample DNA lysates, prepared as described on page 82, including a paraffin negative control (see page 85)
Primer solution (10 µM in each primer)

10mM $MgCl_2$

1M $MgCl_2$ (9.52 g + H_2O to 100 ml)	100 ul
H_2O	9.9 ml

0.5M Tris buffer, pH 8.3

Tris-base	3.52 g
Tris-HCl	3.31 g
H_2O to a final volume of	100 ml

10 X PCR buffer (store in 1-ml aliquots at -20°C):

	stock concentration	final concentration	amount to add
KCl	1.0M	500mM	5 ml
Tris, pH8.3	0.5M	100mM	2 ml
Gelatin		0.1%	10 mg
H_2O			3 ml

10mM deoxynucleoside triphosphates (dNTPs)

(100mM solutions are available from a variety of suppliers):

100mM dATP	10 µl
100mM dCTP	10 µl
100mM dGTP	10 µl
100mM dTTP	10 µl
H_2O	60 µl

Store at -20°C

PROCEDURE:

1. Read the quality assurance section of this chapter before beginning the procedure.
2. Make a lower buffer mix containing the following reagents for each sample, plus four extras (one positive control, two negative controls, and one to compensate for pipetting losses). Use the *NO DNA* pipetters:

10 X PCR buffer	2.5 µl
10mM $MgCl_2$	7.5 µl
10mM dNTPs	1.0 µl
10µM primers	2.0 µl
Deionized water	11.5 µl

3. Place in a rack one PCR tube for each sample plus one positive and two negative controls. Pipet 25 µl of lower buffer mix into the bottom of each microcentrifuge tube. Tap the tubes gently on the bench to move all buffer mix to the bottom of the tube.
4. Place one Ampliwax bead per sample in a clean weigh boat. Using a clean spatula, gently push bead onto the buffer in each tube. Cap the tubes.
5. Place tubes in thermal cycler. Heat to 80°C for 5 minutes. Remove from machine and allow to cool to room temperature (2 or 3 minutes).

6. Make up an upper buffer mix containing the following reagents for each sample plus four extras (use *NO DNA* pipetters):

10 X PCR buffer	7.5 µl
10mM $MgCl_2$.	22.5 µl
Taq polymerase	1.0 µl
Deionized water	48.5 µl

7. Remove caps from PCR tubes.
8. Add 75 µl of upper buffer mixture to each tube.
9. Add 2 µl of a sample DNA lysate to each tube (use DNA pipetters). Add 2 µl of a lysate that is known to contain the target DNA of interest to the positive control tube. Add 2 µl of lysate from the extraction negative control to the first negative control tube. (This tube will indicate whether contamination was introduced during the extraction process.) Add 2 µl of deionized water to the other negative control tube. (This tube will indicate whether contamination was introduced during the PCR process.)
10. Cap the tubes, place them in the PCR machine, and begin the appropriate cycling program.

NOTES:

1. Taq polymerase, while optimally active at 72°C, has some activity at room temperature. If all of the PCR reagents are mixed together at room temperature, it is possible that the primers will bind to nontarget DNA and be extended by Taq polymerase. Once these misprimed sequences are present, they will be amplified during the PCR reaction, resulting in nonspecific bands upon electrophoresis and possibly interfering with the amplification of interest. The procedure given above, known as "hot-start" PCR, avoids this problem by separating the nucleotides and primers from the polymerase and template DNA with a layer of wax. When the temperature becomes high enough to melt the wax, it is also too high for nonspecific priming to occur.
2. Hot-start PCR can be accomplished without Ampliwax beads by modifying the above protocol as follows:

   (a) Make up the lower buffer mix as follows (per sample, plus four extras)

10 X PCR buffer	9.0 µl
10mM $MgCl_2$.	30.0 µl
10mM dNTPs	1.0 µl
10µM primers	2.0 µl
Deionized water	46.0 µl

(b) Make up the upper buffer mix as follows (per sample, plus four extras)

10 X PCR buffer	1 µl
Taq polymerase	1 µl
Deionized water	8 µl

(c) Place 88 µl of the lower buffer mix in each PCR tube.

(d) Add 2 µl of the sample lysate or the appropriate control to each tube.

(e) Layer each reaction with 50 µl of mineral oil, if required by the particular thermal cycler being used.

(f) Place tubes in thermal cycler and begin the cycling program. When the tubes have been at 94°C for 2 minutes, begin adding 1µl of the upper buffer mix to each tube. Be sure that the pipet tip reaches below the mineral oil (if used). Change pipet tips between each sample.

(g) When all tubes have received the upper buffer mix, cap the tubes and allow the cycling program to proceed.

3. Finally, PCR can be accomplished without using a hot-start procedure by simply mixing all reagents except lysate; add the lysate just before cycling, layering with mineral oil (if required by thermal cycler), and cycling as above. There will be some loss of specificity and sensitivity if a hot-start procedure is not used.

AGAROSE GEL ELECTROPHORESIS

PRINCIPLE:

In electrophoresis, the amplified DNA produced in the polymerase chain reaction is loaded into a gel and subjected to an electric current. Since the speed of migration of DNA toward the positive pole is inversely related to its size, this allows the separation of DNA fragments by size. Ethidium bromide, a chemical that intercalates with DNA and fluoresces under UV light, is included in the gel and allows the visualization of DNA fragments after electrophoresis.

MATERIALS AND SOLUTIONS:

Submarine gel electrophoresis apparatus
Power supply
Micropipetter (1-20 µl) NOT the same one used for PCR set-up
Parafilm
Microwave oven or heat block
UV transilluminator and Polaroid camera with red filter and hood
DNA size markers—100 bp to 2000 bp range (an example would be PhiX DNA digested with Hae III, which is available from many suppliers; other marker sets over this size range are also available commercially)
Low-temperature-melting agarose (low melt)
Medium-temperature-melting agarose (med melt)
Ethidium bromide (10 mg/ml)— **CAUTION: POWERFUL MUTAGEN**

10 X TBE, pH 8.0
(0.89M Tris base, 0.89M boric acid, 0.02M EDTA)

Tris-base	108.00 g
Boric acid	55.00 g
EDTA	7.44 g
dH_2O to a final volume of	1,000 ml

Gel loading buffer
(0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in distilled water)

10% bromophenol blue (0.1 g in 1 ml water)	125 µl
10% xylene cyanol (0.1 g in 1 ml water)	125 µl
Glycerol	1.50 ml
dH_2O	3.25 ml

PROCEDURE:

1. Make up 2 liters of 1 X TBE by combining 200 ml of 10 X TBE with 1,800 ml of distilled water.
2. Weigh out 2 g of low-melt agarose and 0.5 g of medium-melt agarose. Combine in a 250-ml Erlenmeyer flask.
3. Add 100 ml of 1 X TBE.
4. Microwave or heat on heat block to boiling. Boil for 10 to 20 seconds or until all agarose has melted.
5. Cool to 50°C to 60°C. Add 10 µl ethidium bromide solution. **Caution: wear gloves and use extreme caution—ethidium bromide is a powerful mutagen.** Swirl to combine.
6. Pour into gel mold and insert comb (follow manufacturer's instructions for particular model).
7. Leave at room temperature until set, about 30 minutes. Flood surface with 1 X TBE and remove comb.
8. Place the gel in the electrophoresis apparatus and fill the apparatus with 1 X TBE, so that the gel is completely submerged. Prepare samples for loading.
9. Cut a 6-inch piece of parafilm with its paper backing and place it on the laboratory bench, film side up.
10. Using a micropipetter, dot 2 µl of gel loading buffer onto the parafilm for each sample, plus one for the markers, keeping the dots well spaced. If radioactive markers are used, place 2 µl of loading buffer in a 1.5-ml microcentrifuge tube.
11. Add 1 µl of (0.5 ug) DNA markers and 17 µl of distilled water to one of the loading buffer aliquots.
12. Add 18 µl of each PCR product to a separate dot (see Fig. 3–2).
13. Load 20 µl of combined loading buffer and PCR product into each well (see Fig. 3–3). Load the markers either in the middle of the gel or at both ends.
14. Put the lid on the apparatus, making sure that the anode lead (usually red) is at the far end, away from the DNA samples.
15. Plug the leads into the power supply, again making sure that the positive lead is attached to the far end of the apparatus.
16. Set the power supply for 100V and turn on. Let the gel run for 1.5 to 2 hours, or until bromophenol blue has migrated approximately 10 cm (see Fig. 3–4).
17. Turn off power supply, remove lid, and carefully lift out gel tray. Slide gel

onto transilluminator, put on protective glasses, and turn transilluminator on. DNA should appear as thin, red bands parallel to the wells. A 250-bp PCR product will migrate approximately with the xylene cyanol (light-blue) dye.

18. If desired, photograph the gel using a Polaroid camera equipped with a red filter.

NOTES:

1. Care must be taken when loading samples to avoid puncturing the bottom of the wells. When this occurs, the sample leaks out of the bottom of the well and spreads out underneath the gel. Make up a new mixture of loading buffer and sample and load into another well.
2. The most common and most easily avoided mistake in this procedure is attaching the electrophoresis leads backwards. When this occurs, the samples migrate backwards out of the wells and into the electrophoresis buffer.
3. If the samples migrate very slowly or the bands are severely smeared, the problem is probably an imbalance in the salt concentrations of the gel and buffer. Both must be 1X in TBE buffer. If either is inadvertently made up in water, the gel will not run properly.
4. The positive control should be chosen so that it gives a consistently visible ethidium bromide signal. This will serve as a control that the PCR reactions worked. If the positive control is not visible, the samples need to be reamplified with fresh PCR reagents; it is not worth proceeding to the Southern blot step.
5. Electrophoresis units are made by several different manufacturers and hold different volumes of gel. The directions above are for making up 100 ml of 2.5% gel. If the volume must be adjusted, use 2% low-melt agarose and 0.5% med-melt agarose in 1 X TBE (weight/volume).

INTERPRETING ETHIDIUM BROMIDE-STAINED GELS:

1. No markers or bands visible:
   (a) Dark background—ethidium bromide not added to gel. Soak gel in 50 ml of distilled water with 10 μl of ethidium bromide (10 mg/ml) for 30 minutes and look again.
   (b) Bright-pink background—too much ethidium bromide added. Soak gel in distilled water for at least 1 hour and look again.
2. Markers visible but no bands (including positive control): PCR reactions failed; repeat PCR.

3. Markers visible, positive control visible, all other lanes blank: Either samples do not contain DNA target or there is not enough product to visualize with ethidium bromide staining. Blot and hybridize to improve sensitivity.

4. Bands in all lanes at correct size (including negative controls): Contamination; repeat from PCR reactions using same lysates. If paraffin negative control is positive and PCR negative control is negative, all samples in that group must be re-extracted from fresh sections.

5. Lanes have more than one band:

   (a) Some or all lanes have bands in the 40 to 60 bp size range (see Fig. 3–9A, lanes 4, 10, and 12)—these bands represent interaction between the two primers and the polymerase (primer-dimers). Try raising the priming temperature or using a hot-start technique.

   (b) Some or all lanes have multiple bands of various sizes (see Fig. 3–9A, lanes 1, 2, 6, 7, 8, and 14)—nonspecific amplification; try a higher priming temperature, a hot-start technique, or blotting and hybridization to detect correct product.

6. Bands appear to be stuck in wells: DNA has not migrated into the gel.

   (a) If all lanes, including markers, exhibit this problem, either the gel or the buffer, or both, was inadvertently made up with water instead of 1 X TBE. Pour a new gel and run again.

   (b) If some samples stuck in the wells, while others ran normally, those samples with DNA stuck in the wells are probably overamplified. Try reamplifying those cases using much less lysate; for example, dilute the lysate 1:10 in water and use 1 ml of the dilution in the PCR reaction.

SOUTHERN BLOTTING, HYBRIDIZATION, AND AUTORADIOGRAPHY

PRINCIPLE:

Often UV visualization of ethidium bromide-stained PCR product is not sufficiently sensitive to detect all positive signals. Much greater sensitivity can be obtained by probing the PCR product with a radioactively labelled probe that is complementary to a sequence between the two primers (see Fig. 3–5 A and B). The DNA is transferred from the agarose gel to a specialized membrane, to which the DNA binds irreversibly. The membrane, or blot, is then placed in a plastic bag with the labelled probe in a hybridization solution. After allowing time for hybridization of the probe to its complementary sequence, the unbound probe is washed off and the blot is exposed to X-ray film. When the film (now called an autoradiogram) is developed, a band will be seen wherever the probe bound to DNA on the blot.

MATERIALS AND SOLUTIONS:

Gloves
Plastic or glass tray (slightly larger than gel and at least 1" deep)
Rocker apparatus
Paper towels
Medium-fine filter paper (Whatman 3)
Nylon membrane (Nytran, Hybond-C, or Sureblot)
Plastic wrap
Glass plate (slightly larger than gel)
2- to 4-lb weight (large book or full one-liter bottle)
Forceps
15-ml tubes
Plastic seal-a-meal-type bags, approximately 6" x 8" in size
Heat sealer for seal-a-meal bags
Water bath, or oven, set at 55°C
X-ray film cassette
Intensifying screen
Kodak XAR film or equivalent
-70°C freezer
1 X Membrane Blocking Solution (diluted from 2.5X Oncor stock)
Hybrisol III (Oncor)
^{32}P-labelled probe (see next section)

Denaturing solution (0.5M NaOH, 0.6M NaCl):

NaOH	20 g
NaCl	35 g
dH_2O to a final volume of	1,000 ml

Washing solution (0.16 X SSC, 0.1% SDS):

20X SSC	8 ml
20% SDS	5 ml
dH_2O to a final volume of	1,000 ml

(20X SSC (sodium citrate) and 20% SDS (sodium dodecyl sulfate) are available from many suppliers.)

PROCEDURE:

Blotting:

1. Put on gloves.
2. Place gel in plastic box and cover with denaturing solution.
3. Place box on rocker and rock gently for 20 minutes. During this time, the DNA in the gel will become denatured (single-stranded). In its single-stranded form it will be able to migrate out of the gel, bind to the nylon membrane, and bind to the labelled probe.
4. Cut a piece of medium-fine filter paper (e.g., Whatman 3) slightly larger than the gel.
5. Cut a piece of nylon membrane exactly the same size as the gel.
6. Place a 3-inch stack of paper towels (towels must be larger than gel) on a laboratory bench where they will not be disturbed.
7. Place the filter paper on top of the paper towels.
8. Gently ease the nylon membrane into the denaturing solution so that it wets evenly. (This can be done on the surface of the denaturing solution in which the gel is soaking.) Lift the wet membrane out by its edges and lay it on top of the filter paper (see Fig. 3–5). Be careful not to introduce bubbles between the paper and the membrane. If necessary, a 10-ml pipet can be used to roll over the membrane and push out any bubbles.
9. Lift the gel out of the denaturing solution and lay it precisely on top of the membrane (see Fig. 3–6). Gently press from the middle to the outer edges of the gel to eliminate any bubbles from between the gel and the membrane. Again, a pipet can be gently rolled over the surface of the gel to remove any bubbles. If any bubbles remain, the DNA will not transfer properly.
10. Place plastic wrap over the whole stack (see Fig. 3–7).

11. Place the glass plate on top of the gel and the weight on top of the glass plate (see Fig. 3–8).
12. Leave undisturbed overnight. During this time, moisture from the gel will be absorbed through the membrane into the stack of paper towels, carrying the DNA with it. The DNA will bind to the membrane.

Hybridization:

1. Dismantle the stack carefully, gently pulling the membrane away from the gel with forceps. The gel should be less than 1/8-inch thick now. Mark the location of the first well by cutting off that corner of the membrane. Use a pencil or ballpoint pen to label and date the membrane in another corner.
2. Place the membrane in a plastic box and cover with approximately 50 ml of 1 X Membrane Blocking Solution (MBS). Rock on rocker for 20 minutes.
3. Remove membrane from MBS and place in a plastic seal-a-meal bag, sealed on three sides. The bag should be approximately 1 cm larger than the membrane on three sides and about 5 cm larger than the membrane on the open side. Roll a pipet over the bag to squeeze out any excess MBS.
4. All subsequent steps involve the use of radioactive reagents. Wear appropriate protective clothing (coat, gloves) and dosimeter as required by workplace regulation. Work behind a shield as much as possible.
5. In a 15-ml tube, place 5 ml (for blots 50 to 150 cm^2) or 10 ml (for blots 150 to 300 cm^2) of Hybrisol III. Add 1 μl of labelled probe for each milliliter of Hybrisol III. Cap and vortex gently.
6. Pour the probe mixture into the plastic bag. Seal the very edge of the open side, trying not to leave too much air in the bag but not worrying about small bubbles.
7. Use a pipet to roll any small bubbles up to the last sealed edge. Seal the bag again between the membrane and the bubbles, trapping the bubbles between the two seals. Roll the pipet firmly over the bag to ensure that there are no leaks.
8. Place the sealed bag in the 55°C water bath and leave it to hybridize for at least 4 hours or overnight.

Washing:

1. Cut the corner of the hybridization bag and squeeze the probe mixture into a 15-ml tube. The probe can be stored in a -20°C freezer and used again up to five times, over a period of two to three weeks.

2. Cut the bag open and gently remove the membrane with forceps. Place it in a plastic box and cover with washing solution. Rock for 5 minutes.
3. Pour the washing solution into radioactive waste. Cover membrane with fresh washing solution and rock for 5 minutes.
4. Repeat Step three, except instead of rocking for 5 minutes, cover and place the box in the 55°C water bath, or oven, for 5 minutes.
5. Remove the membrane and place on a piece of paper towel. Let air-dry for 5 to 10 minutes. There should be no visibly wet patches on the membrane.
6. Transfer the membrane from the paper towel to a flat piece of plastic wrap. Cover the membrane with another piece of plastic wrap, avoiding wrinkles as much as possible.
7. Place the wrapped membrane in an X-ray film cassette, cover with XAR film (in the darkroom) and an intensifying screen. Expose in freezer at -70°C for 2 hours to overnight.

RESULTS:

Developed film will have bands wherever the probe bound to amplified DNA, indicating that those samples contained the target DNA of interest.

NOTES:

1. The most common sources of error in this procedure are failing to denature the gel and failing to remove all bubbles from between the gel and the nylon membrane. If care is taken in these steps, this blotting technique gives very consistent results.
2. When the gel/membrane/paper towel stack is dismantled, check to make sure that the gel has been reduced markedly in thickness. If it has not, something has prevented the absorption of moisture from the gel, through the membrane and into the paper towels. In this case, very little of the DNA is likely to have been transferred to the membrane. Check the organization of the stack for errors and reblot.
3. It is important to remove bubbles from the hybridization bag, since bubbles can prevent the labelled probe from reaching all parts of the blot.

INTERPRETING AUTORADIOGRAMS

1. Autoradiogram is completely blank, even though labelled markers were used.
   (a) Gel was not markedly thinner after blotting—gel stack was constructed improperly; check blotting stack for errors and reblot (see note 3 above).

(b) Gel was markedly thinner after blotting:
Stack was assembled improperly so that moisture from the gel did not flow through the nylon membrane (e.g., membrane placed above the gel instead of below). Run gel again and reblot.

(c) Gel was not denatured properly. Run gel again and denature 20 minutes before blotting.

2. Autoradiogram is completely black—film was exposed to light before development. Allow film cassette to thaw completely and load with a fresh piece of film.

3. Lanes with positive signals have more than one band—occasionally a band will appear slightly below the band of interest (see Fig. 3–10B). This is thought to be single-stranded DNA of the target sequence that migrates at a different rate from double-stranded DNA. This may be caused by one primer of the pair binding to the target more efficiently than the other. As long as this band is always accompanied by a band of the expected size, it should not be a concern.

4. A band that was visible by ethidium bromide is not visible on the autoradiogram:

   (a) The ethidium bromide band represented nonspecific PCR product that is unable to bind to the target-specific probe (see Fig. 3–9B).

   (b) A bubble in the blotting stack prevented the PCR product from binding to the nylon membrane.

5. Autoradiogram is obscured by black blotches and spiderweb-like lines—nylon membrane was not sufficiently dry when wrapped in plastic and exposed to film (see Step 5 under Washing above). Remove the membrane from plastic wrap, place in washing solution, and incubate at 55°C for 3 to 5 minutes. Remove from solution and let air-dry until no visible moisture remains. Wrap in plastic wrap as described in Step 6 under Washing above and reexpose to film.

END-LABELLING OF OLIGONUCLEOTIDE PROBES

PRINCIPLE:

In order to increase the sensitivity of the PCR assay, an oligonucleotide probe complementary to a DNA sequence between the two primers is labelled with a radioactive molecule. When the probe is annealed to the PCR product and exposed to X-ray film, the radioactivity exposes the film so that a black band is seen when the film is developed. The labelling of the probe is a very simple reaction that can be carried out in any laboratory that is equipped and approved for the use of ^{32}P. In the labelling reaction, T4 polynucleotide kinase enzyme transfers the ^{32}P-labelled terminal phosphate from commercially labelled ATP to the 5' end of the oligonucleotide.

The probe itself should be a 30- to 40-base stretch of DNA complementary to a section of the PCR product between the two primers. When choosing the sequence, look for an area without internal complementarity; in other words, an area that will not be able to fold up on itself. There are many vendors that custom synthesize oligonucleotides.

MATERIALS AND SOLUTIONS:

1.5-ml microcentrifuge tubes
Microcentrifuge tube rack
Microcentrifuge floater (rack to hold tubes in water bath)
Water bath set at 37°C
Water bath set at 65°C
Micropipetter (1-20 μl)
Microcentrifuge
Vortex

Oligonucleotide probe at 2.5 micromoles/liter
(2.5 pmol/μl (pmol = 10^{-12}moles/liter))

Adenosine 5'-{gamma–^{32}P}triphosphate, triethylammonium salt—3000 Ci/mmol (gamma–^{32}P-ATP)

5' End-labelling Kit (available from many suppliers)

OR instead of using a kit, you can make up the following reagents

1M $MgCl_2$

$MgCl_2$	9.52 g
dH_2O to a final volume of	100 ml

1.5M Tris, pH 9.5

Tris base ..... 18.16 g
dH_2O to a final volume of ..... 100 ml

Check pH and adjust with concentrated HCl

10 X kinase buffer
(0.5M Tris (pH 9.5), 0.1M $MgCl_2$, 50% glycerol)

	stock concentration	final concentration	amount to add
Tris base (pH 9.5)	1.5M	0.5M	333 µl
$MgCl_2$	1.0M	0.1M	100 µl
Glycerol	100%	50%	500 µl
dH_2O			67 µl

100mM dithiothreitol

DTT ..... 1.54 g
dH_2O to a final volume of ..... 100 ml

(Store frozen in 1-ml aliquots.)

T4 polynucleotide kinase (available from many suppliers)

PROCEDURE:

1. Place the following reagents in a 1.5-ml microcentrifuge tube:

   Oligonucleotide probe (10pmol) ..... 4 µl
   10 X kinase buffer ..... 2 µl
   100mM DTT ..... 1 µl
   T4 polynucleotide kinase ..... 1 µl
   Gamma-^{32}P-ATP ..... 3 µl
   dH_2O ..... 10 µl

2. Vortex and spin down.
3. Place in 37°C water bath for 45 minutes.
4. Place in 65°C water bath for 10 minutes (to inactivate kinase).
5. Spin down briefly and store in -20°C freezer until use.

END-LABELLING DNA SIZE MARKERS

PRINCIPLE:

DNA markers are labelled with ^{32}P so that they will be visible upon autoradiography.

MATERIALS AND SOLUTIONS:

1.5-ml microcentrifuge tubes
Microcentrifuge tube rack
Microcentrifuge tube floater (rack to hold tubes in water bath)
Water bath set at 37°C
Water bath set at 65°C
Micropipetter (1-20 µl)
Microcentrifuge
Vortex
Phi-X DNA digested with Hae III, approximately 1 µg/µl (a very common set of DNA size standards available from many vendors; other marker sets over the size range of 50 to 2000 bp can be used)
Adenosine 5'-{gamma-^{32}P}triphosphate, triethylammonium salt-3000 Ci/mmol (gamma-32P-ATP)
100% ethanol
70% ethanol

Phenol/chloroform/isoamyl alcohol (50 ml phenol:49 ml chloroform:1 ml isoamyl alcohol)—this reagent can be made up in the laboratory or purchased ready-made.

3M sodium acetate (pH 5.2):
anhydrous sodium acetate ..... 24.6 g
dH_2O to a final volume of ..... 100 ml

5' End-Labelling Kit (available from many suppliers, be sure to select a kit that has both phosphatase and kinase treatments)

OR instead of using a kit, you can make up the following reagents

Calf intestinal phosphatase (CIP) diluted to 0.01 units/µl in water

1M Tris buffer, pH 9.0

Tris base ..... 10.94 g
Tris-HCl ..... 1.52 g
dH_2O to a final volume of ..... 100 ml

100mM $ZnCl_2$

$ZnCl_2$ ..... 1.36 g
dH_2O to a final volume of ..... 100 ml

100mM spermidine

Spermidine ..... 1.45 g
dH_2O to a final volume of ..... 100 ml

1M $MgCl_2$

$MgCl_2$ ..... 9.52 g
dH_2O to a final volume of ..... 100 ml

10 X CIP buffer

	stock concentration	final concentration	amount to add
Tris, pH 9	1M	0.5M	500 µl
$ZnCl_2$	100mM	1.0mM	10 µl
$MgCl_2$	1M	10.0mM	10 µl
Spermidine	100mM	10.0mM	100 µl
dH_2O			380 µl

T4 polynucleotide kinase

10 X kinase buffer

	stock concentration	final concentration	amount to add
Tris base (pH 9.5)	1.5M	0.5M	333 µl
$MgCl_2$	1.0M	0.1M	100 µl
Glycerol	100%	50%	500 µl
dH_2O			67 µl

100mM dithiothreitol

DTT ..... 1.54 g
dH_2O to a final volume of ..... 100 ml
(Store frozen in 1-ml aliquots.)

PROCEDURE:

1. Place the following reagents in a microcentrifuge tube:
   5 µl phi-X DNA, digested with Hae III (approximately 5 µg)
   5 µl diluted CIP
   5 µl 10 X CIP buffer
   35 µl distilled water
2. Place at 37°C for 30 minutes.
3. Add 50 µl of phenol/chloroform/isoamyl alcohol. Vortex 30 seconds.
4. Spin at top speed in microcentrifuge for 5 minutes.
5. Remove top layer to fresh microcentrifuge tube. Discard bottom layer.
6. Add 5 µl of 3M sodium acetate and 100 µl of 100% ethanol to upper layer. Vortex briefly.
7. Place at -20°C for at least 30 minutes.
8. Spin at top speed in microcentrifuge (preferably at 4°C) for 20 minutes.
9. Pour off supernatant. Add 500 µl of 70% ethanol. Vortex briefly.
10. Spin in microcentrifuge for 5 minutes.
11. Pour off supernatant. Add 500 µl of 100% ethanol. Gently pour off alcohol. A tiny white pellet may be visible at the bottom of the tube.
12. Let pellet air-dry.
13. Resuspend DNA pellet in 10 µl of distilled water.
14. Add the following reagents to the resuspended DNA pellet:
    5.0 µl 10 X kinase buffer
    2.5 µl polynucleotide kinase
    2.5 µl 100mM dithiothreitol
    6.0 µl gamma ^{32}P-ATP
    24.0 µl distilled water
15. Vortex and spin down briefly.
16. Place in 37°C water bath for 45 minutes.
17. Place in 65°C water bath for 10 minutes (to inactivate kinase).
18. Spin down briefly and store in -20°C freezer until use.
19. Add 1 µl of labelled markers to the marker mix normally loaded onto an agarose gel. Remember to treat the marker tube, pipet tip, gel, and gel running buffer as radioactive when labelled markers are used.

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Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. *PCR Protocols: A Guide to Methods and Applications*. San Diego, Calif: Academic Press; 1990.

Lewin B. *Genes IV*. New York, NY: Oxford University Press; 1990.

Sambrook J, Fritsch EF, Maniatis T, eds. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1989.

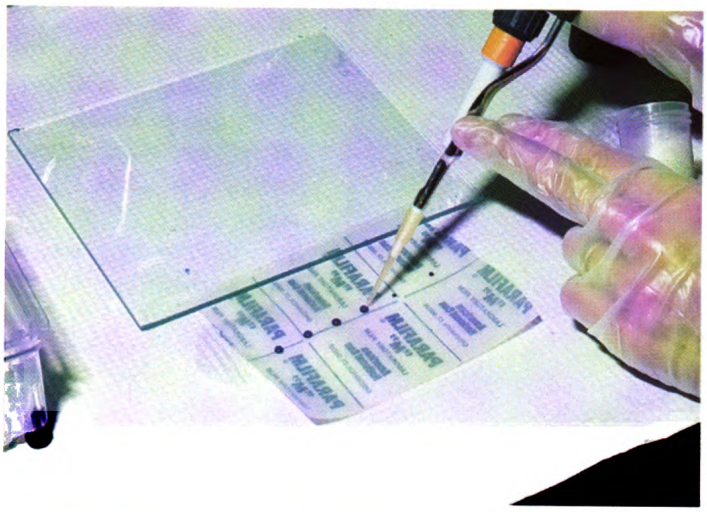


Fig. 3–2. PCR product is added to aliquots of loading buffer that have been dotted onto parafilm.

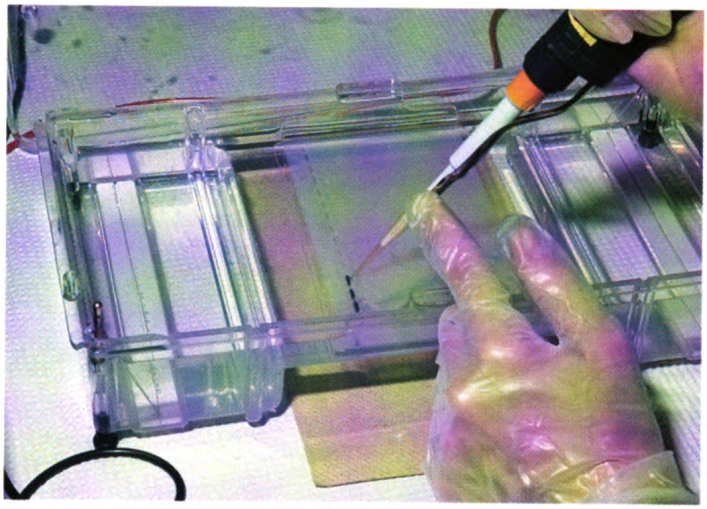


Fig. 3–3. Combined loading buffer and PCR product are loaded into wells in the agarose gel.

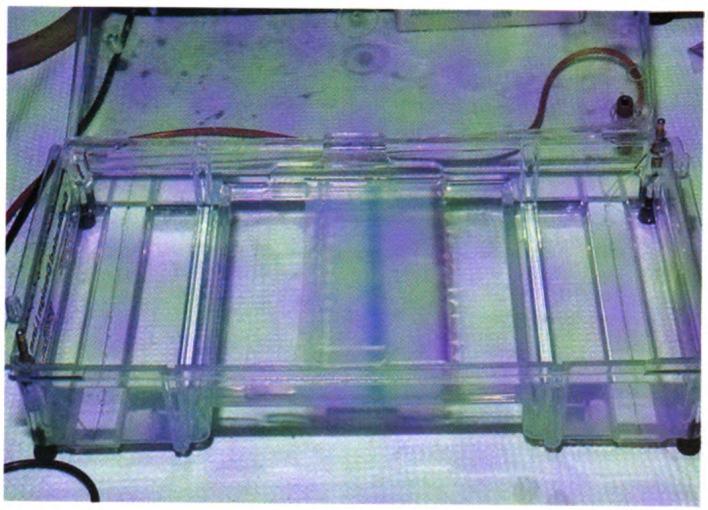


Fig. 3–4. Electrophoresis is complete when the bromophenol blue dye (dark blue) has migrated approximately 10 centimeters.

