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Jan R. Mead
Michael J. Arrowood *Editors*

# Crypto-sporidium

## Methods and Protocols

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# Cryptosporidium

## Methods and Protocols

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Cover illustration: *Cryptosporidium parvum* life cycle stages including scanning electron micrographs of sporozoites, Type I and Type II meronts, and differential interference contrast view of purified oocysts.

# Preface

*Cryptosporidium* is an opportunistic protozoan that infects the intestinal tract. It can cause a diarrhea illness that may be life-threatening in immunocompromised and immunodeficient individuals. More recently it has been recognized as an important disease in young children in developing countries where it may be responsible for growth deficits and cognitive problems. Methods in the early years of cryptosporidiosis research focused on clinical and environmental detection of the parasite, epidemiology, source tracking, and improvements in techniques that would advance understanding of the basic biology of the organism. Recent advancements in cell culture and molecular biology have created opportunities to move cryptosporidial research forward at a faster pace.

The aim of this book is to encompass broad aspects of *Cryptosporidium* research. It includes established methods improved over many years, but perhaps not published in as much detail as previously reported, along with helpful notes not typically included in a standard scientific paper. Among these chapters are methods describing high-throughput cell-based in vitro assays used to evaluate potential anti-cryptosporidial drugs, the use of acute and chronic mouse models of cryptosporidiosis, and a newborn calf model to evaluate clinical and parasitological parameter outcomes. Molecular methods describing the production of recombinant, functional *Cryptosporidium* glycoprotein antigens in *Toxoplasma gondii* are included as well as an updated and detailed guide to using the CryptoDB database.

Additionally, there are standard methods describing genotyping, used primarily for epidemiology studies, and chapters on diagnostic methods. These diagnostic methods include chapters on microscopy, enzyme immunosorbent assays, a nested PCR for the detection of all *Cryptosporidium* spp., environmental detection, and a method for preparing high-quality oocyst DNA from stool samples for subsequent whole genome sequencing (WGS). Evaluation of serological responses using a multiplex bead assay is included in the book. The book also contains methods for the purification of sporozoites and oocysts and an in vitro protocol to examine oocyst inactivation by disinfectants.

*Cryptosporidium* research has been limited by the inability to grow the organism continuously in vitro. *Cryptosporidium* research has made several important advances over the last couple of years with cutting-edge techniques. There are three chapters that describe improved in vitro cell culture techniques. These include the use of stem cells in generating an epithelial cell line, 3D organoid cultures and the use of a hollow fiber bioreactor for parasite production, and evaluation of potential anti-cryptosporidial agents. Additionally, chapters describe the use of CRISPR/Cas9 to generate knockouts and transgenic parasites, silencing of genes in *Cryptosporidium* by transfecting parasites with hybrid complexes, and a method using miRNA to silence genes supporting the study of host-parasite interactions.

We acknowledge and thank the authors that shared the detailed protocols included in this volume. Given the inherent challenges of working with cryptosporidial parasites, these protocols provide a foundation for new investigators to build upon.

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# Chapter 1

# *Cryptosporidium* Diagnostic Assays: Microscopy

## Guy Robinson and Rachel M. Chalmers

### Abstract

Stained microscopy of fecal smears was the cornerstone of *Cryptosporidium* diagnosis for many years, and still provides a low-cost method for detecting oocysts. The development and commercialization of improved enzyme immunosorbent assays (EIA) for coproantigen detection provided an automatable method for mass testing, and rapid diagnostics when incorporated onto a cartridge format. Similarly, immunochromatographic lateral flow assays (ICLF) enable rapid diagnostics. Nevertheless, it is important that positive reactions by EIA or ICLF are confirmed. Here we describe microscopical methods using tinctorial stains for the diagnosis of acute cryptosporidiosis, and using immunofluorescent reagents for diagnosis or for confirmation of EIA or ICLF positive reactions.

**Key words** *Cryptosporidium*, Oocyst detection, Microscopy, Ziehl–Neelsen, Auramine phenol, Immunofluorescent

## 1 Introduction

There are no international Standard Methods for the diagnosis of cryptosporidiosis, but, depending on the setting, both low- and high-tech options are available and those based on stained microscopy of stools are described here. Historically, histological staining and microscopy of thin sections of gut biopsy material was used to diagnose *Cryptosporidium* infection but nowadays the oocyst stage of the life cycle, shed in feces, is the usual diagnostic target. Stained microscopy forms the basis of low-tech methods, as oocysts are hard to identify without staining. It may be necessary to examine stool samples taken over several days until oocysts are seen, or an alternative diagnosis is made, if patients continue to have symptoms. Here we describe the preparation of fecal smears and staining by acid-fast modified Ziehl–Neelsen (Kinyoun) and auramine phenol stains and their subsequent microscopic examination [1–3]. Prior concentration, preferably by formalin-ethyl acetate sedimentation, may be used but is not usually necessary for diagnosis of acute infection unless the samples are submitted in preservative.

While the reagents are cheap, good microscopic examination is labor-intensive. The use of more expensive immunofluorescent stains improves both sensitivity and specificity as well as reduces examination time, and is also described here.

The use of antibodies raised against oocyst antigens has also been incorporated into commercially available enzyme immuno-sorbent assays (EIA) (either in 96-well plate or cartridge format) and immunochromatographic lateral flow (ICLF) assays (in either cartridge or dip stick assays). These do not rely on microscopy skills, vary in their sensitivity and specificity, and should be assessed for suitability for the population being tested. Generally, EIA kits have greater sensitivity and specificity for *Cryptosporidium* than ICLF and modified Ziehl–Neelsen staining (*see* refs. 4 **and** 5 for comparative assessment of methods). They are not described here, as the manufacturer's instructions should be followed, including whether preservatives are compatible. Generally, stools should not be concentrated prior to use of EIA or ICLF as antigens may be lost.

## 2 Materials

*2.1 Preparation of Slides for Stained Microscopy*

1. Glass microscope slides (modified Ziehl–Neelsen and auramine phenol staining) or single- or multiple-well glass microscopy slides (immunofluorescence staining).

2. Sample applicator sticks.

3. Distilled water.

4. Methanol.

5. Diamond marker or pencil.

*2.2 Formalin-Ethyl Acetate Sedimentation for Concentration of Samples Submitted in Preservative*

Commercial devices and reagents are available for the formalin-ethyl acetate concentration of parasites [6, 7], or the following reagents and protocol may be used.

1. 15 mL conical centrifuge tube.

2. Sample applicator sticks.

3. 10% formalin.

4. Sieve with 425 μm aperture, equivalent to 36 mesh British Standard (BS 410-86) or 40 mesh American Standard (ASTM E11-81).

5. Beaker.

6. Ethyl acetate (*see* **Note 1**).

*2.3 Modified Ziehl–Neelsen Reagents*

Commercial supplies are available for all reagents.

1. Strong carbol fuchsin (1–3% basic fuchsin): fully dissolve 20 g basic fuchsin in 200 mL absolute methanol on a magnetic

stirrer. Add 125 mL liquid phenol (general purpose reagent [GPR; 80% w/w in distilled water]) carefully until well mixed, and make up to the final volume with 1675 mL deionized water. Mix thoroughly and filter before use through Whatman No. 1 filter paper to remove debris. Store the stock reagent in the dark in the temperature range 20 ± 5 °C.

2. 1% acid methanol: carefully add 20 mL of concentrated hydrochloric acid to 1980 mL of absolute methanol and mix. Store at ambient temperature.

3. 0.4% malachite green: add 2 g malachite green to 480 mL deionized water and mix on a magnetic stirrer before filtering through Whatman No. 1 filter paper. Store at ambient temperature.

***2.4 Auramine Phenol Stain Reagents***

Commercial supplies are available for all reagents.

1. Auramine phenol: dissolve 3 g of phenol in 100 mL deionized water and slowly add 0.3 g Auramine O before filtering through Whatman No. 1 filter paper. Store in the temperature range 20 ± 5 °C in a light-proof glass bottle with an airtight stopper.

2. 3% acid methanol: carefully add 60 mL of concentrated hydrochloric acid to 1940 mL absolute methanol and mix.