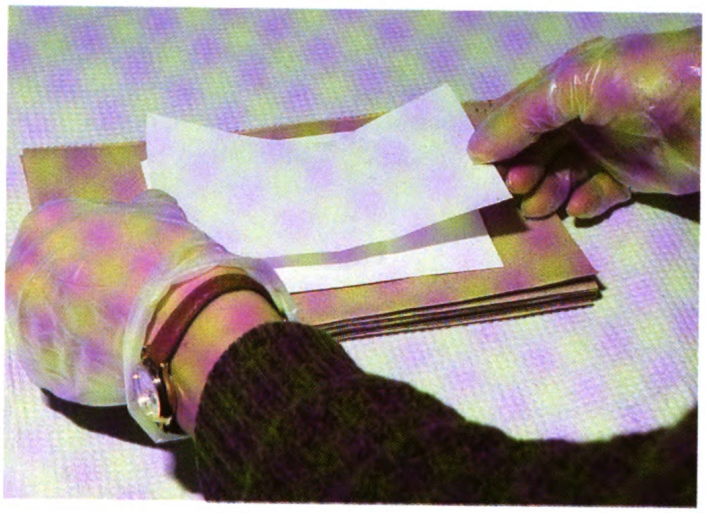


Fig. 3–5. Membrane that has been wetted with denaturing solution is carefully placed on filter paper. Care must be taken to avoid introducing bubbles between the filter paper and the membrane.

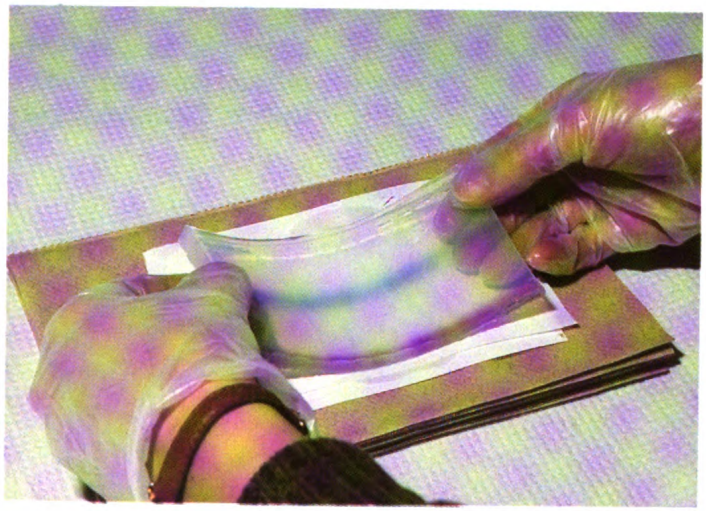


Fig. 3–6. Agarose gel that has been soaked in denaturing solution is carefully placed on membrane. Care must be taken to avoid introducing bubbles between the gel and the membrane.

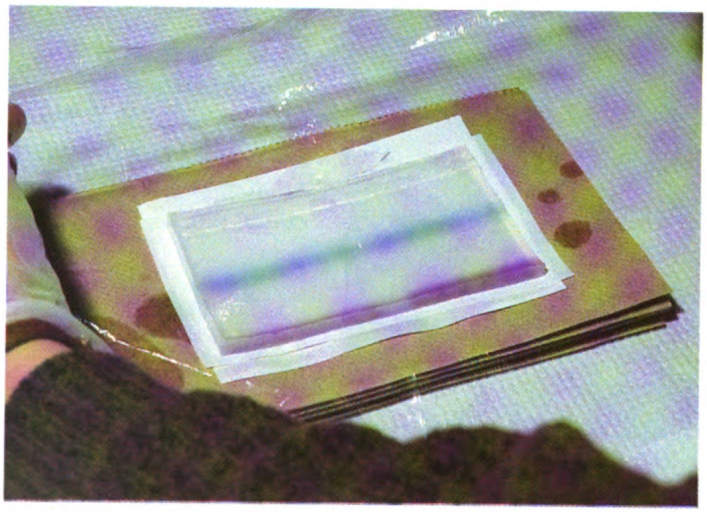


Fig. 3–7. Plastic wrap is placed over gel.

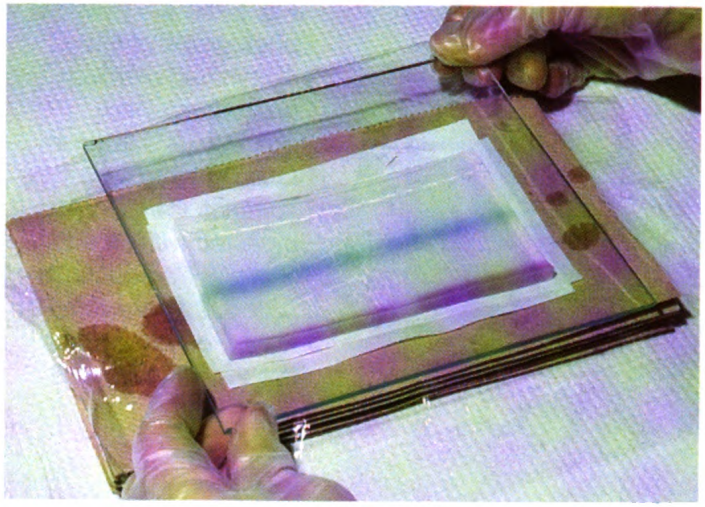
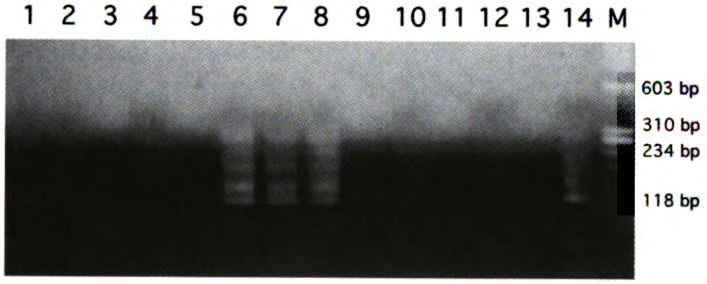


Fig. 3–8. Glass plate is placed over plastic wrap.



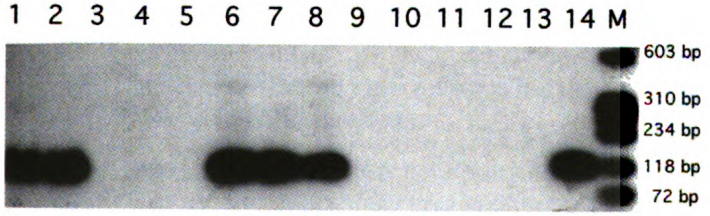


Fig. 3–9 A. Polaroid photograph of UV-illuminated ethidium bromide-stained agarose gel. Marker lane (marked M) contains PhiX DNA digested with Hae III. Sizes of fragments, in base pairs, are listed at right. Lanes 4, 10, and 12 exhibit bands at the 40-60 bp range, which are typical of primer-dimer formation. Lanes 1, 2, 6, 7, 8, and 14 exhibit multiple bands in addition to the target band; these bands indicate non-specific amplification that could be reduced by use of a hot-start technique.

Fig. 3–9 B. Autoradiogram of the same gel as that shown in Fig. 3–9A, after it has been blotted onto nylon membrane and hybridized with an end-labelled probe. Note that the non-specific bands visible on the ethidium bromide- stained gel are not bound by the labelled probe and do not appear on the autoradiogram.

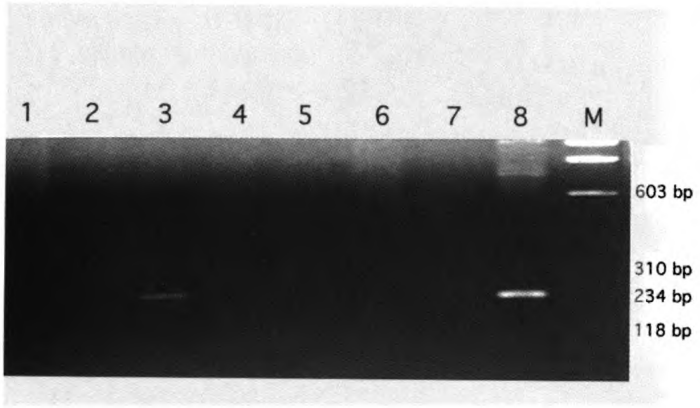


Fig. 3–10 A. Polaroid photograph of UV-illuminated ethidium bromide-stained agarose gel. Marker lane (marked M) contains PhiX DNA digested with Hae III. Sizes of fragments, in base pairs, are listed at right. Positive signals are visible in lanes 3 and 8.

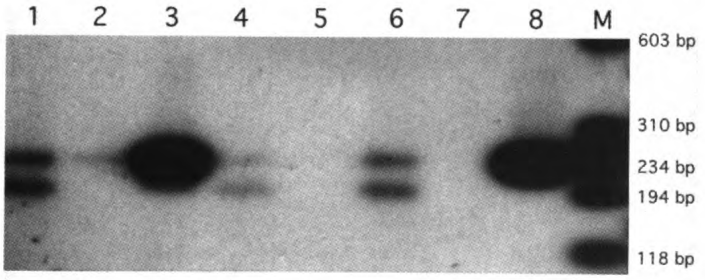


Fig. 3–10 B. Autoradiogram of the same gel as that shown in Fig. 3–10A, after it has been blotted onto nylon membrane and hybridized with an end-labelled probe. Positive signals are visible in lanes 1, 3, 4, 6, and 8. The amount of product in sample 1, 4, and 6 was not sufficient to be seen with ethidium bromide, but is detectable by radiolabelled probe. The second band in lanes 1, 4 and 6 is thought to represent single-stranded product, which electrophoreses at a different rate from double-stranded product.

4

Preparation of Nuclei for Flow Cytometry

Annette Geissel and Joe L. Griffin

INTRODUCTION

Flow-cytometric analysis, in contrast to slower static methods of DNA quantitation, lets us quickly determine the nuclear DNA content of large numbers of cells. DNA flow studies began in 1969 by Van Dilla et al. Initially these studies were limited to the use of hematopoietic tissues, such as peripheral blood, bone marrow cells, and lymphoid tissues. In the 1970's, methods for extraction of whole nuclei from fresh-frozen and/or fixed solid tissues were developed by various laboratories, which in turn lead to experimentation on fixed, paraffin-embedded tissues. Studies on archival material make use of methods developed in the early 1980's for isolating and staining nuclei from paraffin-embedded tissues (Hedley et al. 1983, Hedley 1989, McLemore et al. 1990, Heiden et al. 1991). Recovery of cells from thick paraffin sections allows retrospective studies on collections of tumors and correlation of flow data with clinical history.

The methods described here reflect our experience and include modifications to previously published methods.

DNA measurements by flow cytometry are based on the ability of certain fluorescent dyes to bind specifically and in direct proportion to DNA under certain staining conditions. The principal dye for staining DNA is propidium iodide, which binds by intercalation (insertion) between the double strands of the DNA molecule. Nuclear fluorescence produced by laser excitation is measured and recorded as nuclei flow rapidly in single file through the laser beam. A computer records individual

Fig. 4–1. Flow histogram, diploid cell population.

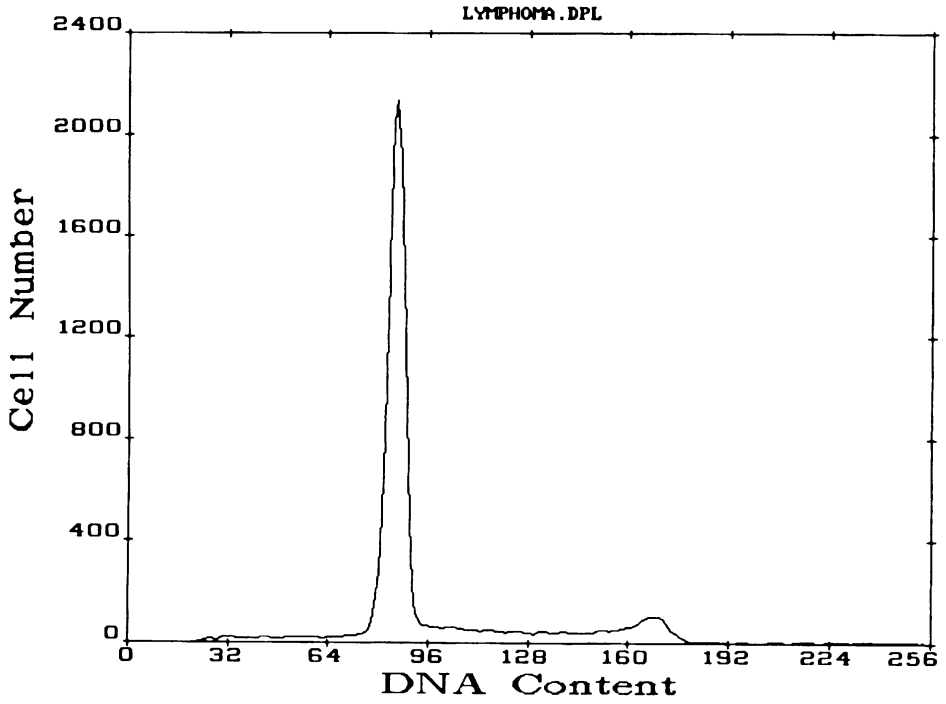
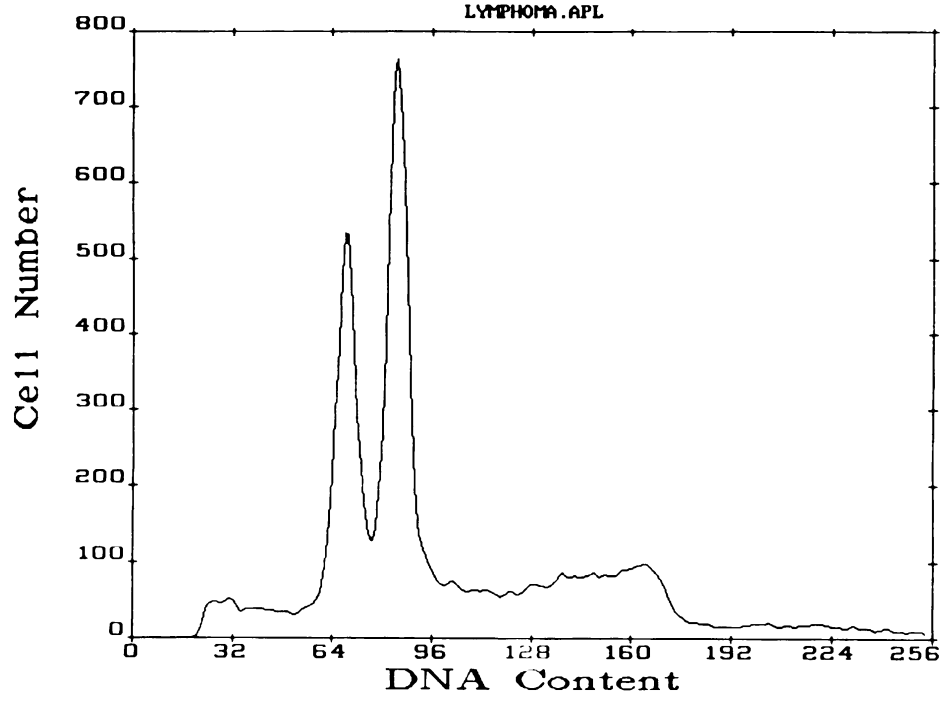


Fig. 4–2. Flow histogram, diploid plus aneuploid populations.



fluorescence levels for each nucleus. Within minutes, data from 20,000 to 100,000 nuclei can be stored for later evaluation.

For analysis, the individual values are accumulated into DNA histograms (Figs. 4–1, 4–2). The height of each channel of a single parameter histogram is proportional to the number of nuclei within a range of fluorescence intensity, which defines that particular channel. For example, a diploid cell population (Fig. 4–1) will segregate into two peaks and an intermediate population. The largest peak, G0-G1, contains nuclei with DNA equivalent to the normal number (2n) of chromosomes. A smaller peak, G2-M, in channels about twice as high as the G0-G1, includes nuclei with a 4n amount of DNA (chromosomes have duplicated and are ready to divide or are dividing). These two peaks are connected by a broad plateau, synthesis phase, containing nuclei with intermediate amounts of DNA (S-phase nuclei, caught while duplicating DNA). In a "normal" histogram of lymphocytes for example, approximately 90% of the cells collect in the G0-G1 peak, while the remaining cells divide between the G2-M peak and S-phase.

Aneuploidy is defined as having a G0-G1 peak separate from, or different from, the normal 2n G0-G1 peak (Fig. 4–2). This second peak represents a population of nuclei with abnormal DNA content. The presence of aneuploidy has been correlated with prognosis (Bauer et al. 1986 and 1993, Koss et al. 1989, Koss 1992). An additional prognostic factor is the relative proportion of proliferative cells in the S-phase and the G2-M peak versus the G0-G1 peak. For example, Fig. 4–1 has a somewhat elevated S-phase (14.6%, Multicycle analysis, debris and clumping compensation) while Fig. 4–2, in addition to the aneuploid peak, shows a high S-phase (24.5% average S), suggesting a high rate of proliferation.

While flow cytometry has the potential to provide diagnostic and prognostic information, flow cytometry technology and chemicals for DNA are often marked — "For research only. Not for use in diagnostic procedures." As of this writing, regulatory agencies have not given formal approval of these systems/methods for diagnostic use.

PRINCIPLE:

DNA specimens to be analyzed by flow cytometry must be in the form of cell suspensions. The preparation methods for fresh, fresh-frozen, and paraffin-embedded tissues are the same in principle but differ in procedural details.

Formalin-fixed, paraffin-embedded tissue is cut into 80-micron sections, deparaffinized, and rehydrated. The tissue is dissociated by digestion with a proteolytic enzyme, to yield a suspension of nuclei with little or no cytoplasm. The nuclei are stained with a fluorochrome, DAPI or propidium iodide, for example. In this chapter we report two different procedures, one with DAPI stain and one with propidium iodide stain. Our preference is the DAPI method because of its simplicity in preparing the sample and the lower coefficient of variation (CV) of DAPI stained nuclear populations. However, the DAPI-stained nuclei are excited under UV light which is not available on all flow cytometers.

ISOLATION AND STAINING OF NUCLEI FROM PARAFFIN-EMBEDDED TISSUES

MATERIALS AND SOLUTIONS:

Microtome (standard, able to cut sections thicker than 50 μm)
Fume hood
Vacuum (aspirator)
Centrifuge (refrigerated 4°C)
Water bath (kept at 37°C)
Vortexer
Microscope
pH meter
Micropipetter
DNA-Prep Station (Coulter, Hialeah, FL) (optional)
13 x 100-mm borosilicate glass tubes
25-ml glass pipets
Disposable plastic transfer pipets
22-gauge spinal tap needle (optional)
3-ml syringe
Pasteur glass pipets
Hemacytometer
Gloves
Aluminum foil
Nylon mesh (Tetco, Inc., Elmsford, NY), 47 to 53 μm (to filter out larger fragments of tissue from nuclear suspensions)

For Isolation of Nuclei:

Hemo-De (Fisher Scientific)

A citrus-based, nontoxic clearing agent used to deparaffinize the sections. Xylene works equally well.

Ethanol solutions: 100%, 95%, 80%, 70%, and 50%

0.1M tris buffer with 0.07M NaCl

Trizma HCl (Sigma, St. Louis) ..... 14.04 g
Trizma Base (Sigma) ..... 1.34 g
NaCl ..... 4.09 g
dH_2O to ..... 1,000 ml

djust pH to 7.2.

0.1% protease

Protease type XXIV (Sigma) ..... 10 mg
Tris buffer ..... 10 ml

For DAPI Procedure:

DAPI staining solution

10μM DAPI in 800 mM disodium phosphate

DAPI (4', 6-diamidino-2-phenylindole dihydrochloride:hydrate) (Sigma) ..... 1.0 mg

Na_2HPO_4. ..... 32.4 g
dH_2O ..... 285 ml

For Propidium Iodide Procedure:

Hank's balanced salt solution (HBSS), pH 7.4, *without* $CaCl_2$ and $MgCl_2$ (Gibco BRL, Gaithersburg, MD)

Use HBSS to wash out the protease if PI staining is used. If HBSS is to be stored after opening, add 1 g/1,000 ml (0.1%) sodium azide as a preservative.

Note: Tumors containing melanin are bleached with 0.5% potassium permanganate and 1% oxalic acid prior to digestion. In the presence of Ca and Mg, oxalate tends to precipitate as crystals that adhere to the nuclei in flow cytometry samples. To avoid this, we use HBSS without calcium and magnesium salts.

Bleaching of melanin containing tissue sections:

1. Immerse sections in 0.5% potassium permanganate, after rehydration, for 60 minutes.
2. Wash briefly in deionized water.
3. Place in 1% oxalic acid for 1 minute.
4. Wash thoroughly in deionized water and continue with the digestion.

Phosphate Buffered Saline (PBS)

NaCl	8.01 g
KCl	0.20 g
Na_2HPO_4.	1.44 g
KH_2PO_4.	0.20 g
Distilled or deionized water to	1,000 ml

Mix and adjust pH to 7.4

NOTE: PBS can also be purchased commercially. We use Dulbecco 10X PBS (Gibco BRL). Prior to use it is diluted 1:10 with deionized water. The pH is checked and adjusted from 7.2 to 7.4.

PBA

Bovine albumin	1 g
Sodium azide	1 g
PBS pH 7.4	1,000 ml

This PBS derivation is primarily used for fresh tissue preparations or cell suspensions. Sodium azide acts as an antibacterial, antifungal agent and is toxic. **Handle with gloves.**

Triton-PBA

Triton X-100 (Sigma, St. Louis)	1 ml
PBA	1,000 ml

The detergent in the buffer relaxes the DNA strands.

RNAse stock solution (Worthington Biochemical Co., Freehold, NJ)

Prepare a solution of 5,000 units/ml in PBS, pH 7.2. As an example, if ribonuclease A has activity of 5,561 units/mg, to make ten 1-ml vials containing 5,000 units each use:

RNAse (5000/5561 x 10)	8.99 mg
PBS	10 ml

To eliminate any residual DNAse heat the solution in a 75°C water bath for 30 minutes. Cool and filter through a 0.22-μm membrane filter. Store 1-ml aliquots of stock solution at -20°C.

RNAse working solution (500 units/ml)

Stock solution	1 ml
PBS	9 ml

Propidium iodide stock solution (Calbiochem-Novabiochem Corp., La Jolla, CA)

Propidium iodide is a carcinogen. Handle with gloves.

Propidium iodide	100 mg
Deionized water	100 ml

Store in a dark bottle at 4°C. Will keep for months.

PI working solution (0.05 mg/ml)

Stock solution	1 ml
PBS	19 ml

PROCEDURE

Tissue Sectioning

Thin sections are obtained immediately before and after the thick sections are cut for flow cytometry. Before cutting the 80-micron thick sections, the top section is stained with hematoxylin and eosin and then reviewed and marked by the pathologist, who determines the amount of tumor present and the areas to be either included or excluded. As a rule, areas of necrosis and excessive fibrosis and/or desmoplasia are omitted. Furthermore, poorly fixed sections and sections from tissues fixed in Bouin's or Zenker's fixatives should not be used. The bottom section, cut after the thick sections, confirms whether the cells of interest are present in the thick sections.

Match the marked slide with the block surface to locate the exact area to be analyzed. Score the block with a sharp probe or needle. When cut, the area of interest will separate from the remainder of the section. Use a brush to separate the two if necessary. Alternatively, thick sections can be mounted on slides and areas of interest dissected away from unwanted areas. Cut sections 50 to 100 μm thick (we use 80-μm sections). The number of sections depends on the cellularity of the tumor and the amount of normal tissue. Estimate one to two sections for cellular tumors, three to four for tumors with extensive connective tissue, and four to five for small foci of tumor. Nonmalignant tissue, from the same case or block (if available), should be cut and run simultaneously as a control. A sample of normal lymph node sections (e.g. from rat, rabbit, or dog) is used as a quality control for the preparation.

Deparaffinization

1. Place sections in a 13 x 100-mm borosilicate glass test tube.
2. Add 3 ml of Hemo-De or xylene and vortex. Leave for 10 minutes and aspirate carefully; repeat twice.
3. After the last Hemo-De application carefully aspirate as much of the clearing agent as possible.

Rehydration

100%	ethanol	x 2, 3 ml - 10 minutes each
95%	"	x 1, 3 ml - 10 minutes
80%	"	x 1, 3 ml - 10 minutes
70%	"	x 1, 3 ml - 10 minutes
50%	"	x 1, 3 ml - 10 minutes

Deionized H_2O - 3 ml - overnight at 4°C.

Aspirate, change, and vortex after each application. (Fig. 4–3)

If the tissue is to be stored for more than 24 hours, leave it in 70% alcohol at 4°C to avoid bacterial contamination.

Protease Digestion

1. Add 1 ml of protease solution to deparaffinized rehydrated section(s) in glass tube.
2. Digest tissue, without applying mechanical means to dissociate cells, in 37°C water bath for 1 to 2 hours. (Digestion time may vary in tissues.)
3. Remove sample from incubator and shake on a shaker/ vortexer for 30 minutes (Fig. 4–4) or pipet with a 5 ml glass Pasteur pipet. If staining with DAPI, count nuclei at this point (see below), then continue with DAPI stain (see page 120).

NOTE: If the yield is low harvest more nuclei by returning the sample to the refrigerator overnight. Pipet cautiously several times the following day and count again.

For PI staining, continue with Step 4.

4. Wash sample by filling tube with Hank's solution (approximately 6 ml), remove section and centrifuge sample at 3,000 rpm for 10 minutes.
5. Aspirate the supernatant and add 1 ml of fresh Hank's solution.
6. Resuspend the nuclei by syringing the pellet thoroughly with a 22-gauge spinal tap needle attached to a 3-ml syringe (Fig. 4–5).
7. Count the nuclei in a hemacytometer. The preferable amount is one to two million cells per milliliter. Proceed with propidium iodide staining.

Count of Nuclei

Use a hemacytometer (Neubauer) and count the nuclei in the four large corner squares, which are divided into 16 small squares. (Fig. 4–6)

The following formula is used to arrive at the number of cells present per ml of solution.

$$N \times 10^4 = \text{cells/ml}$$

N = number of nuclei counted per large corner square x dilution factor (if any). If all four corner squares are counted, divide number by four.

This procedure is slow but provides an approximate nuclear concentration per sample. Concentrated samples may need to be diluted. Cells must be evenly suspended before transferring to the chamber. The minimal number of nuclei for an acceptable flow cytometry assay is usually assumed to be >150,000/ml.

DAPI Staining of Nuclei

After protease digestion and nuclear counting, (see previous page, Step 3), add one milliliter of DAPI staining solution to the sample. Wrap tube in aluminum foil and let stain equilibrate at least for one hour. Filter the sample through 37-μm nylon mesh before analysis.

Result: DAPI is a ultra violet sensitive dye and therefore not suitable for standard fluorescent microscopic demonstration.

Note: This method does not require RNAse digestion or centrifugation, and so conserves time and nuclei. Samples can be stored up to a week at 4°C. Though fluorochrome uptake by nuclei in any specimen is proportional to DNA content, differences in fixation or embedding often cause substantial variation in the G0 / G1 channel number from one specimen to another. In nuclei stained with propidium iodide (PI), the intent is to saturate the DNA with fluorochrome. With this dye, normal tissue from an area of the same paraffin block containing tumor is sometimes used as a diploid standard, though the practice carries some risk. Our experience with the DAPI technique shows that nuclei are not saturated with dye used at the recommended concentration. (Subsaturating concentrations are used because the histogram coefficient of variation increases substantially when more concentrated dye is used.) Hence, variation in G0 / G1 channel placement is greater from specimen to specimen (even for different preparations from the same block) with the DAPI technique.

Propidium Iodide Staining of Nuclei

1. After counting, centrifuge the samples at 3,000 rpm, at 4°C, for 10 minutes.
2. Aspirate supernatant and resuspend nuclei by syringing with a spinal tap needle.
3. Add Triton-PBA, 1 ml/10^6 cells, to each sample, vortex, and keep on ice for 3 minutes.
4. Centrifuge, aspirate, and syringe as in Step 2.
5. Add RNAse working solution, 1 ml/10^6 cells, and incubate in a 37°C water bath for 30 minutes.
6. Centrifuge, aspirate, and syringe as in Step 2.
7. Resuspend in propidium iodide working solution, 0.5 ml/10^6 cells.
8. Vortex, wrap tube in aluminum foil, (Fig. 4–7) and store at 4°C overnight.
9. Filter through 37-μm nylon mesh prior to flow cytometry analysis.

Result

The nuclear DNA, stained with PI, will fluoresce red when viewed under a fluorescent microscope (Fig. 4–8) and when illuminated by appropriate laser light in the flow cytometer. The flow cytometer carries out fluorescence-intensity measurements of individual cells and accumulates the measurements into histograms representing DNA values (Figs. 4–1, 4–2). The peaks in these histograms are then analyzed for ploidy (number of chromosomes).

PROCEDURE AT A GLANCE

1. Deparaffinization and Rehydration

Hemo-De (xylene)		3 ml	x3	10 minutes each
EtOH	100%	3 ml	x2	10 minutes each
"	95%	3 ml	x1	10 minutes
"	80%	3 ml	x1	10 minutes
"	70%	3 ml	x1	10 minutes
"	50%	3 ml	x1	10 minutes
Deionized water		3 ml	x2	1-24 hrs. total at 4°C.

Aspirate and vortex between solution changes

2. Digestion

a. Place deparaffinized rehydrated section(s) in glass tube with 1 ml of protease solution.

b. Digest tissue in 37°C water bath for 1 to 2 hours. (Digestion time may vary between tissues.)

c. Remove tube with sample from incubator and shake tube on a shaker/ vortexer for 30 minutes (Fig. 4–4). For PI staining, proceed to Step 4.

3. DAPI Staining

For DAPI procedure, count and stain at this point by adding 1 ml of DAPI staining solution to the sample. Wrap in foil and refrigerate until analysis.

Note: If necessary, harvest more nuclei by leaving the unstained sample in the refrigerator overnight. Pipet the next day. Repeat the cell count and stain.

4. Wash (For PI Staining)

a. Wash sample by filling tube with Hank's solution (approximately 6 ml), remove section, and centrifuge at 3,000 rpm for 10 minutes.

b. Aspirate the supernatant and add 1 ml of fresh Hank's solution.

c. Resuspend the nuclei by syringing the pellet thoroughly with a 22-gauge spinal tap needle attached to a 3-ml syringe.

5. Count

Count the nuclei in a hemacytometer and if the yield is satisfactory (see NOTE, page 122) proceed with propidium iodide staining.

6. Propidium Iodide Staining

a. Triton PBA

3 minutes on ice - 1 ml/10^6 cells

Centrifuge, aspirate, and vortex/syringe.

b. RNAse working sodution

30 minutes in 37°C water bath - 1 ml/10^6 cells

Centrifuge, aspirate, and vortex/syringe.

c. Propidium iodide working solution

Overnight at 4°C - 0.5 ml/10^6 cells.