3. 0.1% potassium permanganate: add 0.5 g of potassium permanganate to 499.5 mL deionized water and mix using a magnetic stirrer before filtering through Whatman No. 1 filter paper.

***2.5 Immun-fluorescent Microscopy Reagents***

1. Commercially available kits may include: *Cryptosporidium* oocyst positive and negative controls, fluorescently labeled (e.g., with fluorescein isothiocyanate [FITC]) anti-*Cryptosporidium* monoclonal antibodies, and glycerol-based mounting medium containing a photobleaching (quenching) inhibitor.

***2.6 Microscopy***

1. Microscope with bright field and epifluorescence capability.

2. Fluorescence filter blocks: FITC (excitation filter 490 nm; emission filter 510 nm) or UV (excitation filter 355 nm; emission filter 450 nm) filters for phenol auramine. For immunofluorescent staining, the required filter block is dependent on the fluorophore used (the FITC filter noted above is most common).

3. Objective lenses with 10× eyepiece lenses: 40×–50× immersion objectives for screening modified Ziehl–Neelsen and phenol auramine stained samples with 100× immersion objective for examining finer detail; 20× for screening immunofluorescent stained samples, with 40× and 100× immersion for confirmatory examination.

## 3    Methods

Wear appropriate Personal Protective Equipment (PPE) including protective clothing, disposable gloves, and eye protection during all sample-processing steps. Each of these methods is appropriate for examining both human and animal fecal samples.

### 3.1    Preparation of Slides for Stained Microscopy

1. Include a verified positive control on a separate slide each time this procedure is performed.

2. Mark the reference number of the specimen on a clean, dry, glass microscope slide with a diamond marker or pencil (or equivalent), and use a separate microscope slide for each specimen.

3. Fecal samples must be well mixed with the wooden end of a clean applicator stick. Non-diarrheic, very solid or dry feces should be emulsified in oocyst-free, distilled water. Mucoid samples can be digested prior to the preparation of a smear using mucus liquefying agent (e.g., 1:1 (v/v) dithiothreitol (1% (w/v) in water; commercial preparations available) or 0.1% potassium hydroxide) and incubating for 15 min at ambient temperature.

4. *For modified Ziehl–Neelsen and auramine phenol staining*: use a clean applicator stick to prepare a medium-to-thick smear of the well-mixed stool sample, of approximately 4 × 1.5 cm, with areas of varying thickness, on a labeled, clean, dry, glass microscope slide. Allow to air dry. Place slides on a staining rack over the sink and fix by flooding with methanol for 3 min. Drain off and allow to air dry.

5. *For immunofluorescent staining*: smear the homogenized sample, not too thickly (*see* **Note 2**), within the well boundary of a single-well microscope slide, leaving a gap of approximately 1 mm between the edge of the sample and the well. If the sample is fluid, add 5 μL of sample to the well. Allow to air dry. Fix by carefully applying sufficient methanol (approximately 20 μL) to cover each sample without spilling outside of the well. Allow to air dry.

### 3.2    Formalin-Ethyl Acetate Sedimentation for Concentration of Stools

1. Using a sample applicator stick, transfer approximately 0.5–1 g of a well-mixed fecal sample (750 μL if liquid) to a clean 15 mL tube containing 7 mL of 10% formalin.

2. Homogenize the sample with the applicator stick, breaking up any lumps as necessary.

3. Filter the suspension through the sieve into a beaker (*see* **Note 3**) and pour back into the 15 mL tube.

4. Add 3 mL ethyl acetate and cap the tube before vigorously shaking for 30 s.

5. Release the built-up pressure by gently unscrewing before resealing and centrifuging the tube at $1100 \times g$ for 2 min.

6. Using a sample applicator stick, loosen the fatty plug at the top of the sample.

7. Gently pour off the fatty plug and fluid column that is present above the required sample pellet, into a resealable liquid waste container. Allow one or two drops to run back into the tube over the sample pellet.

8. Resuspend the pellet by gentle agitation (*see* **Note 4**) and transfer a subsample onto a microscope slide for drying and staining.

*3.3 Modified Ziehl–Neelsen Staining*

1. Immerse or flood slides on a staining rack over the sink, with cold strong carbol fuchsin and allow to stain for 15 min.

2. Rinse slides thoroughly with tap water (*see* **Note 5**).

3. Immerse or flood slides with 1% acid methanol for 10–15 s to decolorize (*see* **Note 6**).

4. Rinse slides thoroughly with tap water (*see* **Note 5**).

5. Immerse or flood slides with 0.4% malachite green for 30 s to counterstain.

6. Rinse slides thoroughly in tap water (*see* **Note 5**).

7. Allow the slides to air dry.

8. Examine for the presence of oocysts by scanning slides systematically using at least the 40× objective lens of a brightfield microscope. Confirm the presence of oocysts using a high-power (e.g., 100×) objective lens (*see* **Note 7**).

9. Measure the size and shape of the red-stained bodies using a calibrated eyepiece graticule or reticle (*see* **Notes 8** and **9**).

*3.4 Auramine Phenol Staining*

1. Immerse, or flood slides on a staining rack over the sink, with auramine phenol stain for 10 min.

2. Rinse slides thoroughly with tap water (*see* **Note 5**).

3. Immerse or flood slides with 3% acid alcohol for 5 min to decolorize.

4. Rinse slides thoroughly in tap water (*see* **Note 5**).

5. Counterstain by immersing or flooding in 0.1% potassium permanganate for 30 s.

6. Rinse slides thoroughly with tap water to remove excess stain (*see* **Note 5**).

7. Allow the slides to air-dry at room temperature (*see* **Note 10**).

8. Examine for the presence of oocysts, using an epifluorescence microscope equipped with FITC (excitation filter 490 nm; emission filter 510 nm) or UV (excitation filter 355 nm; emission filter 450 nm) filters (*see* **Note 11**), by scanning the slide systematically using at least the 40× objective lens. Confirm the presence of oocysts using a high-power (e.g., 100×) objective lens (*see* **Note 12**).

9. Measure the size and shape of the fluorescent bodies by slowly increasing the voltage (light intensity) of the brightfield light source, so that both fluorescent and brightfield images can be seen concurrently, and using a calibrated eyepiece graticule or reticle (*see* **Note 8**).

*3.5 Immuno-fluorescent Staining*

1. Stain and mount slides according to manufacturer's instructions.

2. Scan the slide for oocysts using a 20× objective lens and confirm using a 40× or greater objective lens of an epifluorescence microscope equipped with an appropriate filter set (e.g., for FITC-labeled stain, excitation wavelength 490 nm and emission wavelength 510 nm) (*see* **Note 13**).

3. Measure the size and shape of the fluorescent bodies using the 100× by slowly increasing the voltage (light intensity) of the brightfield light source, so that both fluorescent and brightfield images can be seen concurrently, and using a calibrated eyepiece graticule or reticle (*see* **Note 8**). If necessary, slides can be stored at 2–8 °C for up to 7 days without loss of fluorescence.

# 4  Notes

1. Diethyl ether was historically used, but now replaced by ethyl acetate which, although safer, is still highly flammable. Caution must be taken to ensure no proximity to naked flames or sparks during use and to avoid prolonged inhalation or skin contact. All precautions outlined in the material safety data safety sheet (MSDS) or safety data sheet (SDS) must be observed.

2. The transparency should be sufficient to read the face of a watch or text of a newspaper through the smear.

3. The sieve should fit comfortably within the rim of the beaker. Both the sieve and beaker must be washed thoroughly in running tap water between each sample. Observe local waste disposal requirements (both chemical and biological). Disposable sieves reduce the risk of cross-contamination between samples.

4. A large pellet indicates that too much sample was processed, insufficient shaking with the ethyl acetate, or a too fast or long centrifuge step was used.

5. Depending on the nature of the feces, some of the sample could wash off the slide at this point; care must be taken to avoid this occurring by adjusting the flow of water to an appropriate level or gently moving the slide while submerged in a water container.

6. Over-decolorizing must be avoided by adhering to the specified timings.

7. The smear can be examined with or without a coverslip. *Cryptosporidium* spp. oocysts stain red on a pale green background (Fig. 1). The degree and proportion of staining varies with individual oocysts. Internal structures take up the stain to varying degrees; some may appear amorphous, while others may contain the characteristic crescent shapes of up to four sporozoites. The presence of "ghost" (unstained) oocysts may be apparent as holes or gaps in the counterstain.

8. Most *Cryptosporidium* spp. oocysts in human samples (e.g., *Cryptosporidium parvum* and *Cryptosporidium hominis*) appear as round or slightly ovoid, 4–6 μm in diameter. In animal samples, larger *Cryptosporidium* oocysts (e.g., *Cryptosporidium andersoni*) may also be seen and appear more ovoid, ~7.5 μm in length by ~5.5 μm in width. Always refer to the positive control to ensure that the size, shape, and color of the putative



**Fig. 1** *Cryptosporidium* oocysts (~5 μm diameter) demonstrating typical features with modified Ziehl–Neelsen staining as seen using (a) 50× objective lens for screening, (b) 100× objective lens for confirmation. Often the sporozoites lay toward the edge of oocysts which gives them the so-called "doughnut" or "erythrocytic" staining (denser around the outside and fainter or clear in the middle)

oocyst are consistent with those of the positive control. However, *Cryptosporidium* spp. cannot be differentiated by microscopy so report as "*Cryptosporidium* oocysts seen."

9. Yeasts and fecal debris can also stain red and correspond to the correct size range. However, the staining is usually more uniform across the body with often more variation in the size and shape of the stained bodies compared with *Cryptosporidium* (Fig. 2). Some bacterial spores may also stain red, but these are too small to cause confusion.

10. Do not blot slides dry, as some blotting papers contain fluorescent fibers.

11. Do not look at the fluorescent light (particularly with the UV filters) around the stage area of the microscope, as this can be harmful to your eyes.

12. The smear can be examined with or without a coverslip and *Cryptosporidium* spp. oocysts exhibit a characteristically yellow/green fluorescence against a dark background. It is the internal sporozoites that absorb the stain and fluoresce brightly, with individual crescent-shaped sporozoites wrapped around each other often visible [8] (Fig. 3).

13. FITC-stained oocysts exhibit a bright apple-green fluorescence under the FITC filter set. Often the fluorescence has an increased intensity around the entire circumference of the oocyst, with no visible breaks in oocyst wall staining. If Evans' blue or Eriochrome Black, which reduce nonspecific fluorescence, is included in the kit, the background fluorescence will be red (Fig. 4a, b).
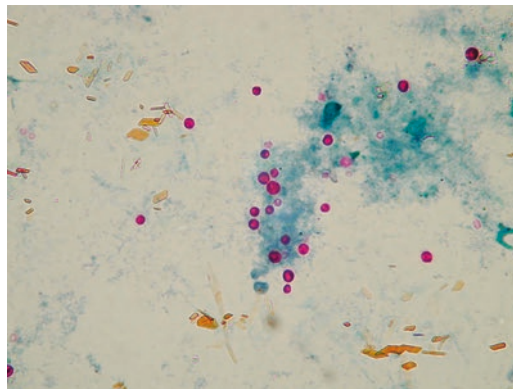


**Fig. 2** Non-*Cryptosporidium* artifacts exhibiting non-specific modified Ziehl–Neelsen staining as seen using a 50× objective
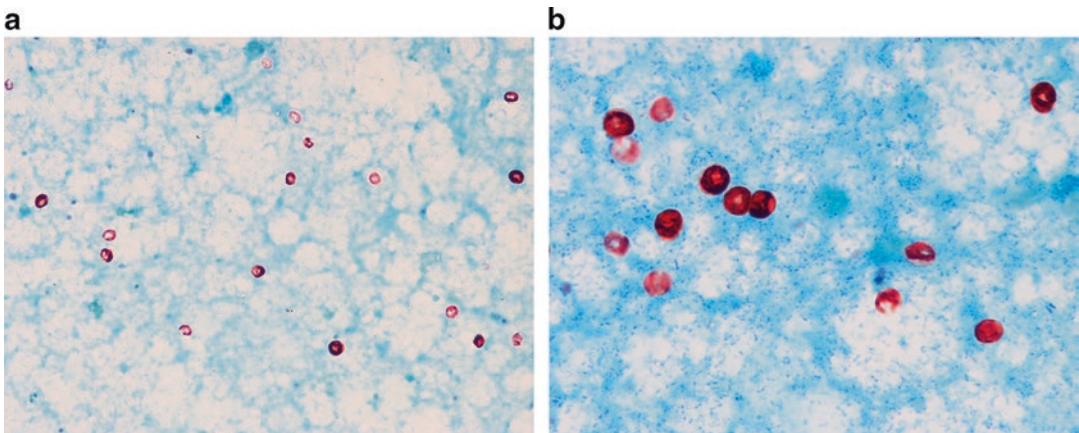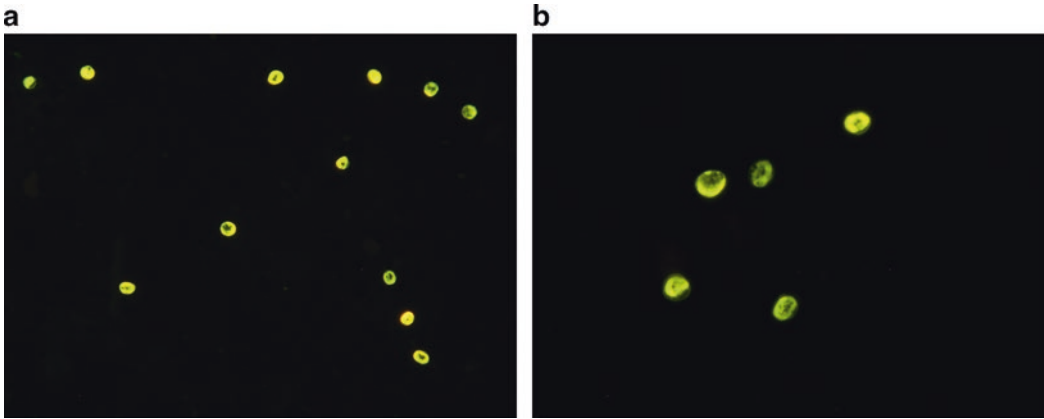
**Fig. 3** *Cryptosporidium* oocysts (~5 μm diameter) in feces demonstrating typical features with auramine phenol staining as seen using (a) 50× objective lens for screening, (b) 100× objective lens for confirmation. The oocyst wall is faintly visible around the internal contents that exhibit the stain. Often the sporozoites lay toward the edge of oocysts which gives them the so-called "doughnut" or "erythrocytic" staining (denser around the outside and fainter or clear in the middle)
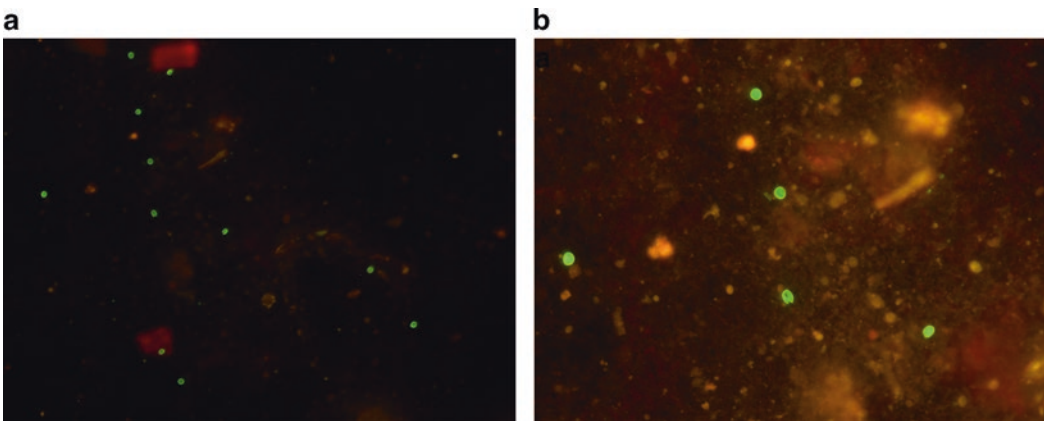


**Fig. 4** *Cryptosporidium* oocysts (~5 μm diameter) in feces demonstrating typical staining with FITC-labeled antibody as seen using (a) 20× objective lens for screening, (b) 40× objective lens for confirmation. The oocyst wall is brightly stained. Note an open oocyst (low center in image) demonstrating the "pac-man" appearance of oocysts that have been split open (excysted)