Wrap in aluminum foil to keep light out.

Note: The minimal amount of nuclei for flow analysis is about 150,000/ml. The optimal amount for DAPI-stained nuclei is between 500,000 and 700,000 / ml while 1–2 million are the desired number for PI-stained nuclei.

Recent results indicate that for some tissues, treatment of formic acid + H_2O_2 will increase the cell yield and lower the CV in the cell analysis (Wang et al. 1993).

HOW TO AVOID PROBLEMS AND AIDS FOR TROUBLESHOOTING

A successful preparation for flow cytometry is a suspension of abundant, clean nuclei, free of debris.

The use of archival tissue has distinct advantages over fresh tissue, such as comparison studies of large numbers of tumors, collection of rare tumors and premalignant lesions, and the ability to sample specific regions of a tumor. However, there are some inherent limitations in the technique. Following is a list of shortcomings and a few suggestions of what can be done to minimize them.

One of the most common problems is excessive cell debris which can result in broad peaks. Assuming that the flow cytometer is properly aligned and giving acceptable results with external controls, such as chick erythrocytes and normal lymphocytes, poor-quality histograms obtained from paraffin blocks are the results of either unsatisfactory starting material or poor technique. The manner in which the specimen was handled prior to embedding is very important but often not known to the receiving laboratory.

Smaller pieces of tissue fixed immediately in 10% neutral buffered formalin are much better fixed than large specimens or autopsy material, where a delay in fixation is often unavoidable. Specialized fixatives, such as Bouin's or mercury-based fixatives, result in uninterpretable histograms (Hedley, 1990).

1. Reasons for rejecting sample blocks for flow analysis are based on the following criteria:
    a. Too little tissue.
    b. Insufficient tumor for analysis.
    c. Excessive amount of necrotic or hemorrhagic tissue.
    d. Histologic evidence of poor fixation, deterioration, or fixation in Bouin's or Zenker's fixatives.
2. An appropriate internal standard, i.e., normal tissue from the same patient, is often not available. If possible, prepare normal tissue from the same block as the tumor and run under identical conditions. To arrive at a diagnostically conclusive histogram, great care needs to be given to localizing and excising the tumor area in the block and to as much separation from the nonmalignant surrounding tissue as possible.
3. The deparaffinization of the sections must be complete. When adding 100% ethanol to the aspirated sample after the third Hemo-De

application, the alcohol should remain absolutely clear. If not, repeat the last immersion in clearing agent. Always vortex after adding the next solution.

4. Excess debris precludes a satisfactory interpretation of histograms.
    a. To reduce the chance for debris and to maximize the total number of whole nuclei, 80-micron thick sections are cut for flow analysis. Wet the block between each section with a tissue soaked in plain water to obtain smooth, cohesive sections.
    b. Some methods may require mincing of the tissue before digestion. Mincing increases the chance for additional debris. This can be minimized with very sharp curved scissors or scalpels.
    c. Filter carefully before analysis to avoid spilling unfiltered suspension into the sample.
5. Poor results not attributable to starting material or flow cytometer performance require a thorough review of the technique. In particular, the digestion step needs to be scrutinized as to grade and activity of the enzyme used. The optimum in concentration and length of digestion time can vary with different tissues and tumors. It is advisable to test for optimal conditions before starting a new study.
6. In the propidium iodide staining procedure, it is important to syringe, preferably with spinal tap needles, between solution changes in order to avoid clumping of nuclei. Some of these appear as doublets in the histogram.
7. Always check your solutions for clarity, date, and correct pH. Commercially obtained solutions have expiration dates. Observe these and any color change that may occur.

Wear gloves during the procedure!

ISOLATION AND STAINING OF NUCLEI FROM FRESH TISSUES FOR FLOW CYTOMETRY

Collect fresh tissue aseptically into ice cold PBA. Keep sample at 2° to 6°C if it is to be processed within 24 hours. Otherwise, remove tissue from medium and freeze at -20°C until processing.

1. Mince tissue with small scissors in a watchglass with a few milliliters of cold PBA (page 116).
2. Force minced tissue through a stainless-steel screen (60 mesh) and flush screen repeatedly with PBA, or (depending on tissue) dilute minced tissue with 5 ml PBA and syringe solution back and forth using a 10-ml syringe with a 20-gauge needle.
3. Let tissue sediment at unit gravity for 10 minutes.
4. Aspirate supernatant into a 50-ml centrifuge tube, add PBA, and spin at 4°C at 1,800 rpm for 10 minutes.
5. Disperse and recentrifuge pellet in PBA.
6. Resuspend pellet in fresh PBA, then filter through 37-μm nylon mesh.
7. Count cells (nuclei) in hemacytometer.
8. Dilute, or concentrate, cells to 2 x 10^6 cells/ml.
9. Fix 3 ml of cell suspension with 7 ml of ice-cold 100% ethyl alcohol (add alcohol drop-wise while vortexing) for 1 hour.
10. Centrifuge cells and resuspend in 3 ml Triton-PBA (see page 116); let stand on ice for 10 minutes.
11. Centrifuge cells and resuspend in 3 ml RNAse, 500 units/ml, in PBA. Incubate at 37°C for 20 minutes.
12. Centrifuge cells and resuspend in 2 ml propidium iodide, 0.05 mg/ml in PBA; stain 15 minutes to overnight.

For an alternative method see Castro et al. (1993).

ISOLATION AND STAINING OF MONONUCLEAR CELLS FROM WHOLE BLOOD FOR FLOW CYTOMETRY

1. Dilute 10 ml of heparinized whole blood 1:4 with PBA (page 116) and layer 10 to 12 ml of diluted blood on top of 3 ml of Histopaque 1077 (Sigma, St. Louis) in 15-ml centrifuge tubes. Centrifuge at room temperature, at 1,500 rpm, for 30 minutes.
2. Discard supernatant down to interface layer between serum and Histopaque and, with a pipet, carefully recover cells trapped in the interface.
3. Wash cells three times in PBA by centrifugation at 4°C.
4. Count cells in a hemacytometer (see page 121).
5. Dilute, or concentrate, cells to 2 x 10^6 cells/ml.
6. Fix 3 ml of cell suspension with 7 ml of ice-cold 100% ethyl alcohol (add alcohol drop-wise while vortexing) for 1 hour.
7. Centrifuge cells and resuspend in 3 ml Triton-PBA (see page 116); let stand on ice for 3 minutes.
8. Centrifuge cells and resuspend in 3 ml RNAse, 500 units/ml, in PBA. Incubate at 37°C for 20 minutes.
9. Centrifuge cells and resuspend in 2 ml propidium iodide, 0.05 mg/ml in PBA; stain 15 minutes to overnight.

QUALITY CONTROL

A quality control program is needed to ensure that staining and instrument use are consistent and reliable on a daily basis. We use nuclei from sections of embedded normal lymph node as staining controls. Nucleated erythrocytes from chicken and trout, for example, can also be used. Commercially obtained standard microspheres (beads) with known fluorescence and light-scatter properties are used as instrument standards. Both beads and nuclei are used to optimize and/or calibrate the instrument for optical alignment, determination of detector and amplifier linearity, and comparison of sensitivity.

ADDITIONAL INFORMATION ON FLOW CYTOMETRY

The following list of references provides a limited view of an extensive literature. For an overview of flow cytometry, see Shapiro (1988). Bauer, Duque, and Shankey (1993) include recent reviews on DNA analysis and other clinical flow use.

The technical details that affect the results of procedures described herein are analyzed by Hitchcock and Ensley (1993), and Heiden et. al. (1991). See also Dressler (1990), Koss et al. (1989), and Koss (1992).

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Fig. 4–3. Exchange of solutions by aspiration.

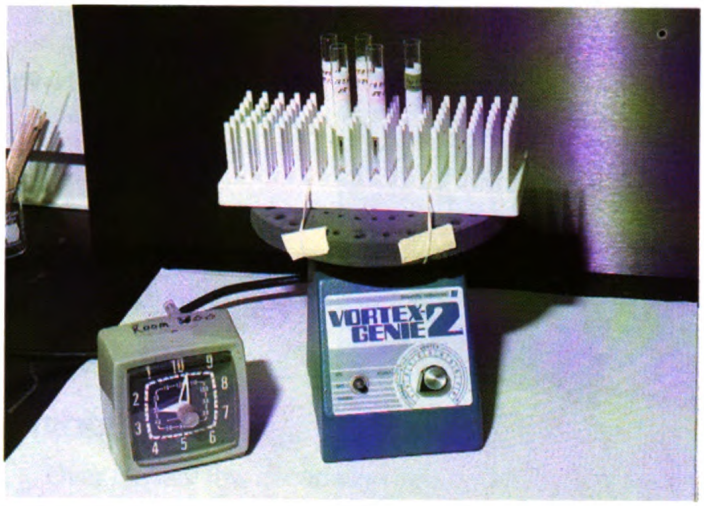


Fig. 4–4. After digestion of samples they are put in racks and shaken for 30 minutes. Here a vortexer, set at low speed, is used.

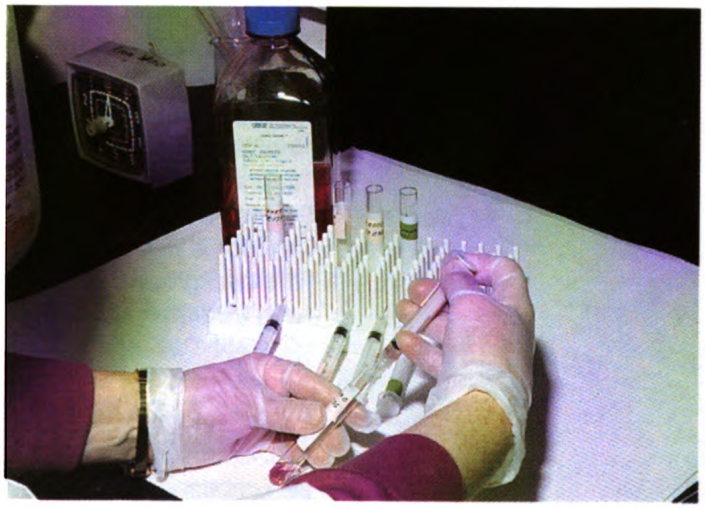


Fig. 4–5. Syringing of digested and shaken sample after wash in Hank's, centrifugation, and aspiration.

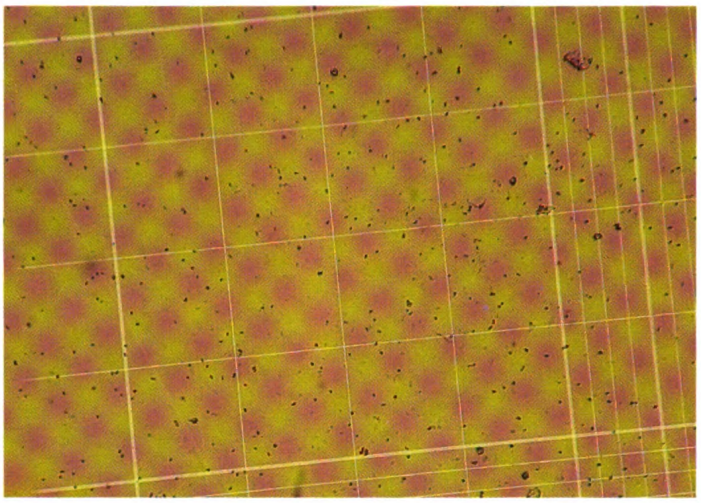


Fig. 4–6. Nuclei visualized in counting chamber. Cells in the 16 squares are counted and the number is multiplied by 10^4 to get number of cells/ml. Note: This preparation is very concentrated and should be diluted to facilitate counting.

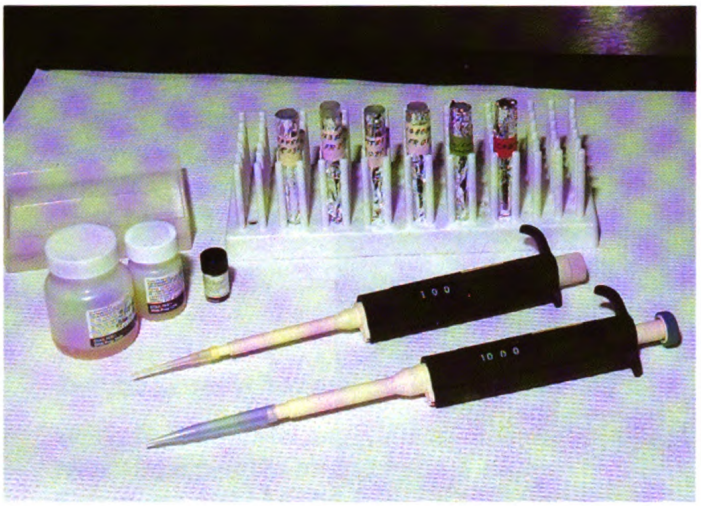


Fig. 4–7. Stained nuclei, to be analyzed, protected from light with aluminum foil.

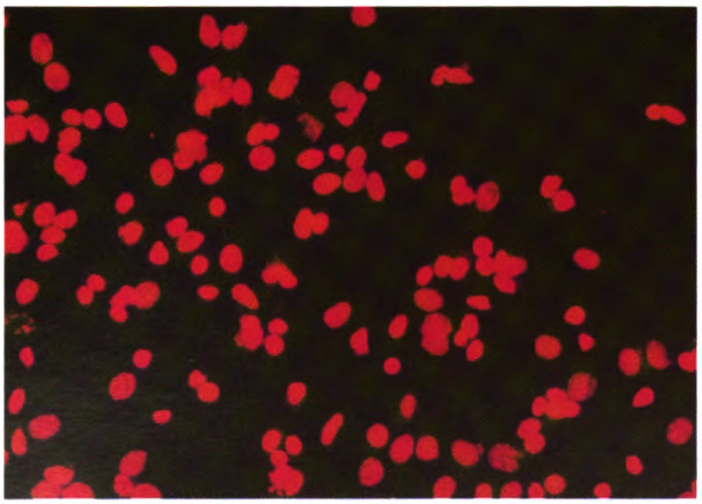


Fig. 4–8. Propidium iodide stained cells, prepared from paraffin-embedded tissue, viewed with a fluorescence microscope. Note there are some cell clumps but the background is clean and free from debris.

5

Quantitative Staining Techniques for Image Cytometry

Ulrika V. Mikel

INTRODUCTION

Over the last few decades image cytometry has become a tool increasingly applied to research in pathology. One of its main objectives is to determine the amount of deoxyribonucleic acid (DNA) in tumor cells in order to detect if the cells are aneuploid (have too much or too little DNA) and to determine how rapidly they are dividing or proliferating. The DNA content is determined by the amount of nuclear stain uptake in cell populations. This is done by measuring the optical density of cells stained with absorbance stains, which bind to nuclear chromatin in proportion to the amount of DNA, or the fluorescence intensity of cells stained with fluorochromes, which also bind stoichiometrically, i.e., in a proportional way. As pathologists have become more familiar with the technique, some of them are known to have used image cytometry as an aid in the diagnosis and prognosis of tumors. It is quite possible that the importance of this technique will grow and, in the future, routinely be applied and used in most histology laboratories.

Absorbance cytometry

The most commonly used stain for quantitative evaluation of nuclear DNA is the Feulgen reaction. This reaction is based upon cleavage of the purine-deoxyribose

bonds by acid hydrolysis to expose reactive aldehyde groups. The aldehyde groups are then stained and become a measure of the DNA content in a nucleus. In the Feulgen reaction, it is Schiff's reagent, classically a colorless, water-clear, fuchsin-sulfurous acid reagent, which covalently binds to the aldehyde groups. Basic fuchsin (pararosaniline) is the dye conventionally used in Schiff's reagent, but other dyes such as acriflavine, thionin, and azure A can be substituted for basic fuchsin. They are of different chemical type, but all have at least one primary amino group.

A number of factors will influence the reactivity of this stain: pH, temperature, age, sulfur dioxide concentration of the stain, length of time in the bleach wash, alcohol rehydration and dehydration, and the fixation method of the tissue.

Fixation will influence the rate of "liberation" of aldehydes during hydrolysis and, thus, the maximum of the Feulgen stain intensity. Neutral formalin and Carnoy's fixative are excellent fixatives for the Feulgen reaction, while chromate- and dichromate-containing fixatives make tissues resist hydrolysis cleavage. Mercury-based fixatives are reported to give weaker staining, while alcohol-fixed material should not be stained at all with the Feulgen reaction. However, alcohol-fixed cells can be postfixed in formalin and will then stain stoichiometrically with the Feulgen reaction. Bouin-fixed tissues stain very weakly if conventional hydrolysis treatment of 5N HCl at room temperature for one hour is used. The optimal time in this case is closer to 30 minutes.

The conventional Feulgen stain, the Schiff's reagent, keeps for 6 months if it is properly stored in a stoppered bottle in a refrigerator. Proper storage prevents oxidation. Still, over time the sulfite concentration is decreased and the solution becomes more acid. To standardize the stain between staining batches, it is necessary to adjust the pH of the staining solution to a pH lower than the initial pH of the freshly made stain. If the stain turns pink or red, the reagent has deteriorated and lost its specificity. Other Feulgen stains, e.g. azure A and thionin, do not keep as long as the classical Schiff's reagent.

Another proven method to quantitatively stain DNA is gallocyanine-chrome alum. Gallocyanine is a basic dye that binds to phosphate groups. At low pH, the staining of proteins will be negligible and only nucleic acids are stained. By treating the sections with RNAse, an enzyme that removes RNA, the stain becomes specific for DNA. The nuclear stain uptake is dependent on several factors such as boiling time of the dye when preparing the stain, pH of the staining solution, and fixation of the tissue. A pH of 1.6 will give optimal staining results. Alcohol and Carnoy's fixation are preferred fixatives. Other fixation methods such as formalin and Bouin's fixation can comfortably be used, although formalin is reported to give higher background staining. The reaction itself is not understood, but it is thought that by boiling gallocyanine and chrome alum new complex dye properties are developed. It is essentially the chromium that is responsible for the binding to the nucleic acids. The nuclei stain steel gray, and the staining reaction is very stable and highly reproducible. Differentiation in water or alcohol has no influence on the color intensity. The age of the staining solution will influence the staining; therefore, the stain should be saved no more

than a couple of weeks. For quantitative purposes, freshly made stains should be used.

Fluorescence cytometry

The DNA content in nuclei can also be determined with fluorescence stains. The advantages of using the fluorescence technique are its simplicity and sensitivity and the low investment needed for equipment. The disadvantages include the quenching and fading of stains, nonspecific and interfering autofluorescence, and the variable quantum efficiency of fluorochrome molecules. For quantitative work, the fluorescence intensity of the nucleus must be proportional to the total fluorochrome uptake of the nucleus. This condition is only true in a restrictive range of concentrations.

The staining mechanisms of fluorochromes vary as they bind to nucleic acids in different ways. Hoechst dyes and DAPI bind to the outer groove of the DNA molecule, while acridine orange, ethidium bromide, and propidium iodide are intercalating dyes. Nonspecific staining may occur when the pH of a specific stain is outside a specific range. Some fluorochromes are affected by organizational differences of the chromatin such as compact versus dispersed. Here, the fixation of the tissue or cells may play a role. For instance, it has been shown that fluorescence intensity is quenched by formalin fixation; therefore, alcohol fixation is preferred. It should also be noted that the autofluorescence on formalin-fixed tissues is so high it is virtually impossible to do fluorometry on cells in tissue sections fixed in formalin. However, it is possible to perform fluorescence measurements on formalin-fixed cell preparations such as cytospins (cells spun onto a slide through centrifugation) or on touch preparations of fresh tissues fixed in formalin.

Instrumentation

Today, computer-interfaced, image-analysis video systems (Fig. 5–1) are most commonly used to measure the optical density of cells. For fluorescence measurements, the equipment is much simpler. All that is necessary is a fluorescence microscope, a photomultiplier tube, and a diaphragm in the microscope's image plane that determines the measuring field. Thus, video systems are quite expensive, while equipment to measure fluorescence can be simple and inexpensive. Scanning microspectrophotometers, less expensive than image-analysis systems but much more tedious to use, can measure both fluorescence intensity and optical density.

For quantitative work, it is necessary to calibrate the measuring instruments in order to be able to compare samples in a study or to aid in the diagnosis of individual cases. For fluorometry, the standard should be suitable for the particular stain used, i.e., the excitation and emission spectrum should be within the same range as the stain used. There are several materials commercially available for this purpose, e.g. uranyl glass, inorganic phosphor particles, and polystyrene spheres with a fluorophore incorporated in the plastic (Kaplan and Picciolo, 1988). The linearity of the photomultiplier tube can be checked with gray filters of different

optical densities. For video systems there should be a match between the optics of the microscope, the video camera, the image-digitizing hardware, and the image-processing algorithm (Jarvis, 1986, and Castleman, 1987).

Data collection

From the quantitatively stained cells and/or tissues, a population of nuclei are measured and analyzed. The measurements themselves will be in arbitrary units; therefore, control cells must be measured to establish a normal population. As the stain uptake is dependent on so many factors (tissue type, fixation, age of the stain itself, etc.), the control cells should be collected from the tissue section or cell preparation being analyzed. Tissue sections seldom contain both tumor cells and normal cells of the same histogenic type. Therefore, as a rule, lymphocytes are used as control cells. In preparations of isolated cells or nuclei, there is no way of distinguishing normal cells from tumor cells; in those cases lymphocytes must be the controls. It should be noted, though, that heterochromatic cells often give lower integrated optical density values than do euchromatic cells such as epithelial cells. The average lymphocyte value, from a population of 25 cells, still gives information about where the average normal DNA value will fall. To analyze a particular tumor, at least 100 to 150 nuclei should be measured. The amount of measured stain uptake tells where in the cell cycle (Fig. 5–2) a measured nucleus will fall, when compared to the control. The values are put into histograms from which the normal or resting phase cells (G0-G1), the DNA synthesis (S) and the post DNA synthesis (G2-M) phases can be extracted. Figures 5–3 and 5–4 illustrate examples of two types of histograms attained through image cytometry measurements. Figure 5–3 shows a histogram of measurements done on Feulgen-stained diploid and tetraploid nuclei from touch preparations of rat liver nuclei. The tetraploid G0-G1 population shows twice the Feulgen value of the diploid G0-G1 population. The histogram in Figure 5–4 depicts measurements done on nuclei, isolated from thick paraffin sections, from a breast tumor. The different phases in the cell cycle are marked. This histogram shows both a euploid (normal number of chromosomes) and an aneuploid (nuclei with more than the diploid number of chromosomes) G0-G1 phase. The S-phase in this example is the proliferative phase of the aneuploid population. Note that low cell numbers in image histograms preclude accurate estimate of S-phase fraction.

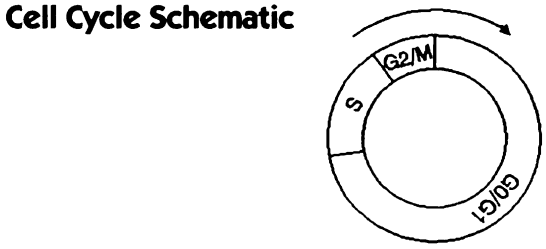


Fig. 5–2. Schematic representation of the cell cycle.

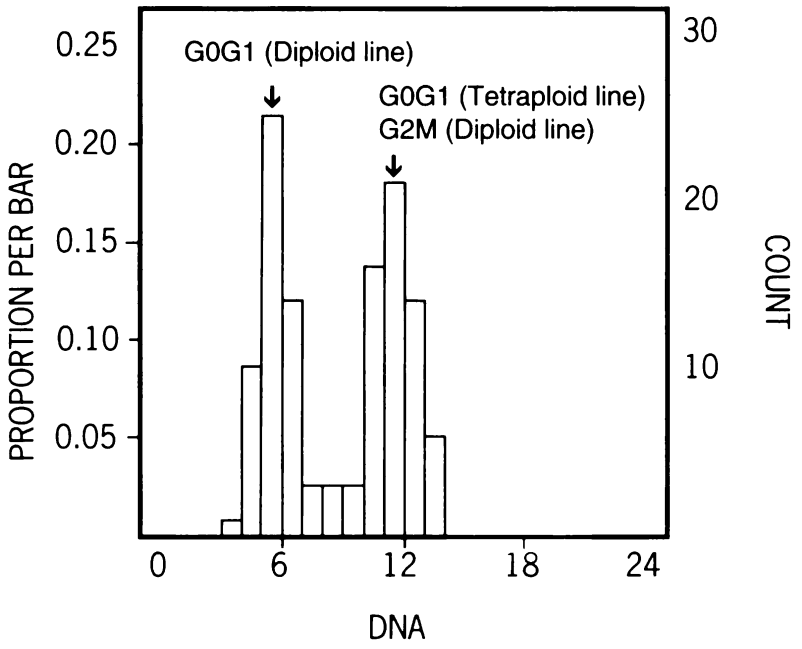


Fig. 5–3. Histogram of Feulgen values from measurements done on diploid and tetraploid rat liver nuclei.

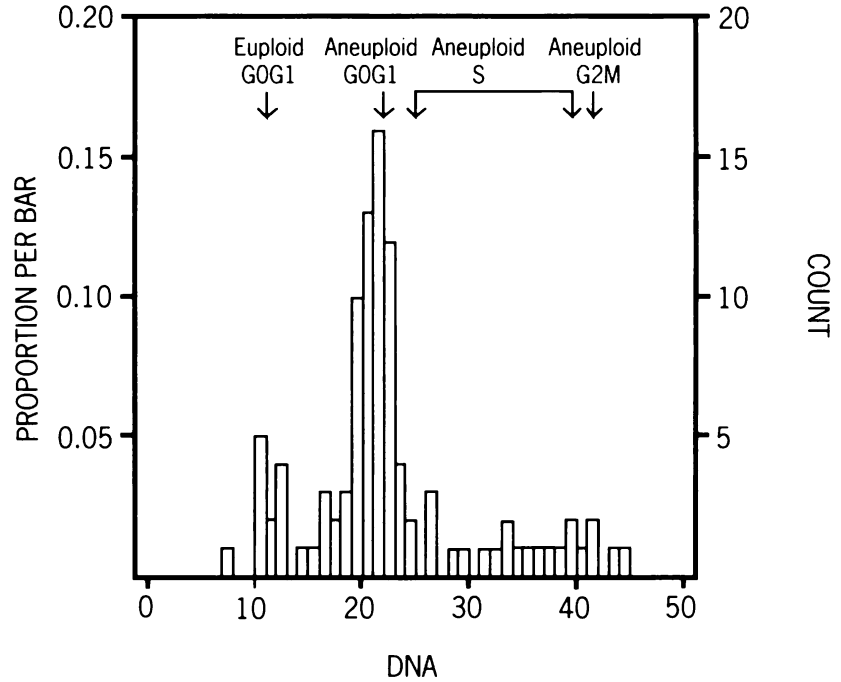


Fig. 5–4. Histogram of Feulgen values from measurements done on nuclei from a breast tumor.

STAINING METHODS FOR ABSORPTION CYTOMETRY

THE CLASSICAL FEULGEN STAIN

PRINCIPLE:

The Feulgen reaction is a nuclear, stoichiometric stain that is used to determine the DNA content in cells. The procedure includes an acid hydrolysis step that cleaves the purine-deoxyribose bonds in nuclear DNA so that reactive aldehyde groups are exposed. The exposed aldehyde groups are then stained with the Schiff's reagent, used in the classical Feulgen reaction, or some other chromogen (see procedures below), and the amount of stain uptake becomes a measure of the DNA content in a nucleus.

SOLUTIONS:

5N HCl

dH_2O (distilled or deionized water) .....56 ml
Conc. HCl .....40 ml

(✋ Always add acid to water)

Schiff's reagent

Dissolve 0.5 g pararosaniline in 100 ml boiling water.
Cool solution to about 50°C.
Add 10 ml 1N HCl and 2 g potassium metabisulfite.
Store in dark at room temperature for 24 hours.
Add 1 teaspoon activated charcoal.
Shake solution vigorously.
Filter until stain is clear.
Store in refrigerator.
Filter before use.

SO_2 water

Potassium metabisulfite ($K_2S_2O_5$) ..... 1 g
dH_2O ..... 190 ml
1N HCl ..... 10 ml

Mix right before use.

Ethanol: 50%, 70%, 95%, 100%

Xylene

Mounting medium

PROCEDURE:

1. Deparaffinize and hydrate slides.
2. Hydrolyze in 5N HCl for one hour.
3. Rinse (one dip) in dH_2O.
4. Stain in Schiff's reagent at pH 1.5 (see NOTES) for one hour.
5. Wash in 3 changes of SO_2 water for 10 minutes each.
6. Wash in running tap water for 5 minutes.
7. Rinse in dH_2O.
8. Dehydrate through 50%, 70%, and 95% ethyl alcohol for 3 minutes each.
9. Absolute ethyl alcohol, 2 changes for 5 minutes each.
10. Clear in xylene, 2 changes for 5 minutes each.
11. Mount in resinous medium.

RESULTS: (See Fig. 5-5, page 157)

Nuclei ..... red to light purple
Background ..... unstained
Absorption ..... max. about 560 nm

NOTES:

Many things will influence the Feulgen reaction (e.g. fixation, temperature, pH, alcohol concentrations, time sequences in the procedure steps); therefore, when this method is used for quantitative measurements, it is important to standardize it as much as possible. For repeatable results, the pH of the stain should always be the same. The reason for choosing a pH lower than the pH of freshly made reagent is because stored reagent turns more acid with time. Choose a low pH,

anywhere from 1.5 to 1.7, and then standardize the staining aliquot at the time of use to the chosen pH. Adjust the pH with 5N HCl. It is also important to standardize the step-time sequences so that the procedure is done the same way for each staining batch. Note that the stain works best on formalin-fixed tissues and the hydrolysis time has to be cut in half if Bouin's fixative is used. When cell preparations are stained, alcohol-fixed cells have to be postfixed in formalin. However, cells fixed in Carnoy's solution stain well in the Feulgen reaction.

Schiff's reagent keeps for 6 months when stored in a stoppered bottle in a refrigerator. If the stain turns pink or red, discard it.

REFERENCES:

Mikel UV, Fishbein WN, Bahr GF. Some practical considerations in quantitative absorbance microspectrophotometry. Preparation techniques in DNA cytophotometry. *Anal Quant Cytol Histol.* 1985;7:107-118.

Stowell RE. Feulgen reaction for thymonucleic acid. *Stain Technol.* 1945;20:45-58.

AZURE A FEULGEN STAIN

SOLUTIONS:

5N HCl

dH_2O (distilled or deionized water) ..... 56 ml
Conc. HCl ..... 40 ml

(✋ Always add acid to water)

Azure A stain

Azure A ..... 250 mg
dH_2O ..... 50 ml
1N HCl ..... 5 ml
Potassium metabisulfite ($K_2S_2O_5$) ..... 1 g

Mix well and filter before use.

SO_2 water

Potassium metabisulfite ($K_2S_2O_5$) ..... 750 mg
dH_2O ..... 142.5 ml
1N HCl ..... 7.5 ml

Mix right before use.

Ethanol: 70%, 95%, 100%

Xylene

Mounting medium

PROCEDURE:

1. Deparaffinize and hydrate slides.
2. Hydrolyze in 5N HCl for 50 minutes.
3. Stain in azure A, pH 1.5 (see NOTES), for 2 hours.
4. Wash in 3 changes of SO_2 water for 5 minutes each.
5. Rinse in dH_2O for 5 minutes.
6. Dehydrate in 70% and 95% ethyl alcohol for 3 minutes each.
7. Absolute ethyl alcohol, 2 changes for 5 minutes each.
8. Clear in xylene, 2 changes for 5 minutes each.
9. Mount in resinous medium.