### References

1. Casemore DP, Armstrong M, Sands RL (1985) Laboratory diagnosis of cryptosporidiosis. J Clin Path 38:1337–1341
2. Smith H (2008) Diagnostics. In: Fayer R, Xiao L (eds) *Cryptosporidium* and cryptosporidiosis, 2nd edn. CRC Press, Boca Raton
3. Anon (2016) Chapter 2.9.4. Cryptosporidiosis. In: Manual of diagnostic tests and vaccines for terrestrial animals. Available at: http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.09.04_CRYPTO.pdf
4. Chalmers RM, Campbell BM, Crouch N, Charlett A, Davies AP (2011) Comparison of the diagnostic sensitivity and specificity of seven *Cryptosporidium* assays used in the United Kingdom. J Med Microbiol 60:1598–1604
5. Chalmers RM, Katzer F (2013) Looking for *Cryptosporidium*: the application of advances in

detection and diagnosis. Trends Parasitol 29:237–251

6. Perry JL, Matthews JS, Miller GR (1990) Parasite detection efficiencies of five stool concentration systems. J Clin Microbiol 28:1094–1097

7. Saez AC, Manser MM, Andrews N, Chiodini PL (2011) Comparison between the Midi Parasep and Midi Parasep solvent free (SF) faecal parasite concentrators. J Clin Pathol 64:901–904

8. Hanscheid T, Melo Cristino J, Salgado MJ (2008) Screening of auramine-stained smears of all fecal samples is a rapid and inexpensive way to increase the detection of coccidial infections. Int J Infect Dis 12:47–50

# Chapter 2

## *Cryptosporidium* Diagnostic Assays: Molecular Detection

**Guy Robinson, Kristin Elwin, and Rachel M. Chalmers**

### Abstract

Molecular diagnostic assays for *Cryptosporidium* are usually based on PCR and may detect the entire genus or target specified species. Of the ~40 species, fewer than half have been reported from humans, and most human cases of cryptosporidiosis are caused by *Cryptosporidium parvum* or *Cryptosporidium hominis*. Here we describe a nested PCR for the detection of all *Cryptosporidium* spp. that can then be differentiated by sequencing the PCR amplicons, and a duplex, real-time PCR for the simultaneous detection and differentiation of *C. parvum* and *C. hominis*.

**Key words** *Cryptosporidium*, *C. parvum*, *C. hominis*, Detection, DNA extraction, Nested PCR, Real-time PCR

## 1 Introduction

Molecular detection of *Cryptosporidium* spp. in feces, or other relevant clinical samples such as bile, small bowel aspirate, sputum, bronchoalveolar lavage, antral washout, or liver biopsy, is usually based on PCR amplification of sporozoite DNA. Molecular methods offer improved sensitivity and detection rates compared with oocyst detection by tinctorial microscopy and antigen detection by enzyme immunoassays (EIA) or immunochromatographic lateral flow assays (ICLF) [1, 2]. A critical stage for molecular detection is the adequate disruption of oocysts for efficient DNA extraction from the sporozoites contained within. Oocysts may be partially purified first, using a flotation or sedimentation technique, or DNA extracted directly from feces [1]. Options for oocyst disruption include bead-beating, freeze–thaw cycles, heating or chemical/enzymatic treatments [1]. Options for subsequent DNA extraction and purification include commercial spin columns, glassmilk, and Chelex resin [1, 3]. Many multi-pathogen gastrointestinal diagnostic PCR assays are now available in "black box" format [4]. Here, we outline *Cryptosporidium* DNA extraction directly from stool and other sample types, and describe two options for PCR

detection. The first is designed to detect any species in the genus *Cryptosporidium* using a nested PCR targeting the small subunit rRNA gene [5–7]. By sequencing the DNA amplicons, the infecting species can be identified. Of the 40 or so species, fewer than 20 have been reported infecting humans, and most cases of human cryptosporidiosis are caused by just two species, *Cryptosporidium parvum* (zoonotic) or *Cryptosporidium hominis* (anthroponotic). The second assay described here is therefore designed for the specific detection and differentiation of *C. parvum* and *C. hominis* using a real-time PCR developed from two previously published assays [8, 9].

## 2    Materials

### 2.1    DNA Extraction

1. Commercially available kits suitable for the extraction of DNA from appropriate specimen types and preparations may vary (*see* **Note 1**). See kit inserts for materials required. Column-based commercial DNA extraction kits usually contain some sort of lysis buffer, proteinase K, wash buffers, and a membrane-based spin column.

2. If semi-purification of the sample is required, see the salt flotation method in Chapter 9 or the sedimentation method in Chapter 1.

3. Control material should be considered carefully (*see* **Note 2**).

### 2.2    Genus-Specific Nested PCR for All Cryptosporidium Species [5–7]

This assay is suitable for testing both human and animal samples, as all known species of *Cryptosporidium* are amplified by the primers used here. If this assay is used as a primary diagnostic, a means of quality-assuring the process needs to be included in each sample (*see* **Note 2**).

1. *Taq* DNA polymerase: various commercial kits are available.

2. 25 mM Magnesium chloride: $MgCl_2$, usually supplied as part of the *Taq* polymerase kit.

3. 10× Reaction buffer (with or without $MgCl_2$): supplied as part of the *Taq* polymerase kit, adjust the $MgCl_2$ in the final master mix according to whether or not the reaction buffer already contains some.

4. 2 mM Deoxyribonucleotide triphosphates (dNTP).

5. 5 mg/mL Non-acetylated Bovine Serum Albumin (BSA).

6. 10 μM 18S primary forward primer (18S1F): standard desalted purification, 5'-TTCTAGAGCTAATACATGCG-3'.

7. 10 μM 18S primary reverse primer (18S1R): standard desalted purification, 5'-CCCATTTCCTTCGAAACAGGA-3'.

8. 10 μM 18S secondary forward primer (18S2F): standard desalted purification, 5'-GGAAGGGTTGTATTTATTAGA TAAAG-3'.

9. 10 μM 18S secondary reverse primer (18S2R): standard desalted purification, 5'-CTCATAAGGTGCTGAAGG AGTA-3'.

10. Nuclease-free (molecular grade) water.

11. Tris–acetate–EDTA (TAE) buffer: Prepare a 1× working solution in oocyst-free distilled water.

12. Agarose gel: 2%. Weigh an appropriate amount of agarose powder (e.g., 1.5 g for 75 mL gel) and add to a bottle/beaker. Pour in the required volume of 1× TAE buffer and heat on a hot plate at a moderate heat setting or in a microwave, periodically stirring/swirling the suspension until the agarose has completely dissolved into the gel solution (*see* **Note 3**).

13. 100 bp DNA size ladder with a range suitable for the detection of a PCR product approximately 830 bp in size (e.g., 100–1000 bp range).

14. DNA loading dye.

15. SYBR Green I (or alternative such as ethidium bromide) DNA stain prepared as per the manufacturer's instructions.

**2.3 Duplex Real-Time PCR for *Cryptosporidium hominis* and *Cryptosporidium parvum***

This assay was validated on human clinical samples at the Cryptosporidium Reference Unit using both the RotorGene 3000 and 6000 (Corbett Research), and RotorGene Q (Qiagen), with the specified reagents (*see* **Notes 4** and **5**). If this assay is used as a primary diagnostic, a means of quality-assuring the process needs to be included in each sample (*see* **Note 2**).

1. TaqMan Environmental Master Mix 2.0 (Applied Biosystems) or suitable alternative commercial real-time master mix if locally verified.

2. *C. hominis* A135 forward primer (CRUA135ChF): standard desalted purification, 10 μM, 5'-CACCAAAGATAATGG ATGTTGTTGAT-3' (2861–2886 XM-661774 TU502).

3. *C. hominis* A135 reverse primer (CRUA135ChR): standard desalted purification, 10 μM, 5'-AATTGCTTCGACAT CGTCCAAT-3' (2964–2985 XM-661774 TU502).

4. *C. hominis* A135 MGB TaqMan probe (CRUA135ChTM): 10 μM, 6-FAM-5'-CAAACGAGCTATTAAAGG-3'-MGB-NFQ (2903–2920 XM-661774 TU502).

5. Lib13 forward primer (CRULib13F): standard desalted purification, 10 μM, 5'-TCCTTGAAATGAATATTTGTGACTCG-3' (458–483 B78618 IOWA).

6. *C. parvum* Lib13 reverse primer (CRULib13RCp): standard desalted purification, 10 µM, 5'-TTAATGTGGTAGTTG CGGTTGAAC-3' (600–623 B78618 IOWA).

7. *C. parvum* Lib13 MGB TaqMan probe (CRULib13CpTM): 10 µM, VIC-5'-TATCTCTTCGTAGCGGCGTA-3'-MGB-NFQ (569–588 B78618 IOWA).

8. Nuclease-free (molecular grade) water.

## 3    Methods

Wear appropriate Personal Protective Equipment (PPE) including protective clothing, disposable gloves, and eye protection during all sample-processing steps.

*3.1    DNA Extraction*    Options for oocyst disruption prior to purification of the extracted DNA include:

1. Bead-beating: This method of oocyst disruption uses zirconia beads shaken very fast on an instrument such as the FastPrep-24 (MP Biomedicals) (*see* **Note 6**).

2. Freeze–thawing: This method usually cycles between freezing in liquid nitrogen and thawing at 95 °C. If liquid nitrogen is difficult to source, a dry ice/methanol slurry can be used as an alternative.

3. Heating: Incubating samples at 100 °C for 10 min to 1 h is sufficient for disrupting the oocysts prior to DNA extraction.

4. Chemical/enzyme treatment: Most DNA extraction kits include a lysis stage that involves incubation with a buffer containing components for chemical or enzymatic digestion.

There are several options for *Cryptosporidium* DNA extraction depending upon the nature of the sample being tested.

1. Directly from stool: Spin column or magnet-based extraction, with constituents to counter the inhibitory substances found in feces, is a common means of extracting DNA directly from stool samples due to their high performance in providing good-quality purified DNA (*see* **Note 7**).

2. From a semi-purified oocyst suspension (e.g., following flotation, method in Chapter 9, or sedimentation, method in Chapter 1) and other liquid sample types (e.g., bile, small bowel aspirate, sputum, bronchoalveolar lavage, antral washout): Column-based kits can also be used to extract DNA from these liquid samples (*see* **Note 8**).

3. From tissue samples: Commercially available tissue extraction kits/protocols are suitable for extracting *Cryptosporidium* DNA from tissue samples known or suspected to contain the parasite (e.g., liver biopsy).

*3.2 Genus-Specific Nested PCR for All Cryptosporidium Species [5–7]*

1. In a DNA-free area, prepare sufficient primary PCR master mix for the number of samples required, plus a no-template control (nuclease-free water) and a *Cryptosporidium* positive DNA control, based on the details for a single reaction in Table 1. While various *Taq* polymerases are commercially available with slightly differing components, this example is based on Qiagen's HotStar *Taq* (*see* **Note 5**).

2. Pipette 45 µL of master mix into a labeled PCR tube for each sample and add 5 µL of test DNA.

3. Transfer the PCR tubes to a thermocycler and run the following three-step cycling conditions (Table 2):

4. Once the primary PCR is complete and again in a DNA-free area, prepare sufficient secondary PCR master mix for the number of samples required and controls, based on the details for a single reaction in Table 3.

5. Pipette 45 µL of master mix into a labeled PCR tube for each sample, and then in a post-amplification area (pre-cleaned with commercial bleach diluted 1:2 with oocyst-free distilled water), add 5 µL of primary PCR product.

6. Transfer the PCR tubes to a thermocycler and run the same three-step cycling conditions used for the primary PCR (Table 2).

7. While the secondary PCR is running, prepare a 2% agarose gel and allow to set with an appropriate comb for the number of samples to be run.

8. Once set, pour TAE buffer into the gel tank until it just covers the agarose (*see* **Note 9**) and carefully remove the comb (*see* **Note 10**).

**Table 1**
**Example of a nested SSU rDNA Primary PCR master mix recipe [5–7]**

| Master mix components | 1× Reaction (µL) |
|---|---|
| 10× reaction buffer | 5 |
| 2 mM dNTP | 5 |
| 5 mg/mL non-acetylated BSA | 5 |
| 10 µM forward primer (18s1F) | 1 |
| 10 µM reverse primer (18s1R) | 1 |
| 5 U/µL HotStar *Taq* polymerase | 0.5 |
| $nfH_2O$ | 27.5 |
| [DNA] | [5] |
| Total reaction volume | 50 |

**Table 2**
**Conditions for the example primary and secondary nested SSU rDNA PCRs**

| Stage | Temperature (°C) | Duration (s) |
|---|---|---|
| Hold (hot start) | 95 | 900 |
| Cycling (40 cycles) | 94 | 45 |
|  | 60 | 45 |
|  | 72 | 60 |
| Final extension | 72 | 420 |
|  | 4 | ∞ |

**Table 3**
**Example of a nested SSU rDNA Secondary PCR master mix recipe [5–7]**

| Master mix components | 1× Reaction (μL) |
|---|---|
| $MgCl_2$ | 3 |
| 10× Reaction buffer | 5 |
| 2 mM dNTP | 5 |
| 10 μM forward primer (18s2F) | 1 |
| 10 μM reverse primer (18s2R) | 1 |
| 5 units/μL HotStar *Taq* polymerase | 0.5 |
| $nfH_2O$ | 29.5 |
| [Primary PCR product] | [5] |
| Total reaction volume | 50 |

9. Prepare a DNA size ladder according to the manufacturer's instructions (e.g., 1 μL of ladder with 9 μL of nuclease-free water) for each row of the gel wells and mix with 2 μL of DNA loading dye.

10. Prepare each sample by mixing 2 μL of DNA loading dye with 10 μL of secondary PCR product.

11. Very carefully, taking care not to stab the gel, add 10 μL of each sample to the wells (*see* **Note 11**) ensuring that each row contains a lane with positive control DNA.

12. Connect the gel tank to the power supply (*see* **Note 12**) and run the gel as per the instructions for that tank (e.g., 110 V for 50 min).

13. Once the samples have run sufficiently, disconnect the tank from the power and place the gel into the stain of choice for the appropriate time according to the manufacturer's instructions (e.g., SYBR green I for approximately 15 min).

14. Using the appropriate gel imaging equipment for the chosen stain, make a permanent photo record of the gel before interpreting and recording the results (*see* **Note 13**).

*3.3 Duplex Real-Time PCR of Cryptosporidium hominis and Cryptosporidium parvum*

The two PCR assays designed to be specific for *C. hominis* and *C. parvum* combined into the duplex real-time assay described here [10] are based on the *C. parvum* assay described by Hadfield et al. [8] and *C. hominis* described by Tosini et al. [9] (*see* **Notes 5** and **14**).

1. In a DNA-free area, prepare sufficient PCR master mix for the number of samples required, plus a no-template control (nuclease-free water), *C. hominis* and *C. parvum* DNA positive controls, based on the details for a single reaction in Table 4.

2. Pipette 22 μL of master mix into each PCR tube and add 3 μL of test DNA.

3. Transfer the PCR tubes to a thermocycler and run the following two-step cycling conditions (Table 5).

4. Once the run has completed, analyze the results by applying a fluorescence threshold of 0.05, checking the positive controls appear in the correct PCR channel, observing the characteristic curves and recording the Ct values from each sample (*see* **Note 15**).

**Table 4**
***C. hominis* and *C. parvum* duplex real-time PCR master mix recipe**

| Master mix components | 1× Reaction (μL) |
|---|---|
| Environmental master mix | 12.5 |
| CRUA135ChF | 2.25 |
| CRUA135ChR | 0.75 |
| CRUA135ChTM | 0.25 |
| CRULib13F | 2.25 |
| CRULib13RCp | 2.25 |
| CRULib13CpTM | 0.25 |
| nfH$_2$O | 1.5 |
| [DNA] | [3] |
| Total reaction volume | 25 |

**Table 5**
**Conditions for *C. hominis* and *C. parvum* duplex real-time PCR**

| Stage | Temperature (°C) | Duration (s) | Comments |
| --- | --- | --- | --- |
| Hold (hot start) | 95 | 600 | |
| Cycling (50 cycles) | 95 | 15 | |
| | 60 | 60 | Acquiring on appropriate channel with calibration based on autogain optimization on first acquisition |

# 4    Notes

1. It is important to choose a kit that incorporates *Cryptosporidium* oocyst disruption. Alternatively, the method may be adapted to ensure adequate disruption of oocysts before sporozoite DNA can be extracted and purified, for example by bead-beating, freeze–thaw cycles, heating, or chemical/enzymatic treatments [1].