RESULTS: (See Fig. 5-6, page 157)

Nuclei	blue
Background	unstained
Absorption	max. about 590 nm

NOTES:

The azure A stain can precipitate. Be sure to filter it before use. Sections stained with azure A fade less over time than sections stained with other versions of the Feulgen stain.

Azure A keeps in a stoppered bottle in a refrigerator for one month. Also see NOTES under classical Feulgen reaction.

REFERENCE:

Mendelsohn ML, Mayall BH. Chromosome identification by image analysis and quantitative cytochemistry. In: Junis JJ, ed. *Human Chromosome Methodology*. 2nd ed. New York, NY: Academic Press;1974:311-346.

THIONIN FEULGEN STAIN

SOLUTIONS:

5N HCl

dH_2O (distilled or deionized water) ..... 56 ml
Conc. HCl ..... 40 ml

(✋ Always add acid to water)

Thionin stain

Boil 250 mg thionin in 125 ml dH_2O for 5 minutes.
Cool to room temperature.
Restore volume to 125 ml with dH_2O.
Add 125 ml tertiary butanol, 37.5 ml 1N HCl, and 2.5 g sodium metabisulfite.
Close flask and stir for 24 hours.
Let stand for 48 hours before use.
Filter before use.

Ethyl alcohol: 50%, 70%, 95%, 100%

Xylene

Mounting medium

PROCEDURE:

1. Deparaffinize and hydrate slides.
2. Hydrolyze in 5N HCl for 1 hour.
3. Rinse in dH_2O.
4. Stain in thionin stain for 1 hour.
5. Wash in running tap water for 20 minutes.
6. Rinse in dH_2O.
7. Dehydrate slides in 50%, 70%, and 95% ethyl alcohol for 3 minutes each.
8. Absolute ethyl alcohol, 2 changes for 5 minutes each.
9. Clear in xylene, 2 changes for 5 minutes each.
10. Mount in resinous medium.

RESULTS: (See Fig. 5-7, page 158)

Nuclei	blue
Background	unstained
Absorption	max. about 580 nm

NOTES:

See NOTES under classical Feulgen stain procedure. For a useful quantitative stain, the thionin stain should not be kept more than a couple of weeks.

REFERENCE:

van Duijin P. A histochemical specific thionine-SO_2 reagent and its use in bi-color method for deoxyribonucleic acid and periodic acid-Schiff positive substances. *J Histochem Cytochem.* 1956;4:55-63.

ACRIFLAVINE FEULGEN STAIN

SOLUTIONS:

5N HCl

dH_2O (distilled or deionized water) ..... 56 ml
Conc. HCl ..... 40 ml

(✋ Always add acid to water)

Acriflavine stain

Acriflavine ..... 20 mg
Potassium metabisulfite ($K_2S_2O_5$) ..... 130 mg
dH_2O ..... 86 ml
0.1N HCl ..... 14 ml

Mix well and filter before use.

Acid alcohol

70% ethyl alcohol ..... 99 ml
Conc. HCl ..... 1 ml

Ethyl alcohol: 95%, 100%

Xylene

Mounting medium

PROCEDURE:

1. Deparaffinize and hydrate slides.
2. Hydrolyze in 5N HCl for 45 minutes at 28°C.
3. Rinse in dH_2O.
4. Stain in acriflavine stain for 20 minutes.
5. Wash in running tap water for 5 minutes.
6. Wash in acid alcohol, 2 changes for 5 minutes each.
7. 95% ethyl alcohol for 5 minutes.
9. Absolute ethyl alcohol, 2 changes for 5 minutes each.
10. Clear in xylene, 2 changes for 5 minutes each.
11. Mount in resinous medium.

RESULTS: (See Fig. 5–8A, page 158)

Nuclei	pale yellow
Background	unstained
Absorption	max. about 450 nm

NOTES:

Some light sources in conventional microscopes, interfaced to image-analysis equipment, do not give sufficient light for quantitative cytometry when an absorption filter of 450 nm is used. However, a regular dark-blue filter works fine (Fig. 5–8B). It should be noted that the acriflavine-stained nuclei can be scanned in a microspectrophotometer using a 450-nm absorbance filter. This gives the most optimum results of quantitative analysis of DNA, although the procedure is very tedious. The stain can also be used for fluorescence cytometry. In this case, the slides should be mounted in Fluormount (Gallard-Schlesinger Industries, Inc., Carle Place, NY), which is miscible with xylene, or in Gel/Mount (Biomeda, Foster City, CA) after step 7.

The stain can be stored in a refrigerator in a dark bottle for months. Also see NOTES under classical Feulgen reaction.

REFERENCE:

Tanke HJ, van Ingen EM, Ploem JS. Acriflavin-Feulgen Stilbene staining: a procedure for automated cervical cytology with a television based system (LEYTAS). *J Histochem Cytochem.* 1979;27:84-86.

GALLOCYANINE-CHROME ALUM STAIN

PRINCIPLE:

Gallocyanine is a basic dye that binds to acidic phosphate radicals in nucleic acids. The stain uptake is dependent on the boiling time of the stain solution, the pH of the stain, and fixation of the tissue. To be specific for DNA, the tissue to be stained has to be treated with RNAse and used at a low pH to make staining of proteins negligible. Most fixatives work with gallocyanine, including Bouin's fixative and alcohol. However, the fixative used will influence the amount of stain uptake.

SOLUTIONS:

Phosphate buffer, pH 6.8

0.1M monosodium phosphate (NaH_2PO_4) about ..... 51 ml
0.1M disodium phosphate (Na_2HPO_4 $2H_2O$) about ..... 49 ml

Adjust pH with appropriate phosphate solution.

OR

Commercial phosphate buffered saline (PBS) adjusted to pH 6.8.

Warm buffer in 37°C oven or water bath before use.

Phosphate buffer with Triton X-100

0.1M phosphate buffer or PBS, pH 6.8, ... 100 ml
Triton X-100 (Sigma, St. Louis) ..... 0.1 ml

RNAse

Ribonuclease A type XII-A (Sigma, St. Louis) ..... 10 mg
Prewarmed phosphate buffer (PBS) ..... 50 ml

Mix right before use

Gallocyanine stain

Boil 150 mg gallocyanine and 5 g chromic potassium sulfate in 100 ml dH_2O (distilled or deionized water) for 10 minutes.
Cool and filter solution.
Readjust solution to 100 ml.
Adjust pH to 1.60.

Ethyl alcohol: 50%, 70%, 95%, 100%

Xylene

Mounting medium

PROCEDURE:

1. Deparaffinize and hydrate slides.
2. Treat in phosphate buffer or PBS with Triton X-100 at 37°C for 5 minutes.
3. Wash in phosphate buffer or PBS, 2 changes for 5 minutes.
4. RNAse at 37°C for 1 hour.
5. Rinse in dH_2O, 2 changes.
6. Gallocyanine stain for 48 hours.
7. Wash in running tap water for 30 minutes.
8. Rinse in dH_2O.
9. Dehydrate through 50%, 70%, and 95% ethyl alcohol for 3 minutes each.
10. Absolute ethyl alcohol, 2 changes for 5 minutes each.
11. Clear in xylene, 2 changes for 5 minutes each.
12. Mount in resinous medium.

RESULTS: (See Fig. 5-9, page 158)

Nuclei	blue-gray
Background	unstained
Absorption	max. about 570 nm

RAPID GALLOCYANINE-CHROME ALUM STAIN

STAIN:

Add 17.5 g chromic potassium sulfate to 100 ml dH_2O.
Gently warm on a hot plate until dissolved.
Add 1 g gallocyanine and boil for 5 minutes.
Filter and restore solution to 100 ml with dH_2O.
Adjust pH to 1.0 with 5N HCl.

PROCEDURE:

1. Deparaffinize and hydrate slides.
2. 0.1M phosphate buffer with Triton-X (see page 145) for 5 minutes.
3. Wash in phosphate buffer, 2 changes for 5 minutes.
4. RNAse (see page 145) at 37°C for 1 hour.
5. Rinse in dH_2O for 5 minutes.
6. Stain for 15 minutes.
7. Running tap water for 10 minutes.
8. Rinse in dH_2O.
9. Dehydrate through 50%, 70%, and 95% ethyl alcohol for 3 minutes each.
10. Absolute ethyl alcohol, 2 changes for 5 minutes each.
11. Clear in xylene, 2 changes for 5 minutes each.
12. Mount in resinous medium.

NOTES:

The staining method of Einarson, modified by Sandritter et al., gives a stronger stain than the rapid method by Husain and Watts. The stains should be made fresh for quantitative analysis.

REFERENCES:

Einarson L. On the theory of gallocyanine-chrome alum staining and its application for quantitative estimation for basophilia: a selective staining of exquisite progressivity. *APMIS.* 1951;28:82-102.

Husain OAN, Watts KC. Rapid demonstration of nucleic acids using "oxidised" gallocyanine and chromic potassium sulfate: Methods and applications. *J Clin Pathol.* 1984;37:99-101.

Sandritter W, Kiefer G, Rich W. Gallocyanine-chrome alum. In: Wied GL, ed. *Introduction to Quantitative Cytochemistry.* New York, NY: Academic Press; 1970:295-349.

STAINING METHODS FOR FLUORESCENCE CYTOMETRY

PRINCIPLE:

Fluorochromes bind to DNA by intercalating in double-stranded nucleic acids, stacking to the phosphate backbone, or covalently joining to the DNA complex. For quantitative cytometry, the fluorescence emitted by the stained cells must be proportional to the amount of DNA. This condition is only fulfilled if the absorption of exciting and emitted light is low. The quantitative relationship of the stains can be tested on alcohol-fixed touch preparations of mouse liver cells, which are polyploid. The average DNA content of these cells is in ratios of 2:4:8:16, and the average fluorescence measurements should convey this relationship.

ACRIFLAVINE FEULGEN STAIN

SOLUTIONS:

5N HCl

dH_2O (distilled or deionized water) ..... 56 ml
Conc. HCl ..... 40 ml

(✋ Always add acid to water)

Acriflavine stain

Acriflavine ..... 20 mg
Potassium metabisulfite ($K_2S_2O_5$) ..... 130 mg
dH_2O ..... 86 ml
0.1N HCl ..... 14 ml

Mix well and filter before use.

Acid alcohol

70% ethyl alcohol ..... 99 ml
Conc. HCl ..... 1 ml

Gel/Mount or Crystal/Mount, (Biomeda, Foster City, CA)

Ethyl alcohol: 95%, 100%

Xylene

Gurr's Fluormount (Gallard-Schlesinger Industries, Inc., Carle Place, NY)

PROCEDURE:

1. Deparaffinize and hydrate slides.
2. Hydrolyze in 5N HCl for 45 minutes at 28°C.
3. Rinse in dH_2O.
4. Stain in acriflavine stain for 20 minutes.
5. Wash in running tap water for 5 minutes.
6. Wash in acid alcohol, 2 changes for 5 minutes each.
7. Wash in dH_2O, 2 changes for 1 minute each.
8. Shake slides to remove excess water.

9. Mount in Gel/Mount or Crystal/Mount.

OR

9. 95% and 100% ethyl alcohol, 2 changes for 3 minutes each.
10. Xylene, 2 changes for 5 minutes each.
11. Mount in Fluormount.

RESULT: (See Fig. 5-10, page 159)

Nuclei fluoresce ..... bright yellow
Background ..... should be black
Absorption ..... max. 450 nm, emission max. 540 nm

NOTES:

The acriflavine Feulgen method is one of the preferred stains for fluorescence microspectrophotometry. Acriflavine binds covalently to the DNA complex, and the fading during measuring is minimal. It should be noted that fluorescence-intensity measurements compare well with optical-density measurements on the same cell population. Keep slides cold and in the dark. This cuts down on fading.

REFERENCES:

Mikel UV, Becker RL Jr. A comparative study of quantitative stains for DNA in image cytometry. *Anal Quant Cytol Histol.* 1991;13:253-260.

Tanke HJ, van Ingen EM, Ploem JS. Acriflavin-Feulgen Stilbene staining: a procedure for automated cervical cytology with a television based system (LEYTAS). *J Histochem Cytochem.* 1979;27:84-86.

ACRIDINE ORANGE STAIN

SOLUTIONS:

Phosphate buffer (or PBS), pH 6.8

0.1M monosodium phosphate (NaH_2PO_4) about ..... 51 ml
0.1M disodium phosphate (Na_2HPO_4 $2H_2O$) about ..... 49 ml

Adjust pH with appropriate phosphate solution.

OR

Commercial phosphate buffered saline (PBS) adjusted to pH 6.8.

Warm buffer in 37°C oven or water bath before use.

Phosphate buffer with Triton X-100

0.1M phosphate buffer or PBS, pH 6.8, ... 100 ml
Triton X-100 (Sigma, St. Louis) ..... 0.1 ml

RNAse

Ribonuclease A type XII-A (Sigma, St. Louis) ..... 10 mg
Prewarmed phosphate buffer adjusted to pH 6.8 ..... 50 ml

Mix right before use.

Citrate buffer, pH 4.0.

0.2M dibasic sodium phosphate Na_2HPO_4 about ..... 80 ml
0.1M citric acid about ..... 120 ml

Adjust pH to 4.0.

Acridine orange stain

Stock solution:

Acridine orange ..... 100 mg
dH_2O ..... 100 ml

Working solution:

Stock solution ..... 3 ml
Citrate buffer ..... 97 ml

Gel/Mount or Crystal/Mount, (Biomeda, Foster City, CA)

Ethyl alcohol: 95%, 100%

Xylene

Gurr's Fluormount (Gallard-Schlesinger Industries, Inc., Carle Place, NY)

PROCEDURE:

1. Deparaffinize and hydrate slides.
2. Treat in buffer/Triton X-100 at 37°C for 3 minutes.
3. Rinse in phosphate buffer or PBS, 2 changes for 1 minute each.
4. Digest in RNAse at 37°C for 1 hour.
5. Rinse in citrate buffer, 2 changes for 1 minute each.
6. Stain in acridine orange working solution for 15 minutes.
7. Wash in citrate buffer, 3 changes for 1 minute each.
8. Rinse in dH_2O for 1 minute.
9. Shake slides to remove excess water.
10. Mount in Gel/Mount or Crystal/Mount .

OR

10. 95% and 100% ethyl alcohol, 2 changes for 3 minutes each.
11. Xylene, 2 changes for 5 minutes each.
12. Mount in Fluormount.

RESULTS:

(See Fig. 5-11, page 159)

Nuclei fluoresce ..... greenish yellow
Background ..... black
For DNA ..... absorption max. 450 nm, emission max. 540 nm

NOTES:

Acridine orange is an intercalating, metachromatic stain that fluoresces green when complexed with DNA and red when complexed with RNA. Although their absorbance and emission maximum are at different wavelengths, there is always an overlap of the green and red fluorescence. Therefore, for quantitative measurements, RNAse treatment will improve the coefficient of variation between the fluorescence readings of acridine orange-stained nuclei. The buffer/Triton X-100 step makes the nuclei more accessible to the RNAse treatment. Acridine orange works well on alcohol-fixed cells, but note that it is not a suitable stain for formalin-fixed tissue sections because of high autofluorescence. Note also that the pyridine and acetic acid anhydride steps in Riegler's original staining method, which supposedly cuts down on nonessential fluorescence, have been left out, as their effect (in our opinion) is minor. Store the stained slides in the dark and cold to reduce fading.

REFERENCE:

Riegler R. Acridine orange staining procedure for DNA. *Acta Physiol Scand.* 1966;67(suppl 67):12-14.

PROPIDIUM IODIDE STAIN

SOLUTIONS:

Phosphate buffer or PBS, pH 6.8

0.1M monosodium phosphate (NaH_2PO_4) about ..... 51 ml
0.1M disodium phosphate ($Na_2HPO_4.2H_2O$) about ..... 49 ml

Adjust pH with appropriate phosphate solution.

OR

Commercial phosphate buffered saline (PBS) adjusted to pH 6.8.

Warm buffer in 37°C oven or water bath before use.

Phosphate buffer with Triton X-100

0.1M phosphate buffer or PBS, pH 6.8, ... 100 ml
Triton X-100 (Sigma, St. Louis) ..... 0.1 ml

RNAse

Ribonuclease A type XII-A (Sigma, St. Louis) ..... 10 mg
Prewarmed phosphate buffer adjusted to pH 6.8 ..... 50 ml

Mix right before use.

Propidium iodide stain

Stock solution:

Propidium iodide ..... 100 mg
dH_2O ..... 100 ml

Working solution:

Stock solution ..... 2 ml
dH_2O ..... 38 ml

Gel/mount or Crystal/Mount (Biomeda, Foster City, CA)

Ethyl alcohol: 95%, 100%

Xylene

Gurr's Fluormount (Gallard-Schlesinger Industries, Inc., Carle Place, NY)

PROCEDURE:

1. Deparaffinize and hydrate slides.
2. Treat in buffer/Triton X-100 at 37°C for 3 minutes.
3. Wash in phosphate buffer or PBS, 2 changes for 1 minute each.
4. Digest in RNAse at 37°C for 1 hour.
5. Wash in phosphate buffer or PBS, 2 changes for 1 minute each.
6. Stain in propidium iodide working solution for 10 minutes.
7. Wash in dH_2O, 2 changes for 3 minutes each.
8. Shake slides to remove excess water.
9. Mount in Gel/Mount or Crystal/Mount.

OR

9. 95% and 100% ethyl alcohol, 2 changes for 3 minutes each.
10. Xylene, 2 changes for 5 minutes each.
12. Mount in Fluormount.

RESULTS:

(See Fig. 5-12, page 159)

Nuclei fluoresce ..... red
Background ..... black
Absorption ..... max. 525 nm, emission max. 620 nm

NOTES:

Cells can be stained with ethidium bromide at the same concentration and in the same manner as propidium iodide. The absorption maximum is then 500 nm and the emission maximum 585 nm.

REFERENCE:

Mikel UV, Becker RL Jr. A comparative study of quantitative stains for DNA in image cytometry. *Anal Quant Cytol Histol.* 1991;13:253-260.

ADDITIONAL SELECTED REFERENCES FOR IMAGE CYTOMETRY

Bjelkenkrantz K. An evaluation of Feulgen-acriflavin-SO_2 and Hoechst 33258 for DNA cytofluorometry in tumor pathology. *Histochemistry.* 1983;79:177-191.

Castleman KR. Spatial and photometric resolution and calibration requirement for cell image analysis instruments. *Appl Optics.* 1987;26:3338-3342.

Cowden RR, Curtis SK. Microfluorometric investigations of chromatin structure. I. Evaluation of nine DNA-specific fluorochromes as probes of chromatin organization. *Histochemistry.* 1981;72:11-23.

Deitch AD, Wagner D, Richart RM. Conditions influencing the intensity of the Feulgen reaction. *J Histochem Cytochem.* 1968;16:371-379.

Jarvis LR. A microcomputer system for video image analysis and diagnostic microdensitometry. *Anal Quant Cytol Histol.* 1986;8:201-209.

Jongsma A, Hijmans W, Ploem JS. Quantitative immunofluorescence: Standardization and calibration in microfluorometry. *Histochemie.* 1971;25:329-343.

Kaplan DS, Picciolo GL. Characterization of instrumentation and calibrators for quantitative microfluorometry for immunofluorescence tests. *J Clin Microbiol.* 1988;27:442-447.

Kasten FH. The chemistry of Schiff's reagent. *Int Rev Cytol.* 1960;10:1-100.

Picciolo GL, Kaplan DS. Reduction of fluorescent reaction product for microphotometric quantitation. *Adv App Microbiol.* 1984:197-234.

Reisfeld R, Honigbaum A, Velapoldi RA. Thallium-doped potassium chloride disks as UV fluorescence standards. *J Opt Soc Am.* 1971;61:1422-1423.

Ruch F. Principles and some applications of cytofluorometry. In: Wied GL, ed. *Introduction to Quantitative Cytochemistry I.* New York, NY: Academic Press; 1966:431-450.

Walker PMB, Richards BM. Quantitative microscopical techniques for single cells. In: Brachet J, Mirsky AE, eds. *The Cell.* Vol I. New York, NY: Academic Press; 1959:91-137.

Wittekind D. Standardization of dyes and stains for automated pattern recognition. *Anal Quant Cytol Histol.* 1985;7:6-30.

Fig. 5–1. Image analyzing system consisting of microscope with high-resolution digital video camera, two display monitors (one shows an image of cells to be selected and analyzed, the other histograms of measurement data), and a computer to drive the whole system.

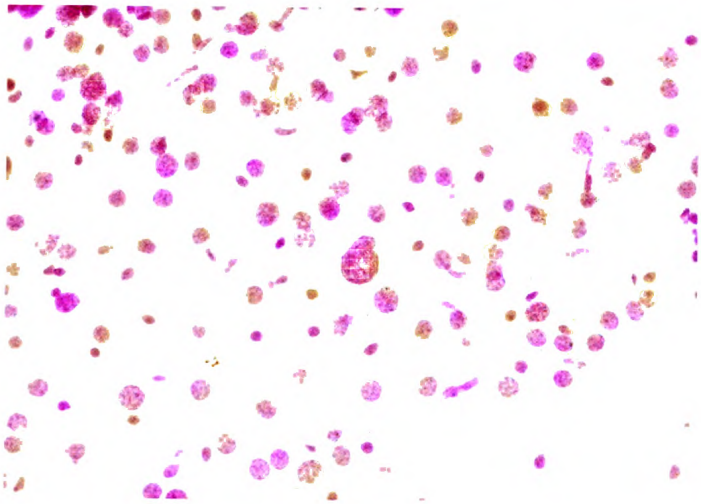


Fig. 5–5. Touch preparation of mouse liver stained with the conventional Feulgen reaction.

Fig. 5–6. Touch preparation of mouse testis stained with azure A Feulgen.

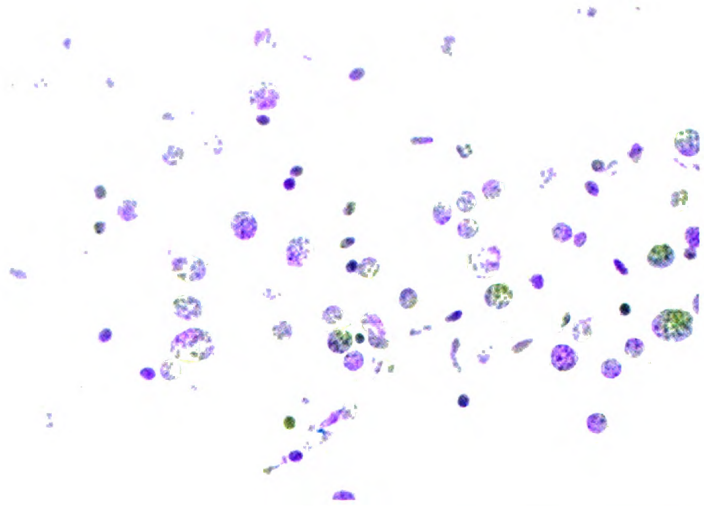
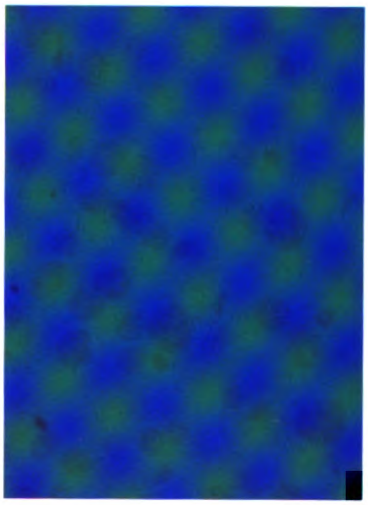


Fig. 5–7. Touch preparation of mouse liver stained with thionin Feulgen.



A B

Fig. 5–8A. Touch preparation of mouse liver stained with acriflavine Feulgen and used as an absorbance stain.

Fig. 5–8-B Acriflavine Feulgen-stained mouse liver cells observed under a broadband, dark-blue filter. Higher magnification than 8A.

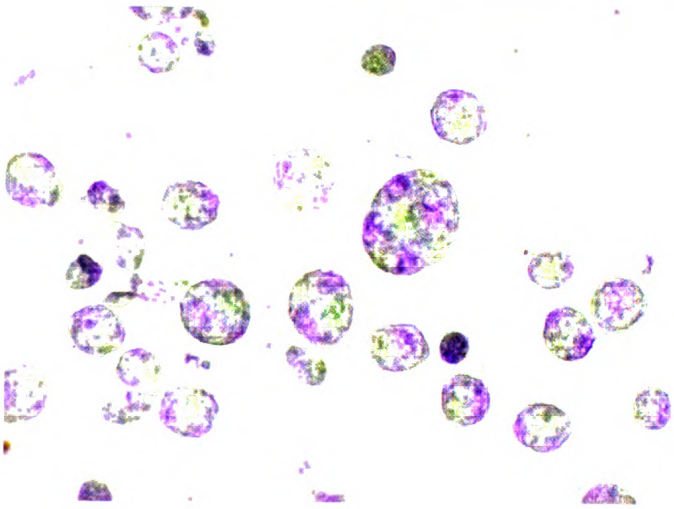


Fig. 5–9. Touch preparation of mouse liver stained with the gallocyanine-chrome alum technique.

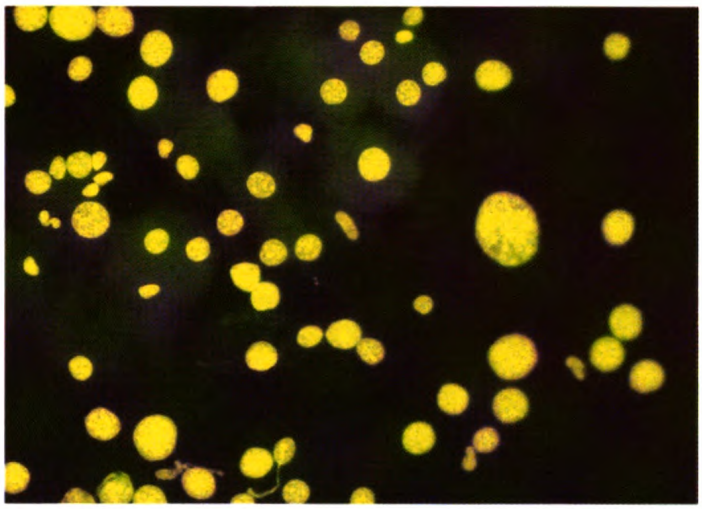


Fig. 5–10. Touch preparation of mouse liver stained with acriflavine Feulgen and used as a fluorescence stain.

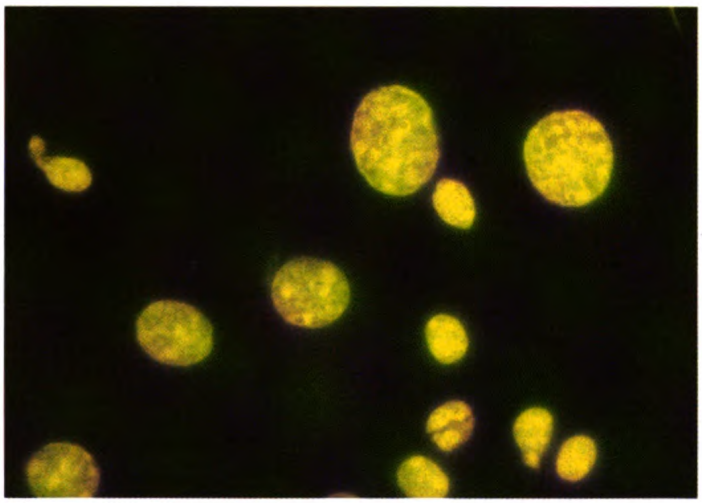


Fig. 5–11. Touch preparation of mouse liver, stained with acridine orange.

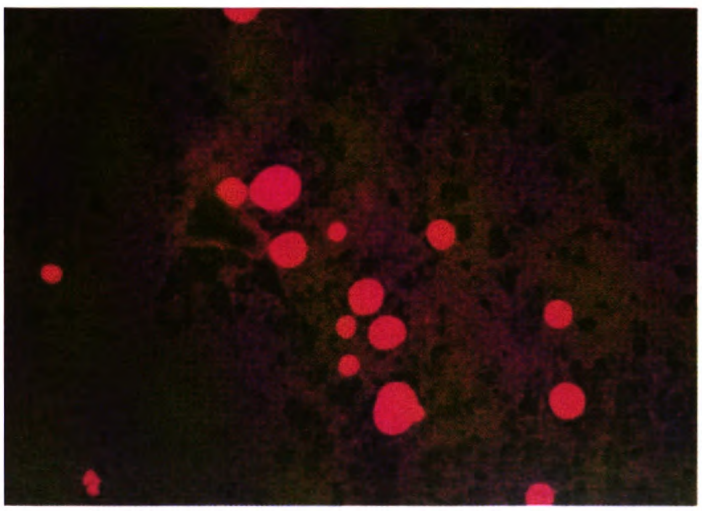


Fig. 5–12. Touch preparation of mouse liver, stained with propidium iodide.

6

Enzyme Histochemistry of Skeletal Muscle

Carole E. Gregory and Joe L. Griffin

INTRODUCTION

This chapter briefly describes methods for staining frozen unfixed sections of muscle biopsies, as applied in the Muscle Laboratory, Department of Neuropathology, AFIP. Enzyme histochemical stains are usually the most useful diagnostic tools for the pathologist interpreting muscle biopsies. Those stains, with a few nonenzymatic stains used for general morphology and localization, are described in this chapter. While our standard biopsy also includes clamped muscle tissue for electron microscopy and for embedding in paraffin after formalin fixation, those topics are not considered here.

Figures 6–1 to 6–12 illustrate artifacts and stains. At the end of the chapter are references to publications that include many photomicrographs of normal and diseased muscle stained by histoenzymatic and related methods.

Some stains answer a yes-no question about a single enzyme to reveal rare deficiency states. Other stains reveal patterns useful for pathologic interpretation, such as NADH and ATPase to show fiber types (type grouping, type predominance, type-specific atrophy, etc.), esterase for denervation (angular atrophic esterase-positive fibers), ATPase for reinnervation (intermediate-density fibers), and alkaline phosphatase for inflammation.

The differences between type 1 and type 2 fibers are summarized in this memory sentence: "One mighty slow, fat, red ox." Type 1 fibers contain more mitochondria, are slow twitch, contain more fat, predominate in what used to be called red

muscles (in animals), and are oxidative rather than glycolytic. Type 2 fibers contain fewer mitochondria, are fast twitch, have less fat, are in "white" muscles, and store and burn more glycogen (less oxidative metabolism). Human muscle contains both type 1 and type 2 fibers. The ratios of fiber types, and other type-specific parameters, are used in pathologic diagnosis.

FLASH FREEZING OF MUSCLE BIOPSY TISSUE

PRINCIPLE:

Flash freezing helps avoid disruption of cellular organization (Figs. 6–1 and 6–2) by ice crystals as water in the muscle tissue freezes. Even freezing directly in liquid nitrogen is too slow, because gaseous nitrogen forms an insulating layer around the tissue. Therefore, isopentane is used as a chemically neutral, heat-conducting medium that removes heat fast enough to prevent significant ice crystal formation.