2. All tests should include a known and characterized *Cryptosporidium* positive sample as a process positive control, which will evidence adequate disruption of oocysts, extraction of DNA, and PCR amplification. In addition, for diagnostic tests, it is good practice to use an internal control within each sample either by amplifying DNA also known to be present in clinical samples (e.g., human DNA targets) or by spiking with exogenous DNA prior to extraction. This provides surety that a negative result is true, due to the absence of *Cryptosporidium* and not due to PCR inhibition or lack of extraction from within that sample. Several products are available commercially that provide this element of quality control. *Cryptosporidium* positive and negative control DNA should also always be included in the PCR to demonstrate that it worked correctly and without reagent contamination. If the same positive control DNA is used each time, assay results can be used for performance monitoring.

3. Do not let the solution fully boil, or burn the agarose powder to the base of the bottle/beaker.

4. The assay is presented in duplex format here, but the targets can be run independently. If *C. hominis* only or *C. parvum* only is sought, the reagents and conditions remain the same, but with only the primers specific for the species of interest. The missing reaction volume from removing the unwanted

primers and probe must be made up with nuclease-free water. *C. parvum* only may be suitable for samples where only this zoonotic species is sought (e.g., young livestock).

5. Use of alternative reagents, conditions, or thermocyclers may alter the assay's performance and will need optimizing and validating to ensure satisfactory sensitivity and specificity.

6. A vortex shaker is not sufficiently vigorous.

7. There are several commercial kits available for the extraction of DNA from stool, feces, or soil, which can all be used for this sample type. Follow the manufacturer's instructions, and ensure adequate disruption of oocysts, for example, by extended heating or chemical/enzymatic treatments [1].

8. For semi-purified suspensions of oocysts, the kits designed for the extraction of DNA from stool or soil are not required due to the much cleaner nature of the samples. Instead, blood or bodily fluid DNA extraction kits and protocols may be used, following adequate oocyst disruption.

9. If too much buffer is added, the current will take the path of least resistance and flow through the buffer above the gel rather than through the gel itself. This will increase the time required for full separation of amplicons and generally results in poorer quality (less sharp) bands.

10. Remove the comb slowly and carefully so as not to break the gel between wells or pull the bottom of the well out with the comb.

11. Pipette the samples into the gel wells slowly so the weight of the loading dye holds the samples within the wells and it does not flow out of the top.

12. Check the cables are plugged in the correct orientation so that the current does not push the samples off the top of the gel rather than run them down it (DNA is negatively charged and therefore moves toward the positive cathode).

13. Samples containing a visible product of 820–864 bp are considered to be *Cryptosporidium* DNA positive (Fig. 1) and can be reported as "*Cryptosporidium* DNA detected." To determine the species present, the PCR amplicons can be sequenced. Those for which no product is visible or for which the product is clearly the wrong size, should be reported as "*Cryptosporidium* DNA not detected."

14. Specificity testing of this assay demonstrated slight amplification of *C. cuniculus* in the *C. hominis* assay and the horse genotype in the *C. parvum* assay, but the real-time profiles of all four species are distinguishable (*see* Figs. 2 and 3 and **Note 15**). Suspected *C. cuniculus* or horse genotype isolates can be confirmed by PCR and sequencing the SSU rRNA gene as described above.
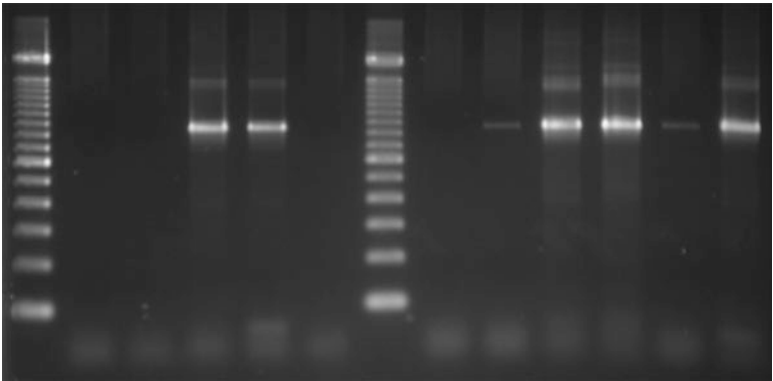
**Fig. 1** Example of *Cryptosporidium* spp. ssu rRNA gene PCR amplicons of about ~830 bp (lanes 4, 5, 9–13) on a 2% agarose gel with a 100 bp size ladder (lanes 1 and 7). Lanes 2, 3, 6, and 8 are negative
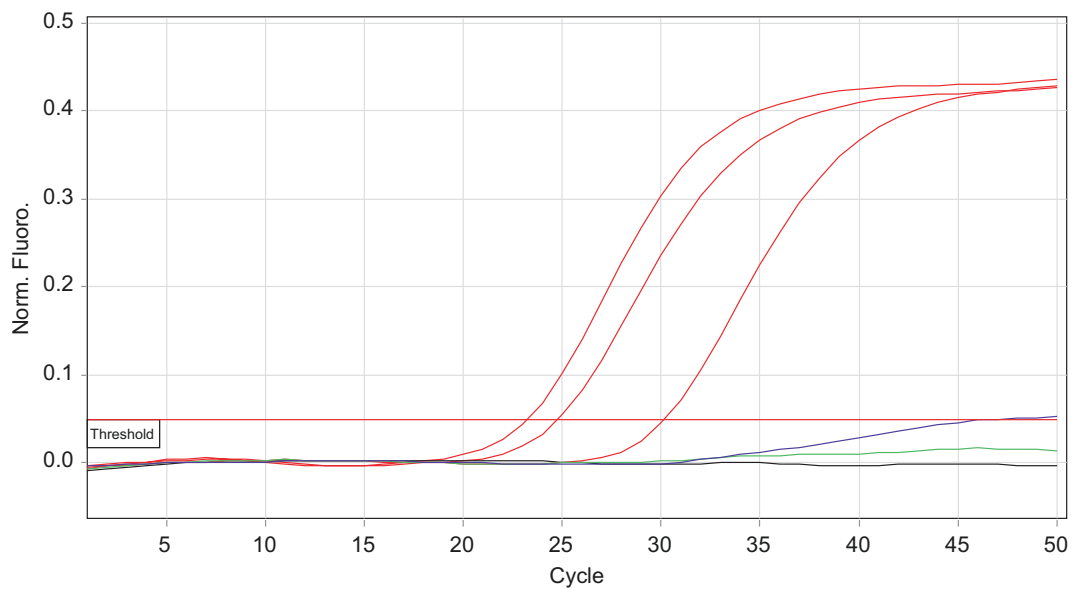


**Fig. 2** Amplification curves produced by the A135 *C. hominis* PCR showing three characteristic *C. hominis* curves (red) and a single *C. cuniculus* (purple) curve. Also shown: Threshold (red horizontal line), *C. parvum* (green), and no-template control (black)

15. All curves should be sigmoidal and reach high fluorescence. Consider using a confirmation assay, such as the nested SSU rDNA-sequencing assay described above, if results are not typical. For example, if a sample tested using the A135 *C. hominis* assay produces a high Ct (or doesn't quite cross the threshold) and very low final fluorescence (green FAM channel), this may indicate the presence of *Cryptosporidium cuniculus* (Fig. 2). If a sample tested using the Lib13 *C. parvum*
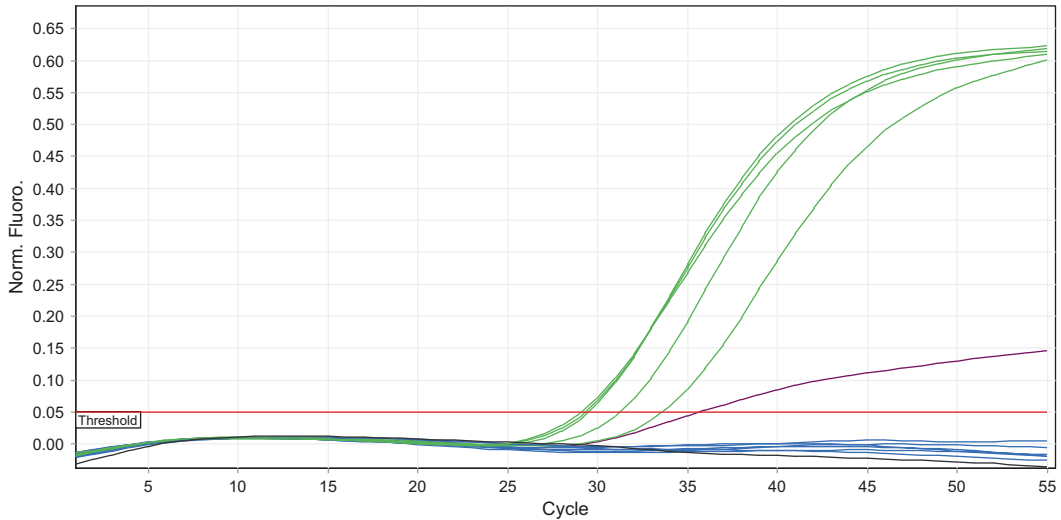
**Fig. 3** Amplification curves produced by the Lib13 *C. parvum* PCR showing five characteristic *C. parvum* curves (green) and a single *Cryptosporidium* horse genotype (purple) curve. Also shown: Threshold (red horizontal line), *C. hominis* (blue), and no-template control (black)

assay has a Ct within the normal range (less than 40), but very low final fluorescence (yellow VIC channel), this may indicate (extremely rarely) the presence of *Cryptosporidium* sp. horse genotype (Fig. 3).

## Acknowledgments

## References

1. Smith H (2008) Diagnostics. In: Fayer R, Xiao L (eds) *Cryptosporidium* and cryptosporidiosis, 2nd edn. CRC Press, Boca Raton

2. Chalmers RM, Campbell BM, Crouch N, Charlett A, Davies AP (2011) Comparison of the diagnostic sensitivity and specificity of seven *Cryptosporidium* assays used in the United Kingdom. J Med Microbiol 60:1598–1604

3. Wiedenmann A, Kruger P, Botzenhart K (1998) PCR detection of *Cryptosporidium parvum* in environmental samples - a review of published protocols and current developments. J Ind Microbiol Biotechnol 21:150–166

4. Binnicker MJ (2015) Multiplex molecular panels for diagnosis of gastrointestinal infection: performance, result interpretation, and cost-effectiveness. J Clin Microbiol 53:3723–3728

5. Jiang J, Alderisio KA, Xiao L (2005) Distribution of *Cryptosporidium* genotypes in storm event water samples from three watersheds in New York. Appl Environ Microbiol 71:4446–4454

6. Xiao L, Escalante L, Yang C, Sulaiman I, Escalante AA, Montali RJ, Fayer R, Lal AA (1999) Phylogenetic analysis of *Cryptosporidium* parasites based on the small-subunit rRNA gene locus. Appl Environ Microbiol 65:1578–1583

7. Xiao L, Alderisio K, Limor J, Royer M, Lal AA (2000) Identification of species and sources of *Cryptosporidium* oocysts in storm waters with a small-subunit rRNA-based diagnostic and genotyping tool. Appl Environ Microbiol 66:5492–5498

8. Hadfield SJ, Robinson G, Elwin K, Chalmers RM (2011) Detection and differentiation of *Cryptosporidium* spp. in human clinical samples by use of real-time PCR. J Clin Microbiol 49:918–924

9. Tosini F, Drumo R, Elwin K (2010) The CpA135 gene as a a marker to identify *Cryptosporidium* species infecting humans. Parasitol Int 59:606–609

10. Moore CE, Elwin K, Phot N, Seng C, Mao S, Suy K, Kumar V, Nader J, Bousfield R, Perera S, Bailey JW, Beeching NJ, Day NP, Parry CM, Chalmers RM (2016) Molecular characterisation of *Cryptosporidium* species and *Giardia duodenalis* from symptomatic Cambodian children. PLoS Negl Trop Dis 10:e0004822

# Chapter 3

# Detection of *Cryptosporidium* Recovered from Large-Volume Water Samples Using Dead-End Ultrafiltration

**Amy M. Kahler and Vincent R. Hill**

## Abstract

The procedure described here provides instructions for detection of *Cryptosporidium* recovered from large-volume water samples. Water samples are collected by dead-end ultrafiltration in the field and ultrafilters are processed in a laboratory. Microbes recovered from the filters are further concentrated and subjected to *Cryptosporidium* isolation or nucleic acid extraction methods for the detection of *Cryptosporidium* oocysts or *Cryptosporidium* DNA.

**Key words** *Cryptosporidium*, Dead-end ultrafiltration, IMS, Microscopy, Real-time PCR, Water

## 1 Introduction

The procedure described here provides instructions for detection of *Cryptosporidium* recovered from large-volume water samples. Water samples are collected using a hollow-fiber, dead-end ultrafiltration method (DEUF). The hollow-fiber ultrafilters used in this procedure have pore sizes on the order of 10 nm (~30 kiloDaltons (kDa)) and thus can be used to recover protozoan parasites, bacteria, viruses, and large toxins from water samples [1–3]. A specific ultrafilter type (REXEED 25S) has been identified in this protocol because of its large filter fiber surface area (2.5 m²) and reported performance effectiveness by multiple research laboratories. However, alternative hollow fiber ultrafilters may be used if they have similar specifications and performance characteristics.

The DEUF method is designed to be performed in the field with minimal equipment and setup. In the DEUF procedure, water flows into the ultrafilter, through the hollow-fiber membranes, and out of the ultrafilter pores while microbes are captured within the hollow fibers. The ultrafilters are capable of filtering 10–50 L of turbid surface water or hundreds of liters of finished drinking water. The volume of water filtered will depend on water quality characteristics and the suspected concentrations of target

microorganisms. After ultrafiltration, the ultrafilter is processed in a laboratory. The ultrafilter is backflushed with 500 mL of a solution containing 0.5% Tween 80, 0.01% sodium polyphosphate, and 0.001% Antifoam Y-30 emulsion to recover the microbes from the ultrafilter. If the concentration of the target microbes is sufficiently high, the resulting backflushed solution can be analyzed directly. If the concentration of the target microbes is low or unknown, the backflushed solution can be further concentrated to achieve a volume that is amenable to downstream detection methods.

For *Cryptosporidium* detection, the sample concentrate may be subjected to immunomagnetic separation (IMS) and immunofluorescence assay (FA) microscopy for observation of *Cryptosporidium* oocysts [4] and/or nucleic acid extraction and real-time PCR for detection of *Cryptosporidium* DNA [5, 6]. The choice of detection methods should be determined by the goals of the study and/or the unique characteristics of the water type being analyzed. The performance recovery efficiency of each methodological step (DEUF, secondary concentration, IMS, nucleic acid extraction) may vary depending on water quality and composition [1, 2]. Therefore, it is recommended that the complete method be evaluated and validated before processing real-world samples to ensure that effective detection can be achieved.

## 2   Materials (*See* Note 1)

*2.1   Dead-End Ultrafiltration*

1. Insulated cooler.

2. Reusable freezer packs.

3. REXEED 25S dialysis filter (Asahi Kasei Medical America Inc., Glenview, IL).

4. Masterflex L/S 36 tubing (platinum-cured silicone or C-Flex ULTRA) (Cole-Parmer, Vernon Hills, IL).

5. Female DIN to 3/8 barb fitting adapters, polypropylene (Molded Products, Inc., Harlan, IA).

6. SNP-8 acetal copolymer tubing clamps.

7. Blood port kidney storage cap (Molded Products).

8. Flow totalizing meter, ½″ schedule 80 PVC NPT turbine, 3.8–38 L/min (Omega Engineering, Inc. Swedesboro, NJ) fitted with two straight barbed to male NPT threaded adapters, ¾″ NPTM × 3/8″ tubing ID.