Muscle biopsy specimens are obtained by a surgeon. The specimen to be frozen is excised as a cylindrical piece of tissue (not clamped), handled with minimal trauma, folded into saline-dampened gauze, sealed in a specimen vial, and placed on ice. Excess saline distorts freezing, so the gauze should be damp with dry corners or squeezed out and excess saline discarded. Transport specimen to the laboratory and freeze within 2 hours (sooner if possible). Optimal size is 5 to 7 mm in diameter and 1.0 to 1.2 cm in length. Larger specimens can not be flash frozen without ice crystal formation in the center of the block, so they are divided into appropriately sized pieces.

Needle biopsies: These small specimens are mounted as other specimens are—naked, held by, but not surrounded by, gum tragacanth. Fiber orientation is usually variable because of distortions caused by the needle, even after checking under a dissecting microscope.

MATERIALS AND SUPPLIES:

Gauze sponges, 4" x 4"
Dental wax
Stainless-steel razor blade
Dissecting microscope
Small blunt-tipped forceps
Embedding block, 1/2" x 1/2"
Hemostat, 10" long
Dewar flask and Pyrex tube (7 1/2" x 1" diameter) ***OR***:
ThermoCup and Pyrex beaker, 50 ml
Ultralow thermometer (to -145°C)
Plastic scintillation vial, wide-mouthed (mouth must be big enough for embedding block)
Cryomarker
Aluminum foil, lightweight
Cryostat set at -20°C, or insulated box containing dry ice
Ultralow freezer (-135°C)

Liquid nitrogen
Isopentane (2-methyl butane, Baker Analyzed)

7% Gum tragacanth (lab grade, Fisher Scientific)

Gum tragacanth ..... 7 g
dH_2O (see Note) ..... 100 ml

Mix by intermittent stirring, no heat, for 30 minutes or until very thick. Good for 3 to 4 weeks when refrigerated. Discard if it becomes moldy or too thin to support muscle tissue.

NOTE: "dH_2O" is used to indicate reagent-grade distilled or deionized water.

PROCEDURE:

1. Write identification of the specimen on an embedding block and on a plastic wide-mouthed scintillation vial.
2. Precool the vial and a square of dry gauze in cryostat.
3. Obtain liquid nitrogen in a Dewar flask and put about 2" of isopentane and an ultralow thermometer in a pyrex tube. Do not cool at this time as isopentane may freeze.
4. Apply a small mound of gum tragacanth to the grooved end of the embedding block.
5. Wearing surgical gloves, remove gauze-wrapped muscle biopsy specimen to dental wax plate on the stage of a dissecting microscope and check for size and orientation.
6. Trim with a new razor blade if necessary, making sharp straight cuts to minimize damage. Prepare a specimen about 5 mm in diameter and 1 cm in length. (Use a millimeter ruler until experience is gained.) Trim away obvious areas of fat and connective tissue, if this can be done without destroying the muscle tissue. Save and freeze excess tissue (see below).
7. Using blunt, smooth-tipped forceps, gently place the muscle specimen on end, to ensure transverse sections, in the gum tragacanth. Surround the bottom of the cylinder of muscle with gum tragacanth, leaving most of the tissue uncovered. The gum tragacanth is for support only. Tissue covered by gum tragacanth during freezing will show severe slow-freeze artifact.
8. Put the Pyrex tube of isopentane into the liquid nitrogen.
9. Clamp the bottom of the embedding block with the muscle specimen in a long hemostat, with the muscle facing directly away from the handles.
10. Once the isopentane tube reaches -145°C, withdraw the thermometer and plunge the block into the isopentane for 8 to 10 seconds. Remove

immediately into the -20°C cryostat. One often hears a single crack or pop. Longer times in isopentane may cause cracking of gum tragacanth and loss of secure support.

11. Blot away isopentane on previously cooled gauze, place specimen in previously labeled scintillation vial, and store at -135°C until ready to cut frozen sections.

12. Freeze extra muscle tissue on foil. Fold a piece of light kitchen-weight aluminum foil, about 1/2" by 2", into a long, narrow V-shaped trough. Place the muscle tissue in the trough, orienting along its length if possible. With the long hemostat, plunge the specimen into -145°C isopentane for 8 to 10 seconds, remove to the cryostat, blot away excess isopentane, and store in the vial with the specimen frozen on the embedding block. DO NOT wrap the extra specimen in foil, as the tightly closed vial is airtight and foil is such an efficient heat conductor there is a danger of thawing the just-frozen tissue and ruining the careful flash freezing.

NOTE: SPECIMEN SAFETY

Precautions by laboratory personnel are particularly important because the tissue is unfixed before sectioning and HIV and other infections may be undetected in patients when biopsied. Swab work surfaces, knives, and tools with bleach, carefully following standard safety precautions.

SECTIONING

MATERIALS AND SUPPLIES:

Cryostat
: A cryostat with a horizontal knife (AO Histostat, Jung) is useful, as sections often fall off vertical knives.

Microtome knives
: B-profile, concave, preferred. New C-profile knives have also been used, but resharpened knives have not worked well for muscle in this lab.

Slide-warming plate (up to 50°C)

Coverslips, 22 x 22 mm, No. 1

Columbia staining dishes for 22-mm square coverslips, Thomas Scientific, Philadelphia

PROCEDURE:

1. See product information for available cryostat.
2. Operate at -20°C.
3. Leave frozen specimens, recovered from storage at ultra-low temperatures, in storage vials in the cryostat for 20 minutes to equilibrate before sectioning.
4. Cut sections using the antiroll plate, at 8 or 10 μm. Sections are easier to pick up from a horizontal knife.
5. Pick up each section on a room-temperature coverslip and air-dry on a slide-warming plate at about 50°C.

NOTE:

Special-purpose material may need to stay frozen. If so, coverslips or slides are coated with poly-L-lysine (see page 44) and are kept at -20°C and not removed from the cryostat. Avoid any possibility of thawing.

STAINING PRECAUTIONS — Maintaining Specimen Identity:

Specific procedures are described for individual stains below. As coverslips can't be easily labeled, standard procedures are needed for running batches of muscle sections to ensure that the identity of specimens is always maintained.

The basic run is done in a Columbia staining dish for 22-mm square coverslips. This jar has 4 grooves on each side and will hold 7 coverslips, angled in zigzag,

with the tissue side of each coverslip facing the front. We assume it will take most of one day to section and stain a standard battery of stains for up to seven biopsy specimens.

Use a master list, section in order, check block and vial labels, put the first section on a labelled slide, space coverslips in sequence on a drying table, keep the same order as coverslips are placed in staining jars, mount and label in sequence, and double-check section shape and microscopic patterns afterward.

STAINING METHODS FOR MUSCLE BIOPSIES

NADH (NADH diaphorase) STAIN

NADH is an important stain for distinguishing type 1 and type 2 muscle fibers. It is usefully correlated with the ATPase procedure, which follows.

PRINCIPLE:

The diaphorase oxidizes NADH (nicotinamide-adenine dinucleotide phosphate) to NAD (nicotinamide-dinucleotide), releasing hydrogen. The H+ is transferred to the tetrazolium salt, Nitro-BT, reducing it and producing an insoluble blue diformazan.

$$\text{NADH} \xrightarrow[\text{Nitro-BT}]{\text{NADH-diaphorase}} \text{NAD} + \text{reduced tetrazolium salt}$$

MATERIALS AND SOLUTIONS:

Phosphate buffer

Solution A: 0.2M potassium phosphate, monobasic (KH_2PO_4), ... 27.2 g/1,000 ml

Solution B: 0.2M sodium phosphate, dibasic, anhydrous (Na_2HPO_4), ... 28.4 g/1,000 ml

Working buffer, pH 7.4 to 7.6

Solution A ..... 15 ml
Solution B ..... 85 ml

0.2% Nitro-BT solution (Nitroblue tetrazolium, crystalline, Grade III, Sigma)

Nitro-BT ..... 200 mg
dH_2O ..... 100 ml

Keep refrigerated. Stable for several months.

Ringer's solution. (Ringer's injection, lactated, USP)

Acetone, 30%, 60%, 90%, and 100%

Glycerol gelatin (Sigma)

NADH staining solution: Make just before use.

NADH (beta-nicotinamide adenine dinucleotide, reduced form, disodium salt, approx. 98%, Sigma)	5.0 mg
Phosphate buffer, pH 7.4 to 7.6	1.4 ml
0.2% Nitro-BT	3.6 ml
Ringer's solution	1.1 ml
dH_2O	1.0 ml

PROCEDURE:

Section thickness: 8 μm.

1. Incubate sections at 37°C in NADH staining solution for 30 minutes.
2. Rinse through quick changes of graded acetone, 30%, 60%, 90%, and 100%. This step increases the contrast between light and dark fibers.
3. Rinse in dH_2O.
4. Mount with glycerol gelatin.

RESULTS:

(See Fig. 6–7.) Blue deposits indicate sites of NADH diaphorase activity. Type 1 fibers are darker; type 2 fibers are lighter. Subsarcolemmal clumps of very dark blue are mitochondria or tubular aggregates. A splotchy appearance is "moth-eaten" fiber. Target fibers, targetoid fibers, and central cores are also visible when present.

REFERENCES:

Scarpelli DG, Hess R, Pearse AGE. The cytochemical localization of oxidative enzymes. I. Diphosphopyridine nucleotide diaphorase and triphosphopyridine nucleotide diaphorase. *J Biophys Biochem Cytol.* 1958;4:747-752.

Wattenberg LW, Leong JL. Effects of coenzyme Q10 and menadione on succinic dehydrogenase activity as measured by tetrazolium salt reduction. *J Histochem Cytochem.* 1959;8:296-703.

ATPase (adenosine triphosphatase) STAIN

The histochemical reaction for adenosine triphosphatase is the primary method of determining distribution, size, and percentage of the fiber types 1 and 2. Intermediate fibers are not usually seen in normal muscle with this reaction, but they do appear in denervation, during fiber-type change after reinnervation. (Griffin and Pezeshkpour 1988).

PRINCIPLE:

According to the Gomori-type phosphatase reaction, calcium ions in the incubating medium precipitate the enzymatic reaction product, orthophosphate, thereby localizing ATPase activity. Treatment with cobalt chloride produces cobalt phosphate, which is visualized, when brought into contact with dilute ammonium sulfide, as black cobalt sulfide deposits.

$$ATP \xrightarrow[H_2O]{ATPase} ADP + H_3PO_4$$

$$H_3PO_4 + CaCl_2 \longrightarrow CaHPO_4 + 2\,HCl$$

$$CoCl_2 + CaHPO_4 \longrightarrow CoHPO_4 + CaCl_2$$

$$(NH_4)_2S + CoHPO_4 \longrightarrow CoS + (NH_4)_2PO4$$

MATERIALS AND SOLUTIONS:

Adenosine 5'-triphosphate (ATP), disodium salt, crystalline, from equine muscle, 99%-100% (Sigma, St. Louis)

0.1M sodium barbital

Sodium barbital (sodium diethyl barbiturate, powder)	20.62 g
dH_2O to a final volume of	1,000 ml

1% calcium chloride ($CaCl_2$)

2% calcium chloride ($CaCl_2$)

Stock barbital solution

0.1M sodium barbital ..... 4 ml
2% calcium chloride ..... 2 ml
dH_2O ..... 14 ml

2% cobalt chloride ($CoCl_2$)

0.2% ammonium sulfide

Ammonium sulfide $(NH_4)_2S$ ..... 1 ml
(20%-24% aqueous, light, certified reagent, Fisher Scientific)
dH_2O ..... 99 ml

Store in fume hood and work in hood. Make this dilution fresh each time.

Ethanol: 75%, 95%, and absolute

Xylene

Mounting Medium

PROCEDURE:

Section thickness: 8 μm.

1. Make stock barbital buffer just before use.
2. Divide stock barbital buffer into two 10-ml portions. Adjust the pH of one portion to 10.4. To the second, add 15.2 mg of ATP. When completely dissolved, adjust pH to 9.4.
3. Preincubate sections in the 10.4 pH solution at room temperature for 10 minutes.
4. Incubate the sections in the 9.4 pH solution (containing the ATP) at 37°C for 30 minutes.

CAUTION: Carry out the remaining steps at room temperature.

5. Place in 1% calcium chloride for 3 minutes.
6. Place in 2% cobalt chloride for 3 minutes.
7. Wash in several changes of deionized water to eliminate the excess cobalt chloride.
8. Develop in 0.2% ammonium sulfide in fume hood for 1 minute.
9. Rinse with several changes of deionized water.
10. Dehydrate through graded ethanol: 75%, 95%, and absolute x 2.
11. Clear in 2 changes of xylene.
12. Mount with resinous mounting medium.

RESULTS:

(See Fig. 6–5.) Black to brown cobalt sulfide deposits indicate sites of adenosine triphosphatase activity. Type 1 fibers stain light brown. Type 2 fibers stain darker brown.

REFERENCES:

Griffin JL, Pezeshkpour G. Myosin ATPase intermediate density fibers for diagnosis of reinnervation. *Muscle Nerve.* 1988;11:915-921.

Padykula HA, Herman E. Factors affecting the activity of adenosine triphosphatases and other phosphatases as measured by histochemical techniques. *J Histochem Cytochem.* 1955;3:161-169.

Padykula HA, Herman E. The specificity of the histochemical method for adenosine triphosphatase. *J Histochem Cytochem.* 1955;3:170-195.

RATPase (reversal of adenosine triphosphatase) STAIN

PRINCIPLE:

A pH of 4.6 inhibits the ATPase activity of type 2 fibers, while the ATPase activity of type 1 fibers is increased. In this way, the usual ATPase activity (i.e., type 2 greater than type 1) is reversed. Subtypes of type 2 can be identified. Sections can be compared directly to the ATPase activity shown by the standard ATPase and to fiber typing shown by NADH.

MATERIALS AND SOLUTIONS:

Adenosine 5'-triphosphate (ATP), disodium salt, crystalline, from equine muscle, 99%-100% (Sigma).

0.1M sodium barbital

Sodium barbital (sodium diethyl barbiturate, powder) ..... 20.62 g
dH_2O to a final volume of ..... 1,000 ml

Acetate buffer, pH 4.55

0.2M sodium acetate ($NaC_2H_3O_2.3\ H_2O$; 27.2 g/1,000 ml) .. 45 ml
0.2M glacial acetic acid (CH_3COOH; 11.55 ml/1,000ml) ..... 55 ml

This solution is stable for several months when kept refrigerated. Check pH weekly.

1% calcium chloride ($CaCl_2$)

2% calcium chloride ($CaCl_2$)

2% cobalt chloride ($CoCl_2$)

When refrigerated, all solutions above except ATP are stable for one year. However, watch for precipitate formation and replace if seen.

0.2% ammonium sulfide

Ammonium sulfide, $(NH_4)_2S$ ..... 1 ml
(light, certified reagent, 20%-24% aqueous, Fisher Scientific)
dH_2O ..... 99 ml

Store in fume hood and work in hood. Make this dilution fresh each time.

Stock barbital solution. Make fresh each time.

0.1M sodium barbital	4 ml
2% calcium chloride	2 ml
dH_2O	14 ml

Ethanol: 75%, 95%, and absolute

Xylene

Resinous mounting medium

PROCEDURE:

Section thickness: 8 µm.

1. Make stock barbital solution just before use.
2. Divide stock barbital solution into two 10-ml portions. Adjust the pH of one portion to 10.4. To the second, add 15.2 mg of ATP. When completely dissolved, adjust pH to 9.4.
3. Preincubate sections in acetate buffer at pH 4.55 for 5 minutes.
4. Place sections in pH 10.4 stock barbital solution for 3 minutes.
5. Incubate the sections at 37°C in pH 9.4 stock barbital solution (containing ATP) for 30 minutes.

NOTE: Carry out the following steps at room temperature.

6. Place in 1% calcium chloride for 3 minutes.
7. Transfer to 2% cobalt chloride for 3 minutes.
8. Wash in several changes of distilled water to eliminate excess cobalt chloride.
9. Develop in 1% ammonium sulfide in fume hood for 1 minute.
10. Rinse in several changes of distilled water to eliminate excess cobalt sulfide.
11. Dehydrate with graded ethanol: 75%, 95%, and absolute x 2.
12. Clear in two changes of xylene.
13. Mount in resinous mounting medium.

RESULTS:

(See Fig. 6–6.) Black cobalt sulfide (CoS) deposits indicate sites of adenosine triphosphatase activity. Type 1 fibers stain dark brown; type 2A fibers stain light brown; and type 2B fibers stain medium brown. For differentiation of type 2C fibers, not considered here, see Brooke and Kaiser and Dubowitz.

REFERENCES:

Brooke MH, Kaiser KK. Some comments on the histochemical characteristics of muscle adenosine triphosphatase. *J Histochem Cytochem.* 1969;17:431-432.

Brooke MH, Kaiser KK. Three "myosin adenosine triphosphatase" systems: the nature of the pH lability and sulfhydryl dependence. *J Histochem Cytochem.* 1970;18:670-672.

Dubowitz V. *Muscle Biopsy—A Practical Approach.* 2nd ed. London: Balliere Tindall: 1985.

NONSPECIFIC ESTERASE STAIN

Esterases are present in inflammatory cells (granulocytes and macrophages), where they are contained in lysosomes, and react strongly at the motor end-plate (acetylcholine esterase). In muscle biopsy specimens, denervated fibers are positive for esterase and can be distinguished from, for example, fibers with type 2 atrophy.

PRINCIPLE:

Esterases catalyze the hydrolysis of esters to produce alcohol and acid reaction products. Naphthol is split from the acetate by enzymatic action, and the free naphthol is diazotized by nitrite and pararosaniline to yield an insoluble red reaction product at sites of activity.

MATERIALS AND SOLUTIONS:

0.1M sodium phosphate

Sodium phosphate, dibasic (Na_2HPO_4) ..... 14.2 g
dH_2O to a final volume of ..... 1,000 ml

1% alpha-naphthyl acetate in acetone

Alpha-naphthyl acetate, crystalline (Sigma, St. Louis) ..... 100 mg
Acetone ..... 10 ml

Pararosaniline stock

Pararosaniline ..... 1 g
dH_2O ..... 20 ml
Conc. HCl ..... 5 ml

Warm gently. Cool to room temperature. Filter and store in refrigerator.

4% sodium nitrite

sodium nitrite ..... 400 mg
dH_2O ..... 10 ml

Incubation medium

Pararosaniline stock ..... 0.40 ml
4% sodium nitrite ..... 0.40 ml
0.1M sodium phosphate, dibasic ..... 10.00 ml
1% naphthyl acetate in acetone ..... 0.25 ml

Mix reagents in order, just before incubation. Adjust pH to 6.2 to 6.7.

Ethanol: 75%, 95%, and absolute

Xylene

Mounting medium

PROCEDURE:

Section thickness: 8 μm.

1. Incubate sections at 37°C in incubation medium for 1 hour.
2. Wash in running tap water for 10 minutes.
3. Dehydrate in graded alcohols: 75%, 95%, and absolute x 2.
4. Clear in xylene.
5. Mount in resinous medium.

RESULTS:

(See Fig. 6–9.) Positive reaction product is dark maroon. Denervated angular atrophic fibers, PMN's, and macrophages stain more darkly than the yellow to pale-orange background; end-plates are a strong reddish brown.

This nonspecific esterase stain was modified and applied to muscle in the laboratory of W. K. Engel, compared to alpha-bungarotoxin labeling by Ringel, Bender, and Engel and referred to Barka and Anderson, p. 261, by Karpati and Engel.

REFERENCES:

Barka T, Anderson PJ. *Histochemistry- theory, practice and bibliography*. New York, NY: Harper and Rowe; 1965.

Karpati G, Engel WK. Correlation histochemical study of skeletal muscle after suprasegmental denervation, peripheral nerve section, and skeletal fixations. *Neurology*. 1968;18:681-692.

Ringel SP, Bender AN, Engel WK. Extrajunctional acetylcholine receptors: alterations in human and experimental neuromuscular disease. *Arch Neurol*. 1976;33:751-758.

ALKALINE PHOSPHATASE STAIN

Alkaline phosphatase is particularly useful in revealing sites of phagocytosis and inflammation in muscle biopsy specimens.

PRINCIPLE:

Alkaline phosphatase catalyzes the breakdown of esters of orthophosphoric acid at alkaline pH. The substrate, sodium alpha-naphthyl phosphate, hydrolyzes rapidly at elevated pH when alkaline phosphatase is present. An insoluble azo dye is produced by the coupling of the diazonium salt, fast blue RR, with alpha-naphthol released by hydrolysis.

MATERIALS AND SOLUTIONS:

Borax-boric acid buffer (0.095M)

Boric acid (H_3BO_3)	3.72 g
Borax, sodium borate ($Na_2B_4O_7.10H_2O$)	13.3 g
dH_2O to a final volume of	1,000 ml

0.1M magnesium sulfate

Magnesium sulfate, anhydrous ($MgSO_4$)	12.04 g
dH_2O to a final volume of	1,000 ml

Incubation medium

Borax-boric acid buffer	9 ml
0.1M $MgSO_4$	1 ml
Alpha-naphthyl acid phosphate (monosodium salt, Sigma)	10 mg
Fast blue RR salt	10 mg

Adjust pH to 8.8. It is normal for the solution to be turbid.

10% neutral buffered formalin

Glycerol gelatin (Sigma)

PROCEDURE:

Section thickness: 8 µm.

1. Incubate sections at 37°C in incubation medium for one hour.
2. Wash in distilled water.
3. Fix in 10% formalin for 10 minutes.

4. Wash in running tap water for 10 minutes.
5. Mount in glycerol gelatin.

RESULTS:

(See Fig. 6–10.) Background is yellow. Abnormal fibers stain black. Black reaction product around muscle fiber bundles indicates inflammation.

Control is internal. Inner surfaces of capillaries between muscle fibers stain grey to black. Engel and Cunningham used 0.2M borate and found arterioles (15 to 35 μm) stained, while capillaries did not. If small blood vessels do not stain, run a normal control.

REFERENCE:

Engel WK, Cunningham GC. Alkaline phosphatase positive abnormal muscle fibers of humans. *J Histochem Cytochem.* 1970;18:55-57.

MYOADENYLATE DEAMINASE STAIN

Adenosine monophosphate deaminase (AMP-DA)

PRINCIPLE:

This stain demonstrates myoadenylate deaminase. Absence of stain reveals enzyme deficiency. The principle involved is that of thiol reduction of the tetrazolium salt to a formazan at the site of substrate hydrolysis, which produces local pH increases to accelerate reduction and deposition.

Incubate with a section from a specimen known to be normal for AMP-DA.

MATERIALS AND SOLUTIONS:

Nitro-BT-AMP

p-nitrotetrazolium blue ..... 100.0 mg
Adenosine 5'-monophosphate (AMP)- ..... 40.0 ml
(sodium salt from yeast, Sigma)
dH_2O ..... 90.5 ml

Dissolve with magnetic stirring and store at 0° to 4° C.

Dithiothreitol solution

Dithiothreitol, 99% ..... 50 mg
dH_2O ..... 3 ml

Subdivide into ten 0.3-ml aliquots in small glass tubes and freeze at -20°C.

3M potassium chloride

KCl ..... 22.37 g
dH_2O to a final volume of ..... 100 ml

Store at room temperature.

Staining solution

Nitro-BT-AMP ..... 9.0 ml
3M KCL ..... 0.7 ml

Add the KCl, with stirring, to the Nitro-BT-AMP solution and bring to room temperature in a water bath. Adjust to pH 6.1 with 0.01M NaOH. Thaw one tube of dithiothreitol; bring to room temperature; then add dropwise, with stirring, to the stain.

Rinsing solution (0.15M KCl, 1.5mM sodium citrate)

Potassium chloride	11.120 g
Sodium citrate, dihydrate	0.441 g
dH_2O to a final volume of	1,000 ml

Adjust pH to 6.0 with 1N HCl.

Glycerol gelatin (Sigma)

PROCEDURE:

Section thickness: 10 μm.

1. Mix staining solution just before use.
2. Stain in staining solution at room temperature for one hour.
3. Wash in two changes of rinsing solution.
4. Rinse in distilled water.
5. Mount in glycerol gelatin.

RESULTS:

(See Fig. 6–11.) A negative reaction, which is abnormal, is indicated by a complete lack of staining. There are also intermediate degrees of reaction, determined by comparison with the normal control section. Type 1 fibers show granular blue densities on a clear cytoplasmic background. Type 2 fibers show a fine reticular blue staining on a diffusely pink cytoplasm. Fiber typing is based on color differences, rather than density differences. This stain is not intended for primary use in fiber typing.

REFERENCE:

Fishbein WN, Griffin JL, Armbrustmacher VW. Stain of skeletal muscle adenylate deaminase. *Arch Pathol Lab Med.* 1980;104:462-466.

MODIFIED GOMORI'S TRICHROME STAIN

This modification of the original Gomori's trichrome stain, applied to fresh-frozen biopsy sections, reveals more structural details than are seen in paraffin-embedded material. The trichrome stain facilitates interpretation of enzyme histochemical stains of the same specimen, as well as being a rapid stain for preliminary evaluation.

PRINCIPLE:

This nonenzymatic stain is based on differential affinity of tissue components for stains of different colors.

MATERIALS AND SOLUTIONS:

Hematoxylin stain, Gill's 2 (Fisher Scientific). Store in a tightly capped container in the dark at room temperature. The solution is stable until the manufacturer's expiration date.

Modified Gomori's stain

Chromotrope 2R, dye content 85% (Sigma, St. Louis)	0.8 g
Fast green FCF	0.3 g
Phosphotungstic acid	0.6 g
Glacial acetic acid	1.0 ml
dH_2O	100 ml

Adjust pH to 3.4. Store at room temperature. This stain is stable for 1 month.

0.2% acetic acid

Glacial acetic acid	1 ml
dH_2O	499 ml

This solution is stable indefinitely.

Ethanol: 50%, 75%, 95%, and absolute

Xylene

Resinous mounting medium

PROCEDURE:

Section thickness: 8 µm.

1. Stain in hematoxylin for 5 minutes.
2. Rinse in deionized water several times, until the water is clear.

3. Stain in the Gomori's stain for 10 minutes.
4. Rinse once in deionized water.
5. Dip in 0.2% acetic acid for 30 seconds.
6. Dip in 50% ethanol for 30 seconds.
7. Dehydrate in rapid changes of ethanol: 75%, 95%, and absolute x 2.
8. Clear in xylene.
9. Mount with resinous mounting medium.

RESULTS:

(See Fig. 6–4.) Myofibrils are stained green and intermyofibrillar material (mitochondria and sarcoplasmic reticulum) is stained red. Interstitial connective tissue is stained a lighter green, easily distinguishable from the myofibrils. Nuclei are red to purple. Myelin of the nerve branches stains a foamy red, while the axoplasm is pale blue.

NOTE:

In the event the muscle tissue is inadvertently exposed to formalin, this stain will show it clearly. The muscle fibers then stain red instead of predominantly green.

REFERENCES:

Engel WK, Cunningham GG. Rapid examination of muscle tissue: an improved method for fresh-frozen biopsy sections. *Neurology.* 1963;13:919-923.

Gomori G. A rapid one-step trichrome. *Am J Clin Pathol.* 1950;20:661-664.

HEMATOXYLIN AND EOSIN STAIN

This hematoxylin and eosin procedure is similar to that used for paraffin sections. Shorter staining times are used for frozen unfixed sections than for paraffin-embedded material.

PRINCIPLE:

Color differentiation is based on the nucleus being more basophilic, with greater affinity for hematoxylin, while the cytoplasm has greater affinity for eosin.

MATERIALS AND SOLUTIONS:

Hematoxylin stain, Gill's 2 (Fisher Scientific). Store at room temperature. Protect from light. The stain is good until the manufacturer's expiration date. Discard used stain.

Eosin stain

Stock solutions:

1% eosin Y solution:

Eosin Y	10 g
dH_2O	1,000 ml

1% phloxine B solution:

Phloxine B	1 g
dH_2O	100 ml

Working solution:

1% eosin Y	100 ml
1% phloxine B	10 ml
95% ethanol	780 ml
Glacial acetic acid	4 ml

Label and store at room temperature. Make fresh each week.

Ethanol: 75%, 95%, and absolute

Xylene

Resinous mounting medium

PROCEDURE:

Section thickness: 8 μm.

1. Stain in hematoxylin for 1 minute.

2. Wash with tap water several times, until water is clear.
3. Stain in eosin working solution for 5 minutes.
4. Rinse twice in 75% ethanol.
5. Dehydrate in ethanol: 95% and absolute x 2.
6. Clear in two changes of xylene.
7. Mount in resinous mounting medium.

RESULTS:

(See Fig. 6–3.) Nuclei are blue. Cytoplasm and most other tissue structures are pink to red.

SUCCINIC DEHYDROGENASE (SDH) STAIN

Sections stained for SDH are similar in appearance to those stained for NADH diaphorase, but SDH more specifically localizes to mitochondria. For example, NADH stains both tubular aggregates and mitochondrial clumps in muscle fibers, while SDH stains only the mitochondria (Figs. 6–7, 6–8).

PRINCIPLE:

Succinic acid dehydrogenase releases H+ from succinate. The acceptance of H+ by the tetrazolium salt, Nitro-BT, produces an insoluble diformazan, visualized as blue deposits.

$$\text{Succinate} \xrightarrow[\text{Nitro-BT}]{\text{SDH}} \text{fumarate} + \text{reduced tetrazolium salt}$$

MATERIALS AND SOLUTIONS:

0.2M succinic acid

Succinic acid, crystalline disodium salt, anhydrous ..... 3.2 g
dH_2O to a final volume of ..... 100 ml

Make fresh each week.

0.2M phosphate buffer, pH 7.4 to 7.6

0.2M potassium phosphate, monobasic (KH_2PO_4) ..... 15 ml
(27.22 g/1,000 ml dH_2O)
0.2M sodium phosphate, dibasic, (Na_2HPO_4) ..... 85 ml
(28.4 g/1,000 ml dH_2O)

0.2% Nitro-BT solution, pH 7.4 to 7.6

Nitroblue tetrazolium, 98% (Sigma, St. Louis) ..... 200 mg
dH_2O ..... 100 ml

Store in a dark bottle and refrigerate. Stable for several months.

Incubation medium

0.2M succinic acid ..... 2.5 ml
0.2M phosphate buffer ..... 2.5 ml
0.2% Nitro-BT ..... 5.0 ml

Saline (0.9% sodium chloride, injection, USP)

Formalin-saline

Sodium chloride	8.5 g
10% neutral buffered formalin	1,000 ml

Ethanol: 15%

Glycerin gelatin (Sigma)

PROCEDURE:

Section thickness: 10 μm.

1. Incubate sections at 37°C in incubation medium for 30 minutes.
2. Wash in saline.
3. Fix in 10% formalin-saline for 10 minutes.
4. Place in 15% ethanol for 5 minutes.
5. Mount in glycerin gelatin.

RESULTS:

(See Fig. 6-8.) Blue deposits indicate sites of succinic dehydrogenase activity within mitochondria.

REFERENCES:

Nachlas MM, Tsou KC, De Souza E, Cheng CS, Seligman AM. Cytochemical demonstration of succinic dehydrogenase by the use of a new p-nitrophenyl substituted ditetrazole. *J Histochem Cytochem.* 1957;5:420-436.

Thompson SW. *Selected Histochemical and Histopathological Methods.* Springfield, Ill: Charles C Thomas: 1966:697-700.

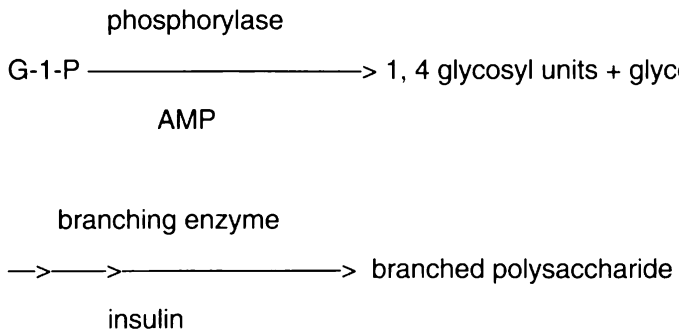
Zugibe FT. *Diagnostic Histochemistry,* St. Louis, Mo: CV Mosby Co; 1970:282.

PHOSPHORYLASE (amylophosphorylase) and BRANCHING ENZYME (1,4-amylo:1,6-transglucosidase) STAIN

PRINCIPLE:

In vitro, phosphorylase is capable of constructing an unbranched amylase-type polysaccharide made of alpha-l, 4 glycosyl units. Branching enzyme joins groups of these glycosyl units to the parent polysaccharide by alpha-l, 6-linkage, producing a branched amylopectin-type polysaccharide.

Glucose-l-phosphate and glycogen are required as primers for the enzymatic reactions. AMP and insulin are activators. The color formed when iodine and a polysaccharide combine is the result of electrostatic binding forces known as dispersion forces. No chemical binding between atoms occurs; rather, the iodine atoms join to form a straight-chain polyiodide around which the polysaccharide molecules form a spiral wrapping. The dispersion forces are created by electrostatic interactions of electrons in the outer shells of the two adjacent atoms.



MATERIALS AND SOLUTIONS:

Insulin (Eli Lilly and Co., regular Iletin I)

Glycogen (Sigma, St. Louis)

Glucose-1-phosphate, crystalline, dipotassium salt, hydrate,approximately 98% (Sigma)

Adenosine-5-monophosphate (AMP) from equine muscle, type 3, Sodium salt, 99+%, crystalline (Sigma)

(Keep reagents 2 through 4 desiccated and below 0°C)

Absolute ethanol

0.2M acetate buffer, pH 5.8 to 6.0

0.2M sodium acetate ($NaC_2H_3O_2.3H_2O$) ..... 94 ml
(27.2 g/1,000 ml dH_2O)
0.2M acetic acid (CH_3COOH) ..... 5 ml
(11.55 ml/1,000 ml dH_2O)

This is stable for several months. Check pH weekly.

Insulin solution

0.2M acetate buffer ..... 10 ml
dH_2O ..... 15 ml
Glycogen ..... 2 mg
Insulin ..... 1 drop

Branching enzyme and phosphorylase solution

Insulin solution ..... 17 ml
Glucose-1-phosphate, hydrate 98% (Sigma) ..... 50 mg
Adenosine 5'-monophosphate (AMP) ..... 10 mg
from equine muscle, sodium salt, 99%, Sigma)

Phosphorylase solution

Branching enzyme and phosphorylase solution ..... 8.5 ml
Absolute ethanol ..... 1.7 ml

Ethanol inhibits branching enzyme activity.

Gram's iodine (IKI) stock solution

Iodine ..... 10 g
Potassium iodide ..... 2 g
dH_2O ..... 300 ml

Working iodine solution

Stock solution ..... 1 ml
dH_2O ..... 9 ml

Gram's iodine glycerin

Gram's iodine stock solution ..... 1 part
Glycerin ..... 5 parts

PROCEDURE:

Section thickness: 8 μm.

1. Incubate three groups of sections in insulin solution (control), branching enzyme and phosphorylase solution, and phosphorylase solution, respectively, at 37°C for one hour.
2. Transfer all sections to working iodine solution for 5 minutes or until blue

to brown color develops. The control will remain straw colored.

3. Mount in Gram's iodine glycerin.

RESULTS:

Figure 6–12 shows phosphorylase treated with ethanol, no branching enzyme. Blue deposits indicate proper functioning of phosphorylase alone. If both phosphorylase and branching enzyme are active, a violet or brownish-purple deposit will be seen. Native glycogen stains reddish brown.

REFERENCES:

Barka T, Anderson PJ. *Histochemistry: Theory and Practice.* New York, NY: Harper & Row; 1963:290-294.

Takeuchi T. Histochemical demonstration of branching enzyme (amylo-1, 4-1,6-transglucosidase) in animal tissues. *J Histochem Cytochem.* 1958;6:208-216.

Takeuchi T. Histochemical demonstration of phosphorylase. *J Histochem Cytochem.* 1956;4:84.

Takeuchi T, Kuriak H. Histochemical detection of phosphorylase in animal tissues. *J Histochem Cytochem.* 1955;3:153-160.

Thompson SW. *Selected Histochemical and Histopathological Methods.* Springfield, Ill: Charles C Thomas: 1966:678-681.

Zugibe FT. *Diagnostic Histochemistry.* St. Louis, Mo: CV Mosby Co: 1970:264-266, 281.

PHOSPHOFRUCTOKINASE (PFK) STAIN

This procedure is used to determine if a glycogen-storage disease is due to a deficiency of phosphofructokinase, rather than of one of the other enzymes necessary for the metabolism of glycogen.

PRINCIPLE:

Several enzymes are involved in the reaction mechanism. Phosphofructokinase catalyzes the conversion of fructose-6-phosphate to fructose-1,6-diphosphate in the presence of ATP. Fructose-1,6-diphosphate is then acted on by aldolase, producing D-glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. Dihydroxyacetone phosphate goes to D-glyceraldehyde-3-phosphate by an isomerase. Glyceraldehyde-3-phosphate dehydrogenase then acts on the latter in the presence of NAD and inorganic phosphate to produce diphosphoglyceric acid and NADH. Diphosphoglyceric acid goes to lactic acid. NADH is oxidized by a tetrazolium reductase. The H+ is transferred to the tetrazolium salt, Nitro BT, reducing it and producing the insoluble blue diformazan.

CONTROL: Use 10-µm frozen sections from a muscle biopsy specimen known to be normal for PFK.

MATERIALS AND SOLUTIONS:

0.2% Nitro-BT

Nitroblue tetrazolium (approx 98%, Sigma, St. Louis) ..... 200 mg
dH_2O ..... 100 ml

Adjust pH to 7.4 to 7.6

Basic incubation solution

0.2% Nitro-BT ..... 10 ml
dH_2O ..... 10 ml
Dissolve in sequence:
Sodium arsenate ($Na_2HAsO_4.7H_2O$, Sigma) ..... 62 mg
Adenosine 5'-triphosphate (ATP) ..... 12 mg
(from equine muscle, disodium salt 99%-100%)
B-nicotinamide adenine dinucleotide (B-NAD) ..... 14 mg
from yeast, approx. 98%, Sigma)

F-1,6-di-P incubation medium

Basic incubation solution ..... 10 ml
Fructose-1,6-diphosphate ..... 10 mg
(tetrasodium salt, 98%-100%, Sigma)
Adjust pH to 8.6.

F-6-P incubation medium

Basic incubation solution	10.0 ml
Fructose-6-phosphate (disodium salt, approx 98%, Sigma)	7.0 mg
Magnesium sulfate ($MgSO_4$)	2.5 mg

Adjust pH to 7.0.

Glycerol gelatin (Sigma)

PROCEDURE:

Section thickness: 10 μm.

1. Incubate sections, with control sections, in F-1,6-di-P and F-6-P incubation media, respectively, at 37°C for one hour.
2. Wash in deionized water.
3. Mount with glycerol gelatin.

RESULTS:

Blue deposits indicate sites of enzymatic activity. Controls and normals should show blue staining in sections incubated in both solutions, indicating normal PFK activity. The absence of blue deposits when the substrate is F-6-P, plus the presence of such deposits when F-1,6-di-P is the substrate, indicates a lack of PFK activity. If blue deposits are absent from both sections, a preceding enzyme is inactive.

NOTE: The pH is critical in this procedure, and the fact that the two incubating solutions are so far apart in pH is deliberate. (See Bonilla and Schotland, page 9, for the explanation; for further information, see Pearse, pages 902-903.)

REFERENCES:

Bonilla E, Schotland DL. Histochemical diagnosis of muscle phosphofructokinase deficiency. *Arch Neurol.* 1970;22:8-12.

Pearse AGE. *Histochemistry, Theoretical and Applied.* 3rd ed. Boston: Little Brown and Company; 1972;2:902.

PERIODIC ACID-SCHIFF REACTION (PAS) STAIN

PRINCIPLE:

The periodic acid-Schiff reaction is used to demonstrate glycogen in muscle. However, this reaction not only shows glycogen but other polysaccharides and several other substances as well. The stain depends upon the oxidation of 1:2 glycol groups (-CHOH-CHOH-) by periodic acid, which results in the formation of dialdehydes. Dialdehydes react with Schiff reagent to produce a reddish-purple stain. Proving the reaction product is glycogen requires digestion with an alpha-amylase.

MATERIALS AND SOLUTIONS:

Schiff's leuco-fuchsin solution

Basic fuchsin	1 g
dH_2O	200 ml
1N HCl	20 ml
Sodium bisulfite	1 g

Dissolve basic fuchsin in hot distilled water. Bring to boiling point. Cool to 50°C. Filter and add the hydrochloric acid. Cool further and add the sodium bisulfite. Solution becomes straw colored. Store in refrigerator.

Test for Schiff's leuco-fuchsin solution:
Put a few drops of Schiff's solution into 10 ml of 37%-40% formaldehyde in a watch glass. If the solution turns reddish purple rapidly, it is good. If the reaction is delayed and the resultant color is deep blue purple, the solution is breaking down.

0.5% periodic acid solution

Periodic acid crystals	0.5 g
dH_2O	100 ml

Normal hydrochloric acid solution

Hydrochloric acid, conc.	83.5 ml
dH_2O	916.5 ml

Diastase solution (Fisher Scientific)

Diastase	0.5 g
dH_2O, sterile	100 ml

Store in refrigerator. Good for one week.

Mayer's hematoxylin

Hematoxylin	1.0 g
dH_2O	1,000 ml
Sodium iodate	0.2 g
Ammonium or potassium alum	50.0 g
Citric acid	1.0 g
Chloral hydrate	50.0 g

Dissolve the hematoxylin in water, using gentle heat if necessary. Then add the sodium iodate, and then the alum. Shake until the alum is dissolved. Add the citric acid, and finally the chloral hydrate. The final color of the stain is reddish violet. Stain will keep well.

Ethanol: 95% and absolute

Xylene

Mounting medium

PROCEDURE:

Section thickness: 8 μm.

1. Place sections in absolute ethanol for 10 minutes.
2. Place in 0.5% periodic acid for 10 minutes.
3. Rinse in distilled water. (Do not use tap water.)
4. Place in Schiff's reagent for 15 minutes.
5. Rinse in running tap water for 10 minutes.
6. Stain in Mayer's hematoxylin for 2 minutes.
7. Wash in tap water for 10 minutes, then rinse in dH_2O.
8. Dehydrate in ethanol: 95% and absolute x 2.
9. Clear with 2 rinses in xylene.
10. Mount in resinous mounting medium.

RESULTS:

Much carbohydrate containing material, particularly glycogen in muscle, shows a positive reaction—rose to purplish red.

NOTE: If PAS reaction with digestion is desired, after Step 1, rinse sections in distilled water and place in 0.5% diastase for 20 minutes. Saliva can also be used in place of the 0.5% diastase. Rinse in running tap water 10 minutes; wash in distilled water; then go on to Step 2 above.

REFERENCES:

Dubowitz V. *Muscle Biopsy—A Practical Approach*. 2nd ed. London: Balliere Tindall; 1985:28.

Gaffney E. Carbohydrates. In: Prophet EB, Mills B, Arrington JB, Sobin LH, eds. *Laboratory Methods in Histotechnology*. Washington, DC: American Registry of Pathology; 1992:151-153.

OSMIUM TETROXIDE-p-PHENYLENEDIAMINE LIPID STAIN

PRINCIPLE:

This stain reveals osmiophilic lipid inclusions. Para-phenylenediamine plus osmium tetroxide produces a more distinct stain than either chemical alone, without staining so strongly that details are obscured.

MATERIALS AND SOLUTIONS:

2% osmium tetroxide solution

Osmium tetroxide, OsO_4 ..... 200 mg
dH_2O ..... 10 ml

(**Caution: Do not inhale vapors. Wear gloves; work in fume hood).** Stable indefinitely. Discard when it turns from clear pale yellow to gray.

1% para-phenylenediamine, (p-Pd)

p-Pd (1,4-phenylenediamine, 97%, Aldrich, Milwaukee) .. 100 mg
dH_2O ..... 10 ml

Handle carefully when weighing and wipe down the work area with a wet paper towel to remove dust, as this stain is almost impossible to remove from clothing and skin. Dissolve in the water overnight. Store in the dark. (Wrap scintillation vial in aluminum foil.) This solution is stable about 1 week. Discard if it turns from pale reddish brown to dark brown.

Mounting medium

PROCEDURE:

1. Wear gloves and work in a fume hood.
2. Place the coverslips on a white background (such as a scintillation vial cap) and place a drop of 2% OsO_4 on the section for 60 seconds.
3. Rinse thoroughly in deionized water. (Pick up the coverslips with forceps and dunk in a container of water ***or*** rinse with a stream of water from a squirt bottle.)
4. Cover sections with a drop of 1% p-Pd for 15 to 20 seconds, (or until color appears).

5. Rinse thoroughly with distilled water.
6. Air-dry thoroughly.
7. Mount with resinous mounting medium.

RESULTS:

This procedure produces clean, brown-stained sections. Type 1 myofibers are darker than type 2. Abnormal lipid deposits and mitochondrial aggregates stain distinctly black. Myelin in peripheral nerve twigs is also prominently stained.

REFERENCE:

Anderson SC, Schochet SS Jr. Osmium tetroxide-p-Phenylenediamine stain for lipid in skeletal muscle. *Arch Neurol*. 1982;39:383.

OIL RED O STAIN IN PROPYLENE GLYCOL

PRINCIPLE:

Oil red O is soluble in lipids. The color differentiates lipids from other cellular constituents.

MATERIALS AND SOLUTIONS:

Oil red O in propylene glycol

Oil red O ..... 0.5 g
Propylene glycol ..... 100 ml

Add a small amount of propylene glycol to the oil red O and mix well. Crush larger pieces. Gradually add the remainder of the propylene glycol, stirring periodically. Heat gently until the solution reaches 95°C. Do not allow the temperature to go over 100°C. Stir while heating. Filter through coarse filter paper while still warm. Allow to stand overnight at room temperature. Filter through a medium fritted glass filter under vacuum. If solution becomes turbid, refilter. Turbidity can also be removed by centrifugation.

85% propylene glycol

Propylene glycol ..... 85 ml
dH_2O ..... 15 ml

Harris' hematoxylin (see *Laboratory Methods in Histotechnology*, p. 56)

Glycerin gelatin (Sigma, St. Louis)

PROCEDURE:

Section thickness: 10 μm.

1. Place sections in absolute propylene glycol for 2 minutes.
2. Stain in oil red O in propylene glycol for 2O to 3O minutes.
3. Differentiate in 85% propylene glycol about one minute. Stir or agitate to prevent folds in tissue.
4. Wash in 2 changes of dH_2O.
5. Stain in Harris' hematoxylin for a few seconds.
6. Wash in 2 changes of dH_2O.

7. Mount in glycerin gelatin.

RESULTS:

Fat is red; nuclei are blue.

REFERENCES:

Allen TC. Hematoxylin and eosin. In: Prophet EB, Mills B, Arrington JB, Sobin LH, eds. *Laboratory Methods in Histotechnology.* Washington, DC: American Registry of Pathology; 1992:56.

Johnson FB. Lipids. In: Prophet EB, Mills B, Arrington JB, Sobin LH, eds. *Laboratory Methods in Histotechnology.* Washington, DC: American Registry of Pathology; 1992:177.

HOW TO AVOID PROBLEMS AND AIDS FOR TROUBLESHOOTING

Flash freezing:

We get fresh specimens from local sources and frozen specimens from remote contributors. Freezing artifacts often occur when directions are not followed, as when someone surrounds muscle with OCT or other freezing media or did not use isopentane (Fig. 6–1). If a thawed specimen is to be refrozen, follow the directions for fresh tissue. A thawed specimen that was placed unfrozen in a freezer rather than flash frozen looks like Figure 6–2.

If you have followed directions and still see ice holes, consider the following:

1. Specimens too long in transit from surgery may show more ice holes, even though the enzymes stain.
2. Specimen may have been in saline or kept on wet rather than damp gauze.
3. Specimen may be too big. Specimens with poor muscle tone may "slump" and spread on the block to become shorter and larger in diameter. The center could then show some ice holes. Holding the specimen upside down briefly may counter slumping.
4. The thermometer could be off or might have touched the glass tube to give a false low reading. Before you assume that, practice a relaxed but fast plunge of the specimen into precooled isopentane. In experiments with good specimens and experienced personnel, we see little ice even when isopentane is only cooled to -70°C in an ultralow freezer.

Sectioning:

1. Tearing, bunching, or compression, if the cryostat is at -20°C, is likely to be due to a specimen with high fat, connective tissue, blood vessels, or nerves. Usually a fatty specimen is fatty throughout and also contains connective tissue, because the muscle tissue has been lost through disease. Surgeons may need to be alerted to avoid end-stage muscle. Tearing, bunching, or compression can also be caused by a dull knife.
2. Soft blocks can be due to improper freezing or high fat. If it is fat, sectioning may be possible if the block is sprayed with a refrigerant spray or the cryostat is lowered to -30°C. We have seen soft blocks that were apparently stored in isopentane or surrounded by warm isopentane and then frozen. Sectioning for enzyme histochemistry was not possible. These specimens were put in formalin and sent for paraffin embedding.

3. Chatter can be caused by too much water in the specimen, a wrong knife angle, a dull knife, a loose block holder, or a loose knife holder.
4. Sections that split when cut can be due to a bad knife nick or the block having cracked during freezing. A cracked block may cut more easily if the block is rotated so that another side first contacts the knife.

Staining:

1. NADH: This procedure usually works well.
2. ATPase and RATPase: These stains are sensitive to pH and can overdifferentiate or underdifferentiate. At times the staining may need to be tuned by shifting the pH of the stain in small increments (0.05 pH units). Make note of specimens that give clear differentiation. These can be pulled and recut to test the staining procedure.

   Precipitate over an otherwise good stain probably is caused by deterioration of the ammonium sulfide. Keep the stock bottle tightly closed. If it darkens to bright yellow, take a new bottle. Precipitate may also form if any of the 3 washes is inadequate. Try more careful washing before changing the sulfide.
3. Esterase: A light precipitate is often present over the section and the surface of the coverslip. The precipitate does not usually hamper visualization when the section is mounted, but the top (nontissue side) of the coverslip may need to be cleaned after the mount sets (gently rub with damp gauze).
4. Alkaline phosphatase: Bubbles sometimes generate after the coverslip is mounted on the slide. If this becomes a problem, the section can be remounted.
5. AMP-DA: If the control does not stain, dithiothreitol (DTT) may have been left out or the pH could be off.
6. Trichrome: If the section stains too blue, the Gomori's mixture is probably too old. Replace the stain monthly. You can correct this somewhat by increasing times in 0.2% acetic acid and 50% ethanol, but watch carefully so as not to decolorize too much.
7. H&E: Too red, too long in eosin; too pale, not long enough.
8. SDH: As with NADH, this procedure usually works well.
9. Phosphorylase: This stain is not permanent, and slides need to be checked immediately. If a record is needed, photograph the result.
10. Phosphofructokinase: For unknown reasons, this stain is not uniformly reproducible.
11. PAS: If the stain does not work, the probable cause is old Schiff's reagent.

12. Os-pPD: If slides are overstained and with precipitate, the pPD solution is probably too odd.
13. Oil red O: In fatty, end-stage muscle, be aware that fat may be moved around by the knife.

ADDITIONAL SELECTED REFERENCES

These references include many illustrations of histoenzymatic stains applied to normal and diseased muscle. The first four reviews show muscle stained in the Muscle Laboratory, AFIP, by the methods described in this chapter.

Armbrustmacher VW. Pathology of the muscular dystrophies and the congenital nonprogressive myopathies. *Pathol Annu.* 1980;15:301-333.

Armbrustmacher VW. Skeletal muscle in denervation. *Pathol Annu.* 1978;13:1-33.

Armbrustmacher VW, Griffin JL. Neuromuscular diseases. In: Schochet SS Jr, ed. *The Clinical Neurosciences: Neuropathology.* New York, NY: Churchill Livingstone; 1983:3:363-416.

Armbrustmacher VW, Griffin JL. Pathology of the inflammatory and metabolic myopathies. *Pathol Annu.* 1981;16:15-60.

Dubowitz V. *Muscle Biopsy—A Practical Approach.* 2nd ed. London: Baillere Tindall: 1985.

Dubowitz V, Brooke MH. *Muscle Biopsy—A Modern Approach.* London: WB Saunders Co;1973.

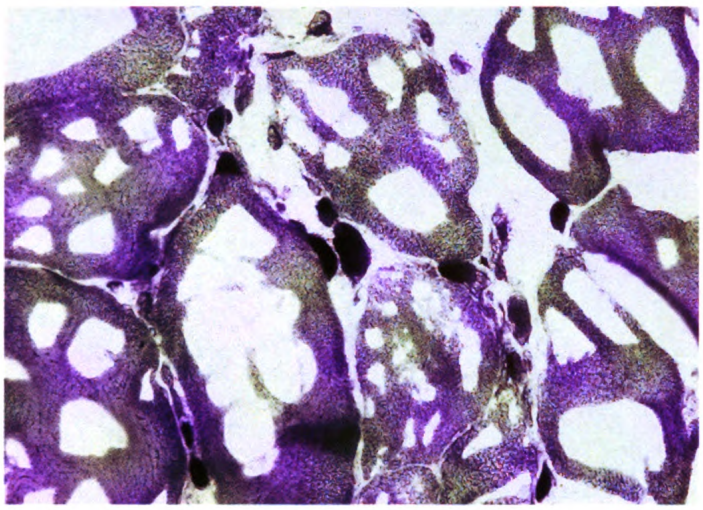


Fig. 6–1. Slow-freeze artifact with internal ice. This sort of artifact is produced by surrounding muscle with OCT, freezing in liquid nitrogen without isopentane, or otherwise slowing the cooling rate. NADH.

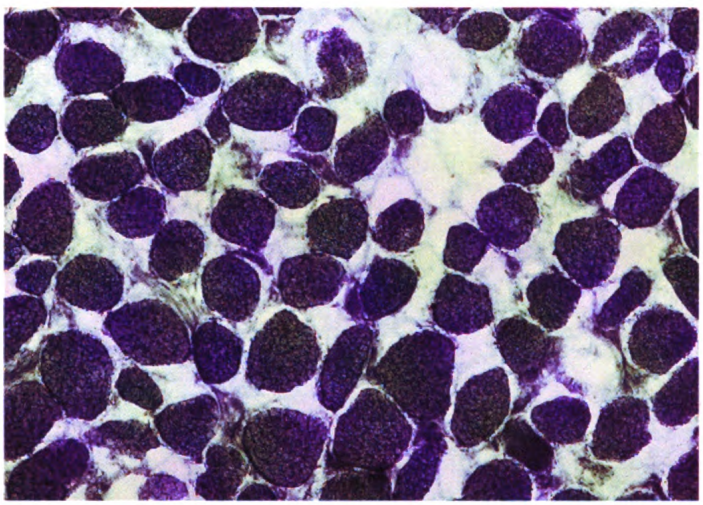


Fig. 6–2. Very slow-freeze artifact. External ice condenses muscle fibers; this happens when a sample is frozen in a freezer or cryostat. AMP-DA.

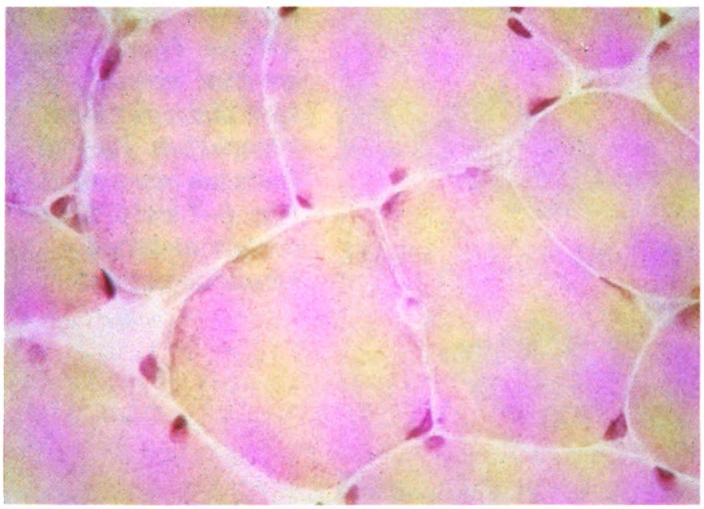


Fig. 6–3. Normal muscle, no freezing artifacts. H&E.

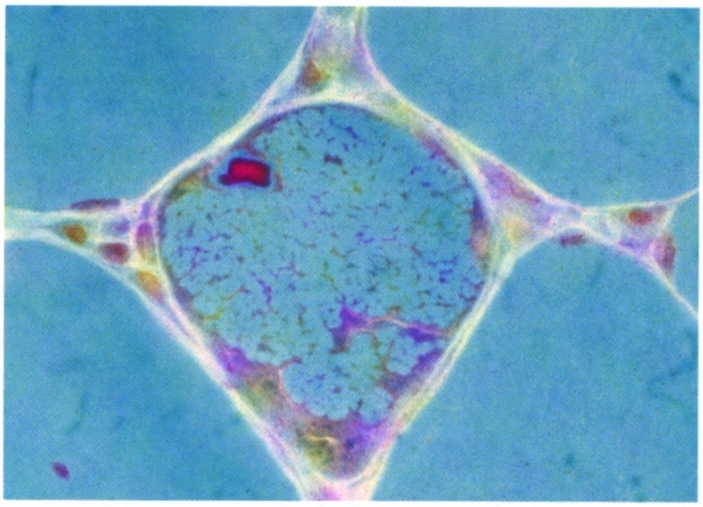


Fig. 6–4. Modified Gomori's trichrome. Large, central "ragged-red" fiber.

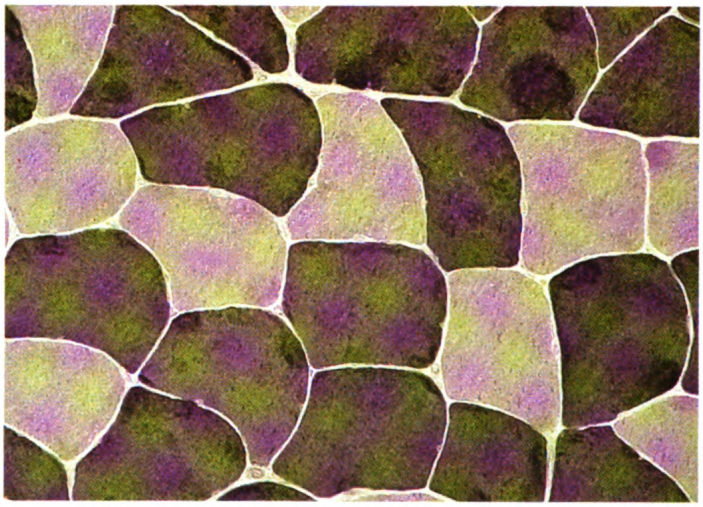


Fig. 6–5. Normal muscle. ATPase. Type 2 fibers are dark; type 1 fibers are light.

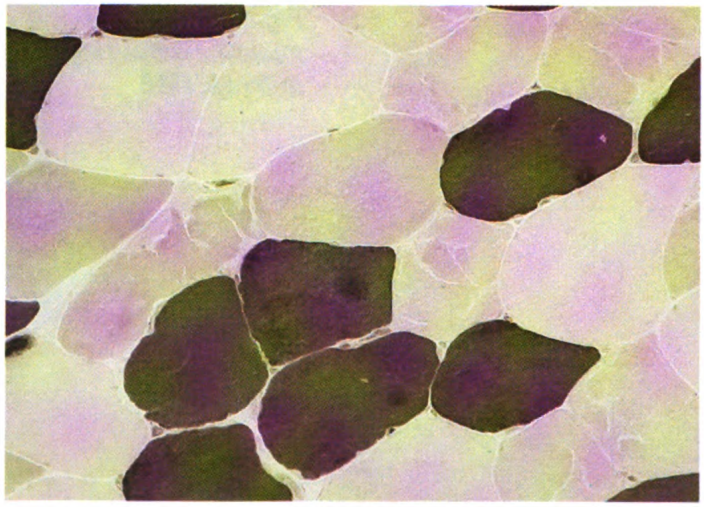


Fig. 6–6. Reverse ATPase. Type 1 fibers are dark; type 2A are light; and type 2B are intermediate. Type 2B fibers are abnormal (see Figs. 6–7 and 6–8) and contain unstained tubular aggregates.

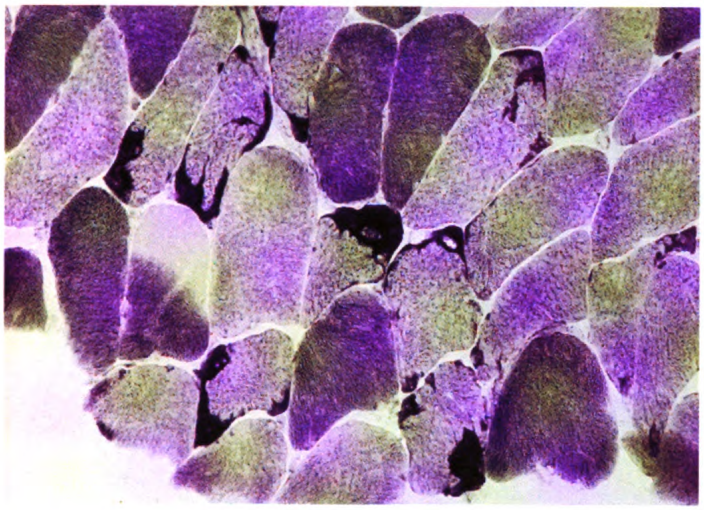


Fig. 6–7. NADH. Type 1 fibers are dark; type 2 are light. Same specimen as in Fig. 6–6. Tubular aggregates are very dark; parts of some type 1 fibers do not stain.

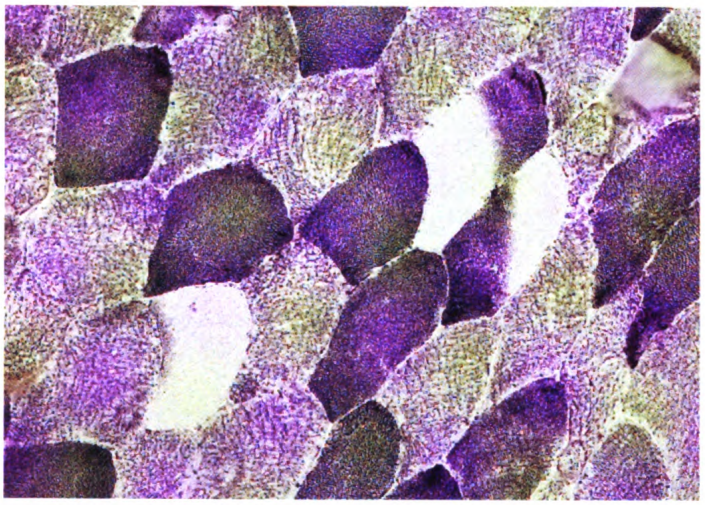


Fig. 6–8. SDH. Type 1 fibers are dark; type 2 are light. Same specimen as in Figs. 6–6 and 6–7. The mitochondrial enzyme SDH does not stain tubular aggregates, but parts of type 1 fibers are unstained (as in Fig. 6–7), a partial absence or failure to stain of mitochondria.

Fig. 6–9. Nonspecific esterase. One angular, atrophic, esterase-positive denervated fiber is near the center.

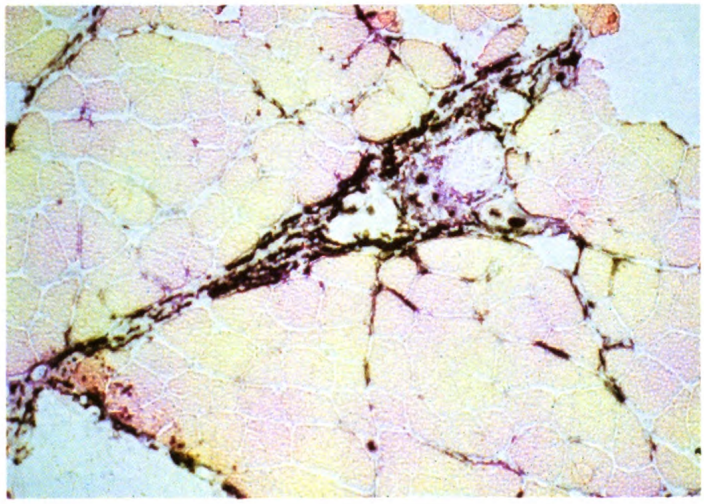


Fig. 6–10. Alkaline phosphatase. Positive reaction (black) around fiber bundles in polymyositis.

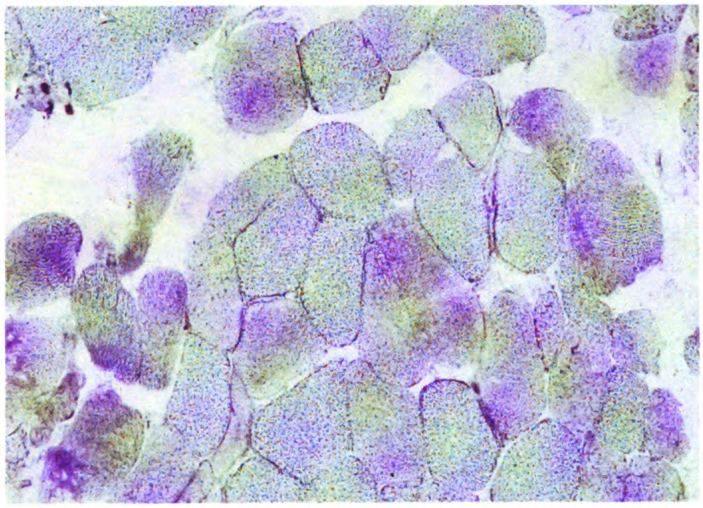


Fig. 6–11. Myoadenylate deaminase (AMP-DA). Positive reaction. Type 1 fibers are more blue; type 2 are more pink.

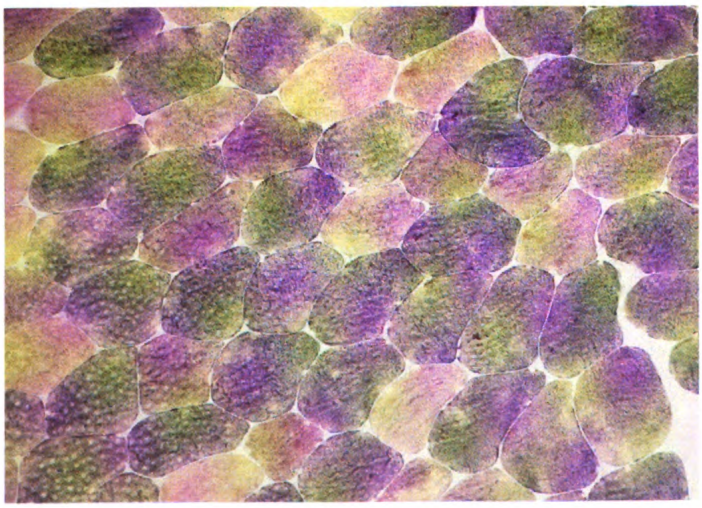


Fig. 6–12. Phosphorylase. Positive reaction, no deficiency.

7

Cytopathology Techniques

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INTRODUCTION

Over the last several decades, cytology has become a widely accepted and integral component of pathology. It is routinely practiced, even in small laboratories where the cytotechnologist and/or cytopathologist may have to rely on help from the histology laboratory. This chapter is intended as a guide of preparatory techniques in cytopathology for histotechnologists, and other technical personnel, who find themselves helping out in a cytology laboratory. Thus, this is an abbreviated version of methods in cytology and not intended for cytotechnologists.

Cytology deals with the structure, function, multiplication, life history, and pathology of cells. Cytopathology implies changes to cells in disease, and cytology samples are examined for their nuclear and cytoplasmic details. Changes in cell patterns help to separate normal and abnormal cells from each other. Cytology preparations can therefore be diagnostic, or helpful in the diagnosis, of disease.

Cell samples can be collected in several ways. The person who handles cytology specimens must be aware that the preparation of cytology samples varies depending upon the type of specimen and the collection method. Gynecologic material (Pap smears) and fine-needle aspirations are smeared and fixed prior to arrival in the cytology laboratory. They are fixed with alcohol-based spray fixative or 95% ethyl alcohol. Watery specimens, or sparsely cellular specimens, such as urines, cerebrospinal fluids (CSFs), and body cavity fluids must be placed on filters or concentrated for optimal cell recovery. Centrifugation concentrates cells into a pellet from which smears, or a cell block, can be made. Cytocentrifugation

concentrates cells in a small area on glass slides for cytospin preparations.

Rapid fixation and, therefore, optimal preservation of cells is paramount for diagnostic interpretation of material. The most common wet fixative for cytology specimens is 95% ethyl alcohol. Specimens fixed in 95% ethyl alcohol are stained with the modified Papanicolaou staining technique. Specimens may also be air-dried and stained with Diff-Quik or a Romanovsky stain. If fluid specimens are collected after operational hours of the laboratory, they should be placed in a refrigerator. To help preservation of these specimens, 50% ethyl alcohol may be added. To prevent clot formation, heparin is added to bloody specimens.

FIXATION OF SMEARS

The main purposes of a cytologic fixative are to penetrate cells rapidly, minimize cell shrinkage, and maintain morphologic integrity. Today, the most commonly used fixative is 95% ethanol, but it can be replaced by other alcohols that give equivalent results, such as 100% methanol, 95% denatured alcohol, 80% isopropanol, and various commercially available spray fixatives. Immediate fixation while the specimen is still wet is essential for optimal preservation, as even minimal air-drying of a sample will alter cellular features. The minimal fixation time is 15 minutes, but prolonged fixation of several days will not alter the appearance of the smear.

Spray fixatives usually consist of an alcohol base and a waxy substance that coats the slide. A small amount of wax, such as Carbowax, is added to the alcohol fixative to provide a protective coat and prevent air-drying artifact. This must be removed prior to staining. Two changes of 95% ethanol, 10 to 15 minutes each, are usually enough to remove this waxy substance. Carbowax is added to the Saccomanno fixative, therefore Saccomanno preparations must also sit in 95% ethanol from 10 to 20 minutes before processing through stains.

Unfixed specimens, to be prepared later, may have 50% alcohol added to the sample as a preservative, or the preservative can be used as a collection fluid. Specimens left for processing until the next day should be collected in 50% ethanol and stored in a refrigerator overnight. Concentrations greater than 50% of this "prefix" solution hardens the sample and makes it more difficult to smear. Heparin should be added to bloody samples to prevent clot formation. This is done at the time of specimen collection.

Fixed cytology samples are, as a rule, stained with the Papanicolaou staining technique. Cell blocks are fixed in formalin, and sections from those are stained with hematoxylin and eosin or other special stains that can be of help in diagnosis. Some cell preparations are by choice air-dried and not fixed. Those preparations are then stained with a Romanovsky stain that contains methanol. The stain itself will then act as a fixative.

SLIDE PREPARATIONS OF CYTOLOGY SAMPLES

Gynecologic samples (PAP smears) and preparations from fine-needle aspirations arrive in the laboratory already fixed and ready for staining. Other samples, some of which are collected in preservative fluid, usually 50% alcohol, are prepared in the laboratory. The preparation method depends on the type and the amount of sample. Sparsely cellular samples can be processed on filters or put on slides with a cytocentrifuge, which concentrates cells in a very small area (cytospins). The preferred cell preparation method, however, is direct smearing of the sample on glass slides. Scant samples can be concentrated through centrifugation and then smeared on glass slides. Leftover material can be prepared into cell blocks. Sections from cell blocks can be stained with special stains and, thus, are helpful in diagnosis.

NOTICE: It is important to be aware that fresh, unfixed specimens can be infectious. Always wear a gown and gloves and handle the samples with care, preferably in a laminar flow hood. Wipe the outside of specimen containers with 70% isopropanol or 10% Clorox solution. Decontaminate all equipment used in cell preparation, such as microscope, centrifuge, blender and vortex by wiping them with alcohol or Clorox solution.

MEMBRANE FILTERS

PRINCIPLE:

Filter preparations are used to prepare sparsely cellular specimens and are substitutes for cytospins. Cerebrospinal fluids, urines, bronchial washes, and transudates are specimens that lend themselves to this type of preparation. To determine the cellularity of a sample, a toluidine blue test is performed. For sparsely cellular or small-volume specimens, filters offer the potential of total cell recovery. The processing of filters is the same for all specimens, and the procedure must be closely adhered to or unsatisfactory preparations will result. Three types of filters (discussed below) may be used in cytologic preparations.

MATERIALS AND SOLUTIONS:

Filter
Filter support holder
Filter clips
Filter forceps
Disposable syringes - 10 ml
Glass slides
95% ethyl alcohol
Papanicolaou stain

PROCEDURE:

Figs. 7–1 and 7–2.

1. Remove filter from box using filter forceps and place in holder.
2. Expand filter by injecting holder with several drops of 95% alcohol.
3. Inject specimen into holder using a 10-ml syringe.
4. Add 1 ml of 95% alcohol using a 10-ml syringe.
5. Remove filter from holder using forceps and cut filter to fit slide. (If using a large filter, cut it in half and use 2 slides.)
6. Attach filter halves with clips, each to one slide, and drop in Coplin jar containing 95% alcohol.
7. Stain in Papanicolaou stain (see page 224).
8. Mount on glass slides, with resinous mounting medium, with cell side up, taking care not to allow air bubbles to get between slide, filter, and coverglass.

NOTES:

There are three types of filters that are currently used in filter preparations of cytologic specimens, namely Millipore, Gelman, and Nucleopore filters. The main differences between them are the type of material from which they are made and their pore size. All three require a modified Papanicolaou staining procedure.

Millipore filters are plastic filters that consist of cellulose-mixed esters with coarse pores. These filters should be expanded with 95% ethyl alcohol to prevent wrinkling when exposed to cytologic specimens. When staining them, sufficient times in the staining solutions, accompanied by alcohol rinses, allow the stains to be absorbed by the cells and not by the filter itself. Special precautions must be taken when mounting Millipore filters. They become transparent only when mounted in a mounting medium of similar refractive index. A common mounting medium is Permount (Fisher Scientific). An appropriate amount of medium must be used so that xylene is not trapped in the filter pores causing the filter to dry out within days after cover slipping; however, too much medium may make microscopic examination impossible under high dry magnification.

Gelman filters have physical and optical properties similar to the Millipore filters but are made from cellulose triacetate, a polymer that is more resistant to ethanol. In comparison to Millipore filters, Gelman filters expand less when exposed to alcohol. The stain uptake by this filter is markedly reduced, which allows the stained cells to stand out from the background. Gelman filters have a refractive index of 1.47 to 1.48 and for good microscopic clarity they also need a mounting medium with the same or similar refractive index. Permount (Fisher Scientific) may be used.

Nucleopore filters are made of a polycarbonate that is not affected by most solvents. For this reason, it is not necessary to preexpand the filter with 95% ethanol. They do not take up stain and thus have a clear background; however, they have two refractive indices and are birefringent, making it impossible to eliminate the image of the pores with a mounting medium. It should also be noted that if they are exposed to xylene for more than 15 minutes, they tend to curl. This makes coverslipping and microscopic evaluation difficult. Nucleopore filters, though, can be dissolved in chloroform or ethylene chloride, which eliminates the curling effect of the filter and the image of distracting pores. However, removing the filter chemically may produce several artifacts that can cause problems when evaluating the specimen.

TOLUIDINE BLUE TEST

PRINCIPLE:

Cellular, nongynecologic specimens (i.e., body cavity fluids) have a high potential for cross contamination and should be stained separately (CLIA-88 regulation). To determine the cellularity of a sample, a toluidine blue test can be performed. Toluidine blue-stained cell samples show clearly if the specimen contains large numbers of cells and cell clusters. Clusters of cells indicate that the fluid is abnormal and should be processed separately.

MATERIALS AND SOLUTIONS

Toluidine blue	0.5 g
95% ethyl alcohol	20 ml
Distilled water	80 ml

Dissolve toluidine blue in alcohol. Add distilled water to bring the final volume to 100 ml. Filter before each use.

PROCEDURE:

1. Place one drop of toluidine stain on a clean slide.
2. Place one drop of the fluid sample next to the stain, allowing contact to be made.
3. Place a small coverslip over the specimen.
4. Scan the slide under a microscope for cellularity.
5. Process samples with high cellularity and clusters of cells separately. Figure 7–3 illustrates a sample with clusters of cells.

DIRECT SMEARS

PRINCIPLE:

Direct smearing of cells is the preferred method of cell preparation. Cell-rich specimens are used directly, and less cellular specimens are concentrated through centrifugation. Sputa, prepared according to the Saccomanno method, are prefixed, mixed in a blender, and concentrated through centrifugation before being smeared on glass slides. Direct smears are prepared using the "two slide pull" method. A couple of drops of sample are put on a slide; a second slide is put on top; and the two slides are gently pulled apart, giving two slide preparations.

MATERIALS AND SOLUTIONS:

Centrifuge
Centrifuge tubes - 50 ml
Vortex mixer
Glass pipettes
95% alcohol
Poly-L-lysine (PLL)-coated glass slides (see Chapter 2, page 44)

PROCEDURE:

Figs. 7–4 to 7–6.

1. Concentrate specimen through centrifugation, if necessary.
2. Decant most of supernatant and vortex sediment for a few seconds.
3. Transfer a small amount of sediment to center of one PLL-coated slide with a glass pipette. Place another slide on top of the sediment and gently move the slides back and forth to disperse cells into a thin layer. Then, pull slides apart quickly.
4. Immerse slides in 95% alcohol for Papanicolaou staining or air-dry them for Diff-Quik staining.

NOTE:

Sputa, body cavity fluids, and fine-needle aspirates are the types of specimens from which direct smears are usually made.

SACCOMANNO METHOD FOR PREPARATION OF SPUTA

PRINCIPLE:

The Saccomanno technique is an alternate method of preparing a sputum specimen. This technique removes debris and mucoid material that may conceal diagnostic cells.

MATERIALS AND SOLUTIONS:

Small blender
Centrifuge
Centrifuge tubes - 50 ml
Vortex mixer
Pipettes
Poly-L-lysine (PLL)-coated glass slides (see Chapter 2, page 44)

Saccomanno fixative

95% ethyl alcohol ..... 1,900 ml
Distilled water ..... 2,100 ml
Carbowax (1540) (DuPont) ..... 92 g

Combine distilled water and alcohol in a one-gallon container. Melt Carbowax in a water bath at 37°C. Add melted Carbowax to solution in gallon container and agitate for 30 seconds.

PROCEDURE:

1. Add Saccomanno fixative to sputum specimen and pour into a 250-ml capacity semi-micro blender container. The total volume of fixative and specimen should be about 100 ml. (If specimen is scant, add enough fixative to equal 30 ml.)
2. Blend the mixture at low speed for 2 seconds and high speed for 5 to 30 seconds. Grossly, fine threads of material should not be visible. If fine threads are seen, blend the mixture for another 15 to 20 seconds or until they disappear.
3. Divide the suspension between two centrifuge tubes and balance the tubes.
4. Centrifuge the specimen for 5 minutes at 1,500 RPM.
5. Decant the supernatant until 2 to 3 ml overlie the cell concentrate.

6. Resuspend the cell concentrate by agitation on a vortex mixer.
7. Aspirate about one milliliter of suspension into a pipette. Add two drops to a clean PLL-coated glass slide. For sparsely populated specimens, 3 to 4 drops or more may be needed.
8. Perform the direct smear method.
9. Lay the slides face up on a level surface and allow the material to air-dry.
10. Immerse the slides after air-drying in 95% ethyl alcohol for 10 minutes to remove the precipitated Carbowax.

NOTES:

The direct sputum smear is used for fresh specimen, while the Saccomanno technique allows the specimen to be refrigerated for processing the next day. The Saccomanno fixative contains Carbowax, which must be removed before staining.

REFERENCE:

Gill G, Plowden K. *Laboratory Cytopathology: Techniques for Specimen Preparation.* Baltimore, Md: Johns Hopkins University Press; 1984.

CELL BLOCK PREPARATION

PRINCIPLE:

A cell block is an added diagnostic procedure for cytologic evaluation. It may be prepared from any fluid, washing, brushing, or needle aspiration. The advantage of a cell block is that it captures any tissue that may be present in the specimen and thus may show some of the tissue architecture. A cell block also allows additional sections to be cut for special stains that can be of help in the diagnosis of a case.

MATERIALS AND SOLUTIONS:

Centrifuge
Centrifuge tubes - 10 ml
2% gelatin in a glass tube melted by immersing tube in boiling water
Heated glass pipet to transfer hot gelatin
10% neutral buffered formalin
Ethyl alcohols
Xylene
Paraffin

PROCEDURE:

1. Centrifuge specimen into a pellet.
2. Decant supernatant.
3. Add about 0.25 ml of hot 2% gelatin carefully and with an applicator stick lift pellet so that gelatin can flow under the cells.
4. Put tube in ice to quickly solidify the gelatin.
5. Remove gelatin button with applicator stick and put in formalin.
6. Fix for at least one hour.
7. Process as tissue for embedding in paraffin.

NOTES:

Some cell specimens, e.g. body cavity fluids, contain blood and will clot in the absence of heparin. Diagnostic material is then trapped within a fibrin network and is not available for evaluation. However, these clots can be put into lens paper, fixed in formalin, and processed as tissue. In these cases, concentration of the sample through centrifugation and embedding in gelatin is not necessary.

REFERENCE:

Gill G, Plowden K. *Laboratory Cytopathology: Techniques for Specimen Preparation*. Baltimore, Md: Johns Hopkins University Press; 1984.

CYTOSPIN PREPARATION

PRINCIPLE:

For sparsely cellular or small-volume specimens, such as cerebrospinal fluids or transudates, centrifugation of samples in a cytocentrifuge onto glass slides (cytospins) captures a larger cell concentrate in a small area. The cytospin eliminates most proteinaceous debris, leaving a clean background. The manufacturers of this type of equipment supply information on materials and solutions needed and on operation of the instrument.

MATERIALS AND SOLUTIONS:

Hemacytometer
Cytospin centrifuge (Shandon Cytospin 2, Shandon Inc., Pittsburgh, PA)
Cytospin chambers
Cytospin filter pads
Poly-L-lysine (PLL)-coated glass slides (see Chapter 2, page 44), OR regular microscope slides if PLL slides are not available
Cytospin fixative or Saccomanno fixative
95% alcohol
Pipettes

PROCEDURE:

1. Count cells in the specimen under a microscope in a hemacytometer or approximate the number (see NOTES below).
2. Dilute cell-rich samples to 2 x 10^6 cells/ml and concentrate sparsely cellular samples through centrifugation to the same number, if possible.
3. Place a filter pad and a PLL-coated slide into each cytospin chamber with the thick side of the filter away from the slide. The hole in the filter pad should be at the bottom of the chamber.
4. Place the chambers inside the cytospin centrifuge.
5. Put a few drops of 95% alcohol in the chamber well to expand the filter.
6. Place 3 drops of sample (or more if sample is sparsely cellular) in the horizontal portion of the reservoir, followed by several drops of cytospin fixative.
7. Cap the reservoir so that no specimen is lost.
8. Place the lid on the cytospin and close the hood.
9. Set the speed (1,500 rpm) and time (2 minutes).

10. Centrifuge.
11. Let the machine stand a moment before opening.

NOTES:

This procedure is recommended for Shandon Cytospin 2, Shandon Inc. Pittsburgh, Pa., and may have to be modified for other cytocentrifuges.

The number of drops of cells to be used can also be determined by placing a drop of the cell suspension on a glass slide, covering it with a coverslip, and approximating the number of cells in a field under a 40x objective. The cell count is then divided into 60, and the answer equals the number of drops to use for each slide. For example, if the cell count is 20, 3 drops are used. When the 10X scan reveals almost no cells, use 20 drops. A few drops of saponin can be added before adding the cytospin fixative, if the specimen is bloody. The saponin lyses red blood cells, thus removing this artifact.

It should be mentioned that there are now devices available to help prepare thin monolayers of cells on glass slides. These devices vary from simple, inexpensive collection devices to expensive machines.

STAINING PROCEDURES

The most common stain for fixed cytology preparations is the Papanicolaou method. Modifications of this technique vary from laboratory to laboratory. It is advisable to standardize the method as much as possible to achieve reproducible results. Air-dried preparations are stained with a Romanovsky stain, usually Diff-Quick or Giemsa. The use of other stains in routine diagnostic cytology has been limited. Special stains, routinely performed in most histology laboratories, are sometimes applied. We refer to AFIP's *Laboratory Methods in Histotechnology* for those special stains.

PAPANICOLAOU STAIN

PRINCIPLE:

The Papanicolaou staining procedure was devised for optimal visualization of cancer cells exfoliated from epithelial surfaces of the body. It is a polychrome staining reaction, consisting of a water-based nuclear stain and two alcohol-based cytoplasmic counter stains, designed to display the many variations of cellular morphology and to show degrees of cellular maturity and metabolic activity. The stain is not specific for any substance found in cancer.

SOLUTIONS:

Hematoxylin

Distilled water	730 ml
Ethylene glycol	250 ml
Hematoxylin Gill II (Lerner, Pittsburgh, PA)	2 g
Sodium iodate	0.2 g
Aluminum sulfate	17.6 g
Glacial acetic acid	20 ml

Combine the ingredients in sequence by stirring them with a magnetic bar for one hour at room temperature. The stain may be used immediately, but filter before each use. The hematoxylin used in this recipe is anhydrous hematoxylin. If crystalline hematoxylin is used, it is necessary to use 2.36 g. One gram of citric acid may be substituted for 20 ml of glacial acetic acid.

Hydrochloric acid bath (used only for filters)

Distilled water ..... 1,000 ml
Conc. HCl ..... 2 ml

(Prepare fresh each day.)

Ammonium hydroxide (blueing bath)

Distilled water ..... 1,000 ml
Conc. NH_4OH ..... 1 ml

(Prepare fresh each day.)

Orange G stain

Stock solution: 10% Orange G
Orange G (Lerner) ..... 10 g
Distilled water ..... 100 ml

(When preparing stock solution, take dye content into consideration. Example: If purity of the dye is 80%, use 12.5 g.)

Working solution:
Stock solution ..... 20 ml
Phosphotungstic acid ..... 0.15 g
95% ethanol ..... 980 ml

Eosin-65 stain

Stock solutions:

20% eosin Y
Eosin Y, aqueous (Lerner) ..... 20 g
Distilled water, 70° to 80°C ..... 100 ml

3% Light-green SF
Light-green SF (Lerner) ..... 3 g
Distilled water, 70° to 80°C ..... 100 ml

(Solutions may be used immediately, but filter before each use. Take dye content into consideration when preparing stock solutions.)

Working solution: EA-65

Stock solution of eosin Y ..... 20 ml
Stock solution of light-green SF ..... 10 ml
Phosphotungstic acid ..... 2 g
95% ethyl alcohol ..... 700 ml
Absolute methyl alcohol ..... 250 ml
Glacial acetic acid ..... 20 ml

Ethanol: 95%, 100%

Xylene

Mounting medium

PROCEDURE:

1. 95% ethanol ..... 10 dips (see note below)
2. 95% ethanol ..... 10 dips
3. dH_2O ..... 10 dips (see note below)
4. dH_2O ..... 10 dips
5. Hematoxylin ..... 3 minutes
6. dH_2O ..... 20 dips
7. Blueing bath ..... 20 dips
8. dH_2O ..... 20 dips
9. 95% ethanol ..... 10 dips
10. 95% ethanol ..... 10 dips
11. Orange G, working solution ..... 1 minute
12. 95% ethanol ..... 10 dips
13. 95% ethanol ..... 10 dips
14. 95% ethanol ..... 10 dip
15. Eosin-65, working solution ..... 5 minutes
16. 95% ethanol ..... 10 dips
17. 95% ethanol ..... 10 dips
18. 95% ethanol ..... 10 dips
19. 100% ethanol ..... 10 dips
20. 100% ethanol ..... 10 dips
21. 100% ethanol ..... 10 dips
22. Xylene ..... 10 dips
23. Xylene ..... 10 dips
24. Mount in resinous medium

PAPANICOLAU STAIN FOR FILTERS

1. 95% ethanol ..... 10 dips
2. dH_2O ..... 10 dips
3. dH_2O ..... 10 dips
4. Hematoxylin ..... 2 minutes
5. Running tap water ..... 20 dips
6. Acid bath, 1 dip per second for ..... 1 minute
   (or until filters turn pale yellow)
7. dH_2O ..... 20 dips
8. Blueing bath ..... 2 minutes
9. 95% ethanol ..... 10 dips
10. 95% ethanol ..... 10 dips
11. Orange G, working solution ..... 10 seconds
    (do not dip filters after this point, let them sit in the solutions)
12. 95% ethanol ..... 1 minute
13. 95% ethanol ..... 1 minute
14. 95% ethanol ..... 1 minute
15. Eosin-65, working solution ..... 8 minutes
16. 95% ethanol ..... 4 minutes
17. 95% ethanol ..... 2 minutes
18. 95% ethanol ..... 1 minute
19. 100% ethanol ..... 1 minute
20. 100% ethanol ..... 1 minute
21. 100% ethanol ..... 1 minute
22. Xylene ..... 1 minute
23. Xylene ..... 1 minute
24. Mount on glass slides with resinous mounting medium

RESULTS:

See Figs. 7–7 to 7–10.

NOTES:

When following the Papanicolaou procedure, note that all smears that have been fixed by spray fixatives and all Saccomanno preparations must sit in 95% ethanol for at least 10 minutes before processing through the stains. "dH_2O" indicates reagent grade distilled or deionized water.

During staining, substantial numbers of cells are detached from their slides or filters. It is not uncommon for these "floaters" to become reattached to another specimen. To avoid the likelihood of this cross-contamination occurring, certain guidelines have been established. All solutions are filtered daily. Cases least likely to shed cells into the staining solutions are stained first; those most likely to shed are stained last. The staining order on the nongynecologic samples is as follows: (1) spinal fluids; (2) smears and cytospins; and (3) all filters. It must be noted, however, that the "floater" problem cannot be totally eliminated.

Events preceding the application of mounting medium to a slide influence the results. These events include the thickness of the cell spread and the presence of water in the absolute alcohol and xylene. For thick cell spreads, or for filters, excessive amounts of mounting medium should be used. Splotchy covering of the preparation by mounting medium produces air bubbles and thus air-drying in spots. This causes an artifact called "cornflaking" (Fig. 7–11). If this occurs, remove the coverslip by immersing the slide in xylene, then recoverslip. Another problem occurs if water gets into the absolute alcohols and is transferred into the xylenes, which then turn cloudy. The result is poorly dehydrated, nonclear images (Fig. 7–12). If water is noted in xylene, IMMEDIATELY change the xylene as well as the absolute alcohols.

Overall, a satisfactory cytology preparation is dependent not only on the staining but also on the collection of the sample, its preparation, and its fixation. A perfect stain may not improve scant cellular samples, poorly fixed degenerated cell samples, or samples where cells are obscured by "lakes" of polymorphonuclear leukocytes (PMNs). Such preparations may have to be called unsatisfactory.

REFERENCES:

Gill G, Plowden K. *Laboratory Cytopathology: Techniques for Specimen Preparation.* Baltimore, Md: Johns Hopkins University; 1984.

Prophet EB, Mills B, Arrington JB, Sobin LH, eds. *Laboratory Methods in Histotechnology.* Washington, DC: American Registry of Pathology; 1992.

QUALITY CONTROL OF THE PAPANICOLAOU STAINING TECHNIQUE

Technical assessment of stained slides is performed to determine optimal staining quality for cytologic evaluation. A daily record of the cellular staining quality must be maintained. This record should include the date and a statement reflecting acceptable or unacceptable staining quality. Corrective action must be documented when staining quality is unacceptable.

Optimal staining depends upon the quantity, quality, and freshness of the actual stains or dyes. Hematoxylin, the nuclear stain, should be reddish, blue, blue-black, or deep purple. A brown color signifies overoxidized hematoxylin and generally yields unsatisfactory staining results. The hematoxylin should not block the uptake or adulterate the colors of the subsequently applied counterstains. For nuclear staining quality, the nuclei of intermediate squamous cells are evaluated. Their chromatin should appear granular, crisp, distinct, and light purple in color. Intact polymorphonuclear leukocyte nuclei may also be evaluated for the assessment of nuclear staining quality. They should stain intensely dark blue to purple and should also give a sharp distinct image of the chromatin.

Counterstains Orange G and Eosin-65 should render vivid colors, cytoplasmic transparency, and differential counterstaining. In ideally preserved cells, several distinct cytoplasmic colors are identified. Eosin Y supplies the pink-red color in mature squamous cells and stains nucleoli red; light-green SF, the blue-green color of metabolically active cells; and Orange G, the orange or yellow of keratinized cytoplasm.

Thick tissue fragments or multileveled accumulations of cells sometimes result in improper dye penetration and colors that are not normally expected. If at all possible, thinner layers are desirable. Care must be taken to assess the morphology of the cells, rather than a cursory assessment of the coloration and perhaps a mistaken diagnostic evaluation.

DESTAINING OF SLIDES

PRINCIPLE:

Occasionally it may be necessary to destain a cytology slide, either to restain with the Papanicolaou stain or to request a special stain that may assist in the diagnosis.

MATERIALS AND SOLUTIONS:

Coplin jars
Xylene
100% EtOH
Acid alcohol (1% HCl in 70% EtOH)
Blueing bath
95% EtOH

PROCEDURE:

1. Place slide in Coplin jar containing xylene and allow coverslip to detach spontaneously; do not pull off. Slides that have been coverslipped for an extended period may take several days for the coverslip to detach.
2. Remove all traces of mounting medium by dipping slide in two changes of xylene for one minute each.
3. Place slide in two changes of 100% EtOH for one minute each.
4. Place slide in acid alcohol until decolorization is complete. The time will vary in this step.
5. Immerse slide in blueing bath for 3 or 4 minutes.
6. Rinse slide in two changes of tap water.
7. Place slide in Coplin jar containing 95% EtOH. Slide is now ready for restaining in the Papanicolaou stain or in a special stain.

DIFF-QUIK STAIN

PRINCIPLE:

This stain is bought commercially and is a modified Wright-Giemsa stain. It was originally intended as a quick blood smear stain, but it has also become a valuable stain for evaluation of air-dried cytologic material.

SOLUTIONS:

Diff-Quik fixative
Diff-Quik Solution I
Diff-Quik Solution II

PROCEDURE:

1. Diff-Quik fixative ..... 5 dips
2. Diff-Quik Solution I ..... 8-10 dips
3. Diff-Quik Solution II ..... 8-10 dips
4. Running tap water ..... 8-10 dips
5. Air-dry stained slide
6. Xylene ..... 5 dips
7. Mount in resinous mounting medium

Restaining of Diff-Quik stained slides can be done with good results, if necessary.

RESTAINING PROCEDURE:

1. Place slide in xylene and gently remove coverslip when it loosens.
2. Soak slide in fresh xylene to remove all mounting medium.
3. Completely air-dry slide.
4. Place slide in Diff-Quik fixative for 45 minutes.
5. Restain slide with solutions I and II, as above.

RESULTS:

See Fig. 7–13.

NOTE:

Filter solution II daily or before each use. Replenish or change stains frequently. Slides stained in Diff-Quik must NEVER be placed on a slide warmer to dry. Diff-Quik slides may be restained if the specimen is overstained or understained.

REFERENCE:

Gill G, Plowden K. *Laboratory Cytopathology: Techniques for Specimen Preparation.* Baltimore, Md: Johns Hopkins University; 1984.

GIEMSA STAIN

PRINCIPLE:

This stain is a one-step Romanovsky-type stain that requires methanol fixation of air-dried cytology smears.

SOLUTIONS:

1/15M phosphate buffer, pH 6.8

Solution A

$Na_2HPO_4.2H_2O$ ..... 5.93 g
Distilled water to a volume of ..... 500 ml

Solution B

KH_2PO_4 ..... 4.53 g
Distilled water to a volume of ..... 500 ml

Working buffer, pH 6.8

Solution A ..... 50 ml
Solution B ..... 50 ml
Distilled water ..... 400 ml
Set pH to 6.8 with either solution A or B.

Giemsa stain

Giemsa (Merck) ..... 10 ml
Working buffer ..... 90 ml

Methanol

Xylene

Mounting medium

PROCEDURE:

1. Fix slide in methanol for 15 minutes.
2. Rinse in working buffer, pH 6.8.
3. Stain in Giemsa stain for 20 minutes.
4. Rinse in running tap water until water runs clear.

5. Air-dry.
6. Clear in xylene for 5 minutes.
7. Mount in resinous mounting medium.

RESULTS:

See Fig. 7–14.

NOTES:

Depending upon the type of specimen, different staining times are used. Most cell types are stained for 20 minutes, but CSFs generally take only 5 minutes. Smears must be well dried in air before fixed in methanol, as methanol fixation of incompletely dried smears gives rise to blue artifacts on the nucleus that may mimic enlarged nucleoli.

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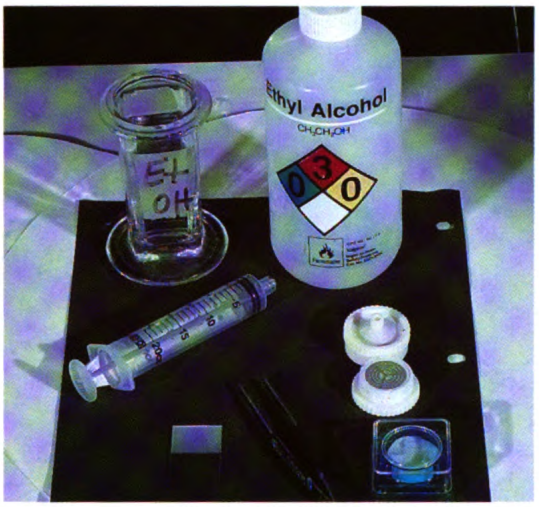


Fig. 7–1.
Materials used for filter preparations.

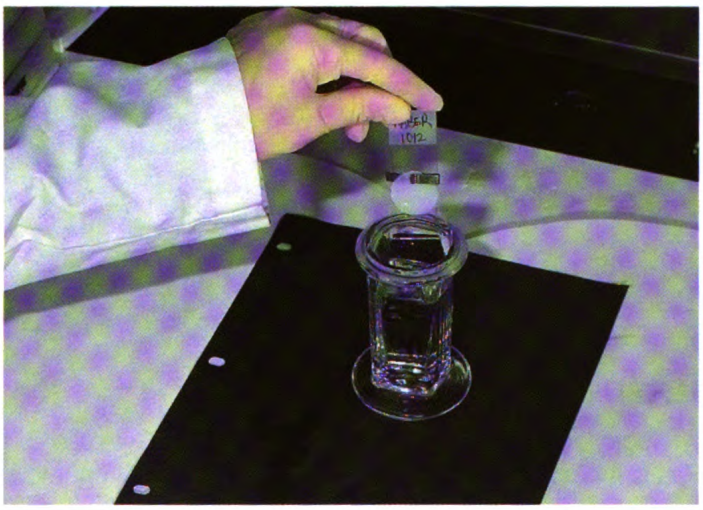


Fig. 7–2. Filter, with specimen attached to glass slide with a clip, and immersed in 95% ethanol for fixation of cells before staining.

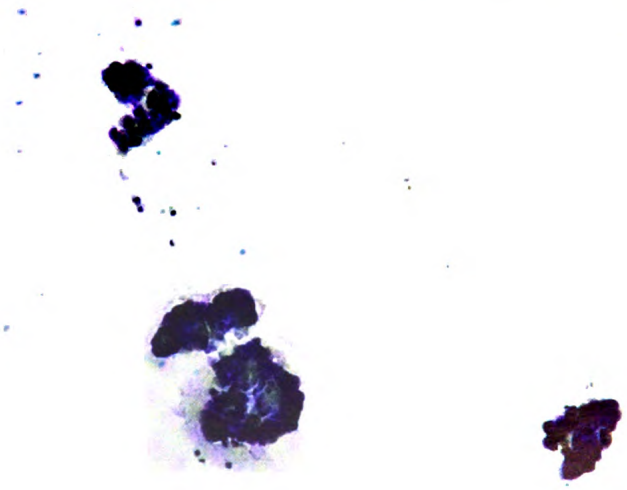


Fig. 7–3. Toluidine blue test showing cell clusters.

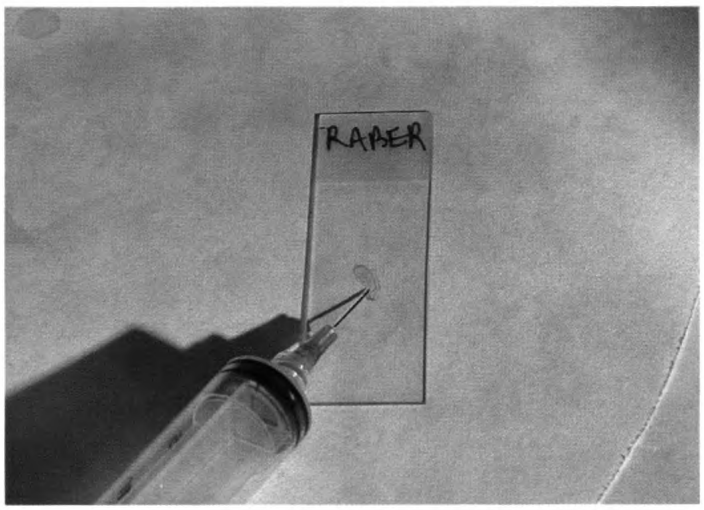


Fig. 7–4. Drop of cell sample placed on glass slide with a syringe. Can also be done with a pipet.

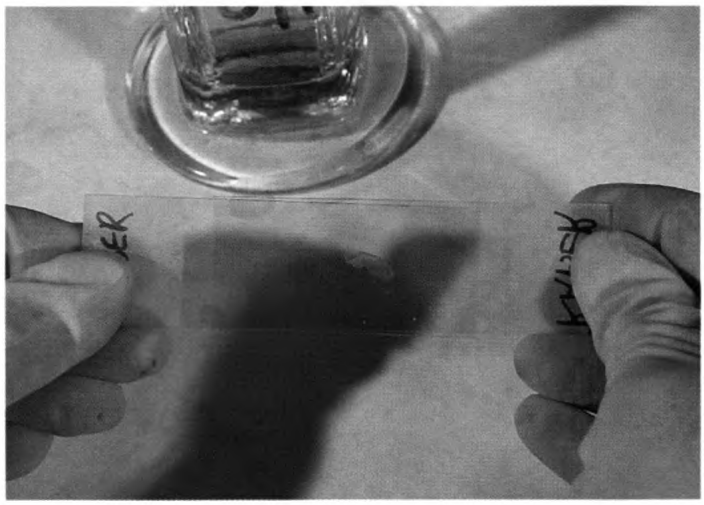


Fig. 7–5. Second slide placed on top and gently moved back and forth to disperse cells into a thin layer.

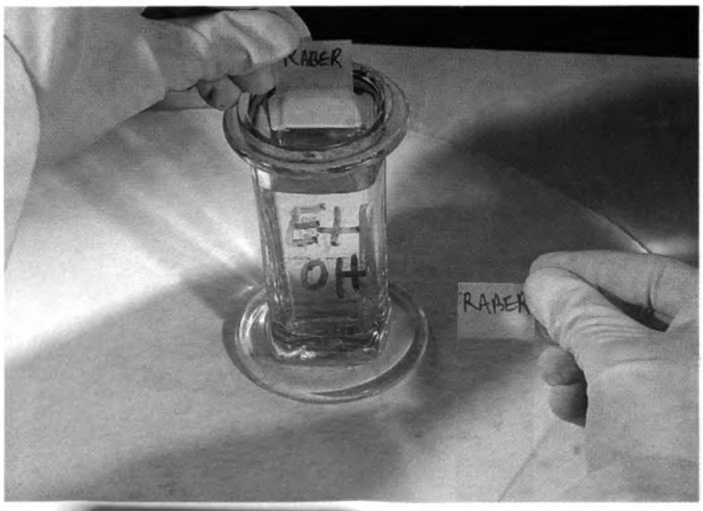
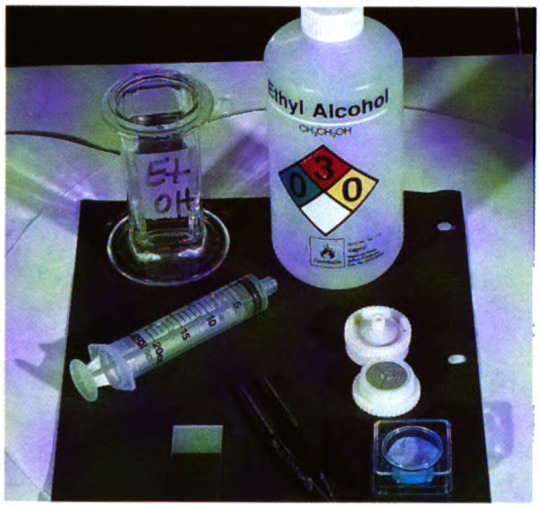


Fig. 7–6. The slides from Fig. 7–5 have been pulled apart and one is immersed in 95% ethanol, one is air-dried.



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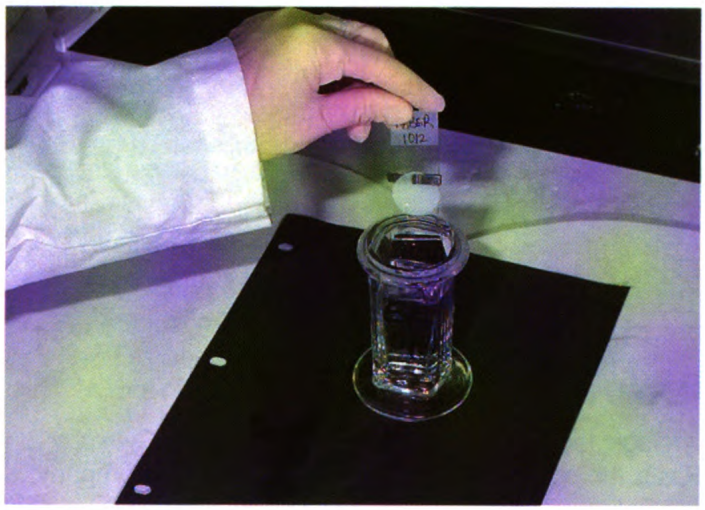

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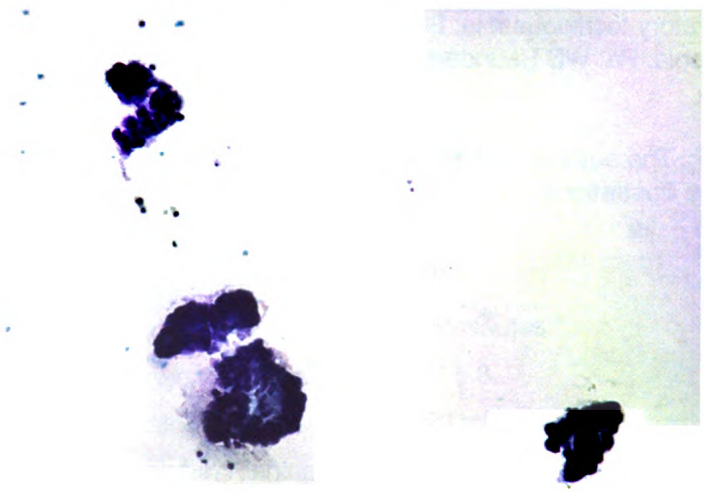

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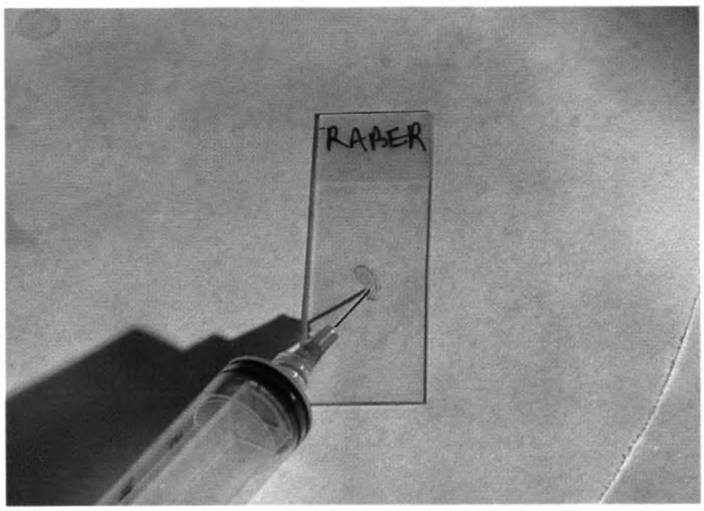

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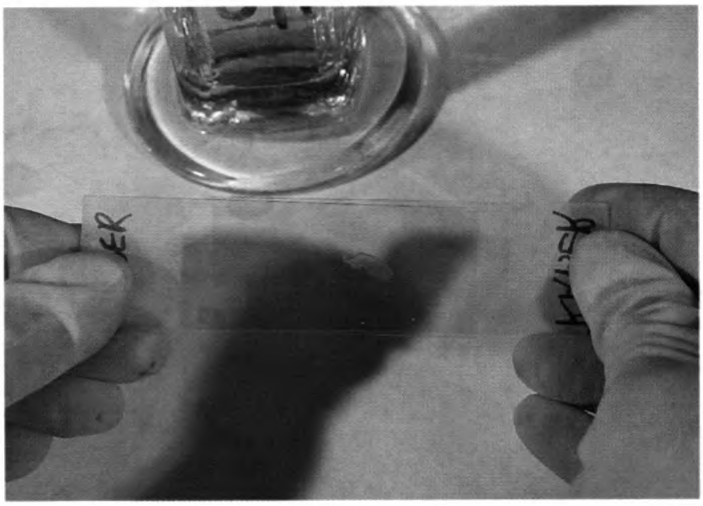

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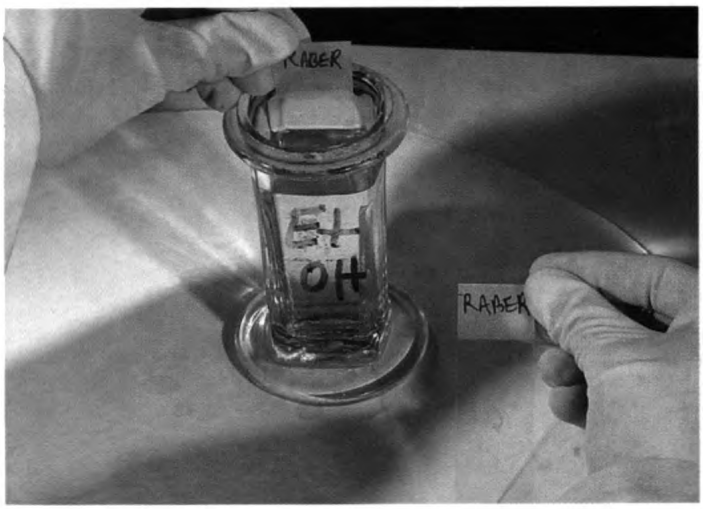

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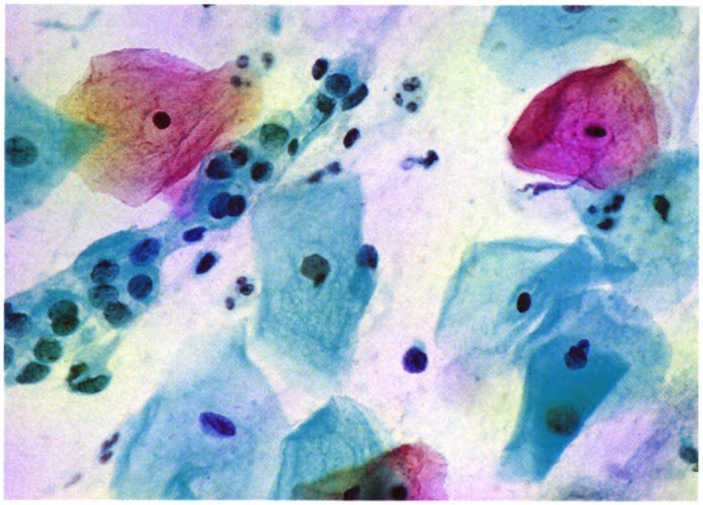


Fig. 7–7. Papanicolaou-stained cervical smear. Squamous, intermediate and glandular cells.

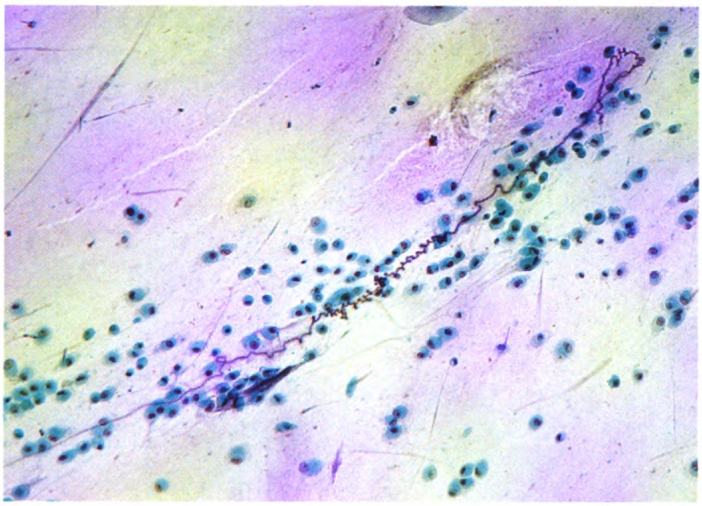


Fig. 7–8. Papanicolaou-stained sputum. Macrophages and Curschmann spiral (coiled dense core of inspissated mucus).

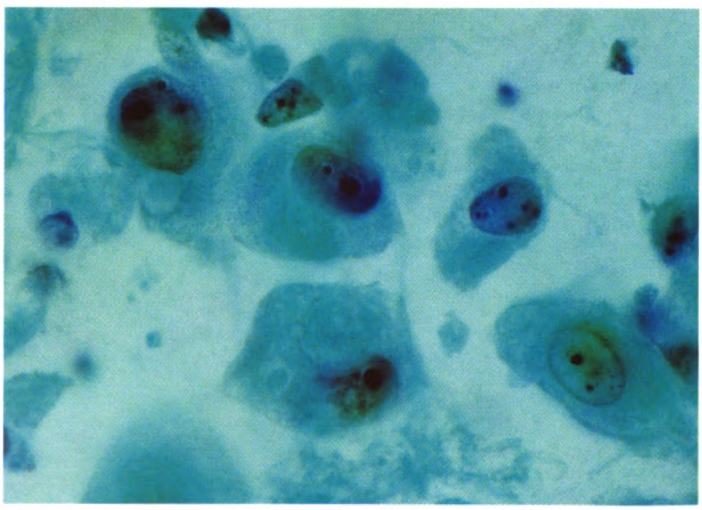


Fig. 7–9. Papanicolaou-stained filter preparation of bladder wash. Transitional cells.

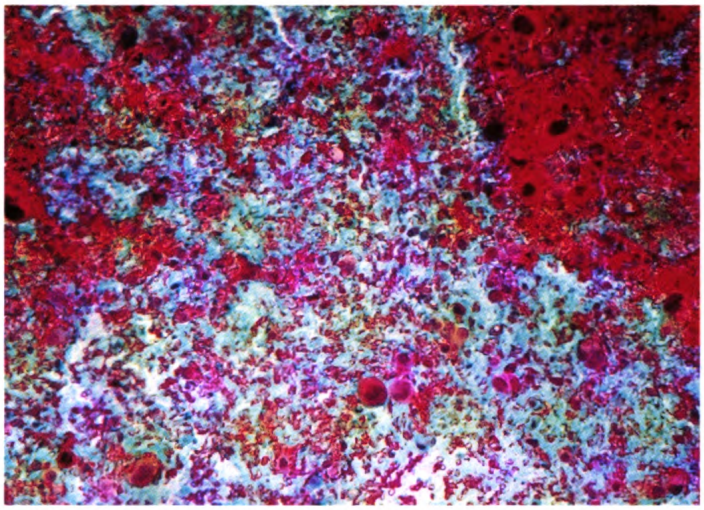


Fig. 7–10. Papanicolaou-stained fine needle aspiration of neck mass.

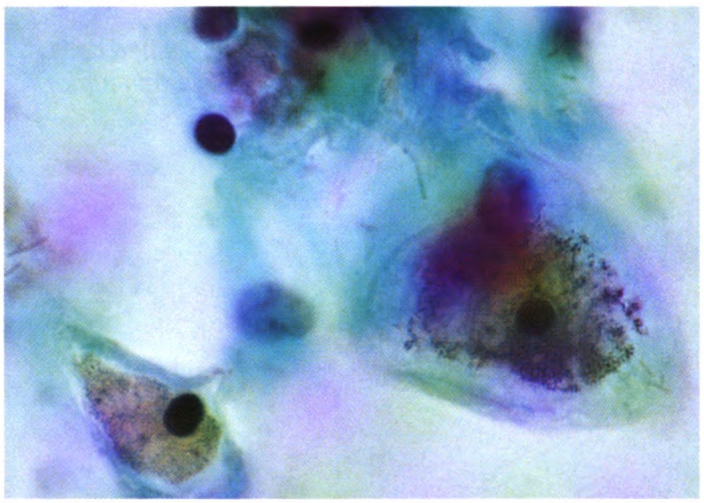


Fig. 7–11. Pap smear exhibiting "cornflaking," an air-drying artifact.

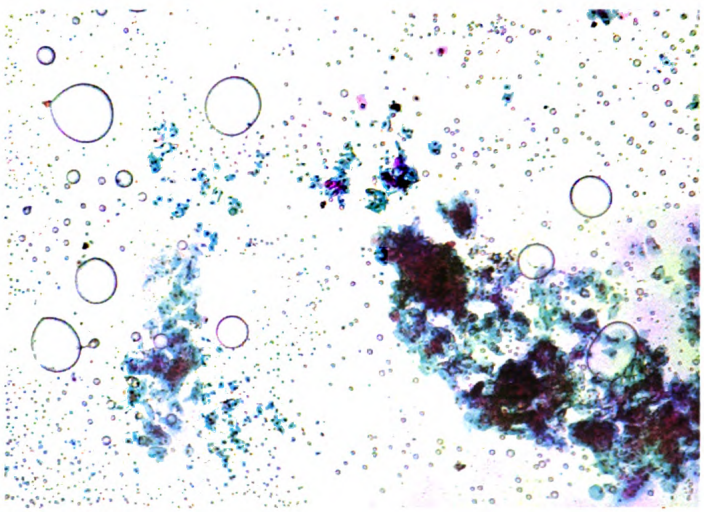


Fig. 7–12. Pap smear exhibiting water in the xylene.

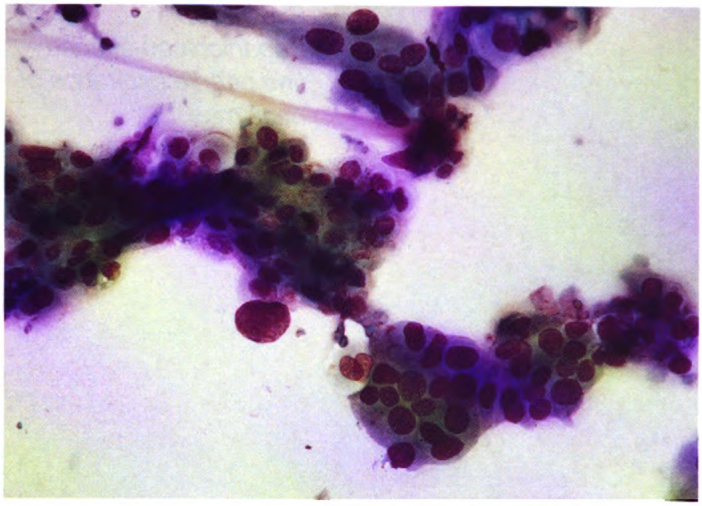


Fig. 7–13.
Diff-Quick-stained fine needle aspiration cell sample from a papillary carcinoma of the thyroid.

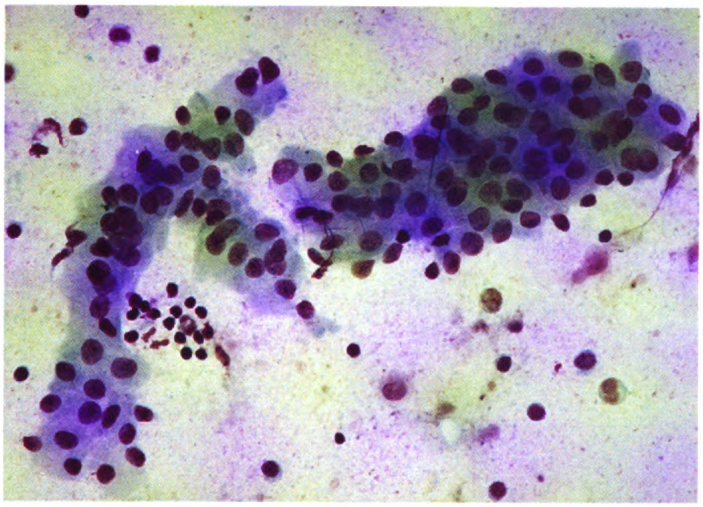
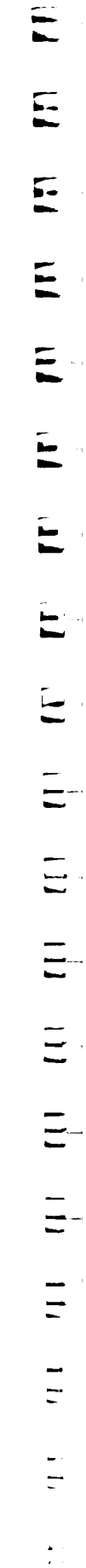


Fig. 7–14.
Giemsa-stained cells from a Warthin tumor.



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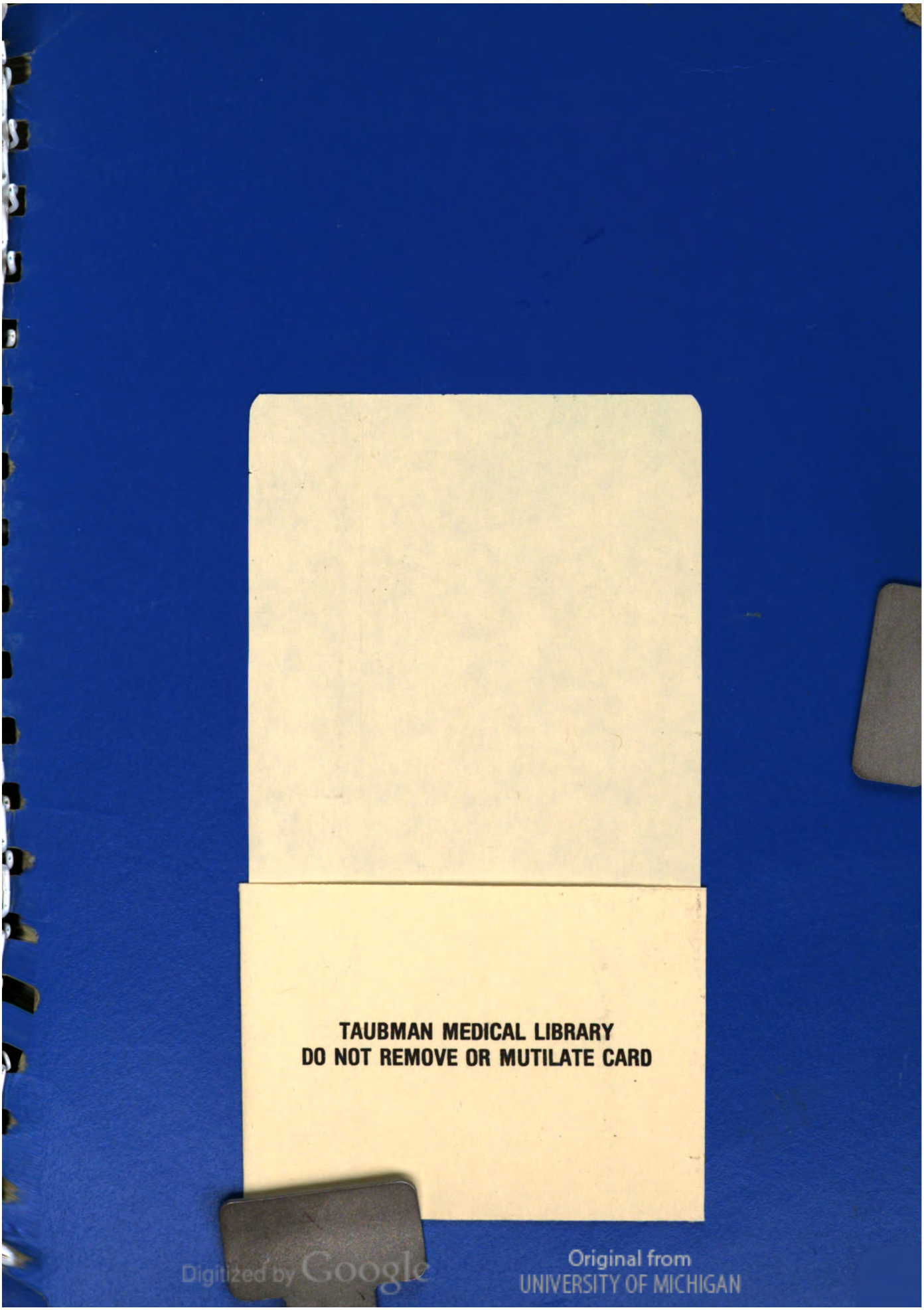

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