9. 20 L collapsible cubitainers, optional.

10. Long nose pliers.

11. Scissors.

12. 500 mL bottles (Nalgene).

13. Sodium thiosulfate.

14. Geotech Geopump peristaltic pump (Geotech Environmental Equipment, Inc., Denver, CO) with EZ-Load II pump head placed on the Series 2 drive (for non-pressurized water sources).

15. Alcohol wipe or equivalent.

16. 60 mL syringe.

17. 1/2″ ID hose × swivel FGHT nylon swivel female insert.

18. SNP-12 acetal copolymer tubing clamps.

19. Masterflex I/P 89 platinum-cured silicone tubing (Cole-Parmer).

20. Reducing connector 5/8″ to 3/8″.

21. SNP-28 acetal copolymer tubing clamps.

22. SNP-24 acetal copolymer tubing clamps.

23. SNP-19 acetal copolymer tubing clamps.

*2.2   Backflushing*

1. Masterflex L/S digital pump system with Easy-Load II pump head, 600 rpm, 115/230 V (or use the same pump as for field collection) (Cole-Parmer).

2. Lab jack with 10″ × 10″ top.

3. Ultra flex support with base plate, 18″ arm length.

4. Masterflex L/S 36 tubing (Platinum-cured silicone or C-Flex ULTRA) (Cole-Parmer).

5. Blunt-edge forceps.

6. Faceshield.

7. Long nose pliers.

8. SNP-12 or SNP-14 acetal copolymer tubing clamps.

9. 500 mL bottle.

10. 1 L bottle.

11. 10 mL plastic serological pipette.

12. Deionized or distilled (DI) water.

13. Tween 80.

14. Sodium hexametaphosphate.

15. Antifoam Y-30 emulsion.

*2.3   Secondary Concentration*

1. Centrifuge capable of accepting 200–500 mL conical centrifuge tubes.

2. 200 mL or 500 mL conical centrifuge bottles.

3. 50 mL plastic serological pipette.

4. 0.01 M PBS, pH 7.2–7.4 (1×) or equivalent buffer solution for resuspension.

*2.4 Immuno-magnetic Separation (IMS) and Fluorescent Antibody (FA) Microscopy*

1. Glass Leighton tubes or Dynabeads L-10 tubes (Applied Biosystems Inc., Beverly, MA).

2. Deionized or distilled (DI) water.

3. Anti-*Cryptosporidium* magnetic beads (Dynabeads anti-*Cryptosporidium* or Dynabeads GC-Combo (Applied Biosystems).

4. Rotary mixer for immunomagnetic beads (Dynabeads rotary mixer, Applied Biosystems).

5. Magnet for magnetic bead capture in Leighton tubes or 10–30 mm tubes (MPC-6 magnetic particle concentrator, Applied Biosystems).

6. 1 mL or 2 mL plastic serological pipettes.

7. 1.5 mL nuclease-free (NF) microcentrifuge tubes (Invitrogen Corp., Carlsbad, CA).

8. Magnet for magnetic bead capture in microcentrifuge tubes (MPC-S magnetic particle concentrator (Applied Biosystems).

9. 0.01 M PBS, pH 7.2–7.4 (1×).

10. 0.1 N HCl.

11. 1 N NaOH.

12. Two-well microscope slides with adhesive coating (SuperStick slides, Waterborne Inc., New Orleans, LA).

13. Heat block or slide warmer.

14. EasyStain *Cryptosporidium* oocyst labeling reagent (bioMérieux Inc., Durham, NC).

15. Coverslips, 22 × 60 mm.

16. Clear nail polish.

17. Fluorescent microscope with FITC filter.

*2.5 Nucleic Acid Extraction and Real-Time PCR*

1. Molecular grade ethanol, 200 proof.

2. Nuclease-free (NF) water (or molecular grade water).

3. 0.2 mm zirconium oxide beads, $Y_2O_3$-stabilized, 95% (Union Process, Inc., Akron, OH).

4. 0.5 mm zirconium oxide beads, $Y_2O_3$-stabilized, 95% (Union Process, Inc.).

5. 0.1 N HCl.

6. Oven.

7. 0.5 mL nuclease-free mL tubes.

8. Double-ended micro-tapered stainless steel spatula.

9. Twist ties.

10. FastPrep-24 bead beater (MP Biomedicals, LLC, Santa Ana, CA).

11. FastPrep compatible 2 mL empty bead beating tubes (MP Biomedicals).

12. FastPrep compatible caps for 2 mL beating tubes (MP Biomedicals).

13. UNEX lysis buffer (Microbiologics, Inc., St. Cloud, MN).

14. Proteinase K, ≥600 mAU/mL.

15. Silica HiBind RNA minicolumn RNACOL (Omega Bio-tek, Inc., Norcross, GA).

16. OneStep PCR inhibitor removal (Zymo Research, Irvine, CA).

17. 2 mL collection tube.

18. 1.5 mL nuclease-free microcentrifuge tubes.

19. Tris-EDTA (TE) buffer, pH 8.0, molecular biology grade.

20. TaqMan Environmental Master Mix 2.0 (Life Technologies Corp., Carlsbad, CA).

21. Oligonucleotides

    **Forward primer**: ATG ACG GGT AAC GGG GAA T

    **Reverse primer**: CCA ATT ACA AAA CCA AAA AGT CC

    **Probe**: 6FAM-CGC GCC TGC TGC CTT CCT TAG ATG-BHQ1

22. T4 gene 32 protein (gp32).

23. Bovine serum albumin (BSA, molecular biology grade).

24. TaqMan Exogenous Internal Positive Control Kit (Life Technologies Corp.).

25. Aerosol barrier pipette tips.

26. Applied Biosystems 7500 real-time PCR thermocycler (Life Technologies Corp.).

27. MicroAmp optical 96-well reaction plate or MicroAmp optical 8-tube strip (Life Technologies Corp.).

28. Optical adhesive film (MicroAmp 96-well format) or MicroAmp optical 8-cap strip (Life Technologies Corp.).

29. Laminar flow cabinet or similar PCR-compatible workstation.

30. Plate or strip spinner.

31. RNase AWAY surface decontaminant (Thermo Fisher Scientific, Inc., Norcross, GA).

## 3  Methods

### 3.1  Reagent Preparation

1. Sodium thiosulfate solution (1% w/v): add 5 g sodium thiosulfate to a 500 mL Nalgene bottle, add 500 mL DI water or ultrafilter effluent as described in Subheading 3.2, **step 15**.

2. Backflush solution (0.5% Tween 80, 0.01% sodium polyphosphate (NaPP), and 0.001% Antifoam Y-30 emulsion): add 10 mL DI water to a screw-cap tube, add 1 g NaPP and 100 µL Antifoam (*see* **Note 2**) to the DI water (10% NaPP, 1% Antifoam), shake vigorously to dissolve the NaPP (*see* **Note 3**). This stock solution can be kept at room temperature or at 4 °C. Add 500 mL DI water to a 500 mL bottle. Add 2.5 mL Tween 80 and 500 µL of the NaPP/Antifoam stock solution to the DI water. Swirl to dissolve the Tween 80. If desired, the backflush solution can be prepared a day in advance and stored at 4 °C. Warm to room temperature before use.

3. BSA/gp32 solution (10 mg/mL BSA, 500 µg/mL gp32): add 10 mL TE buffer to 100 mg of BSA and dissolve by swirling. Aliquot 1 mL into 1.5 mL microcentrifuge tubes and store at 4 °C. Thaw gp32 and add 950 µL BSA solution and invert to mix, store at 4 °C.

### 3.2  Ultrafiltration

1. Prepare the flow totalizer: screw in a tubing adapter to each end of the totalizer, take a length of L/S 36 tubing and cut in half, push tubing onto both sides of the meter, and no tubing clamps are needed.

2. Label ultrafilter with an indelible marker.

3. Fill out pertinent information on chain of custody form or sample collection bench sheet: pertinent information may include: sample location and/or sample ID, measured water quality parameters, water type, site description, etc.

4. Remove one end port cap and screw in a DIN adapter (this is the influent port).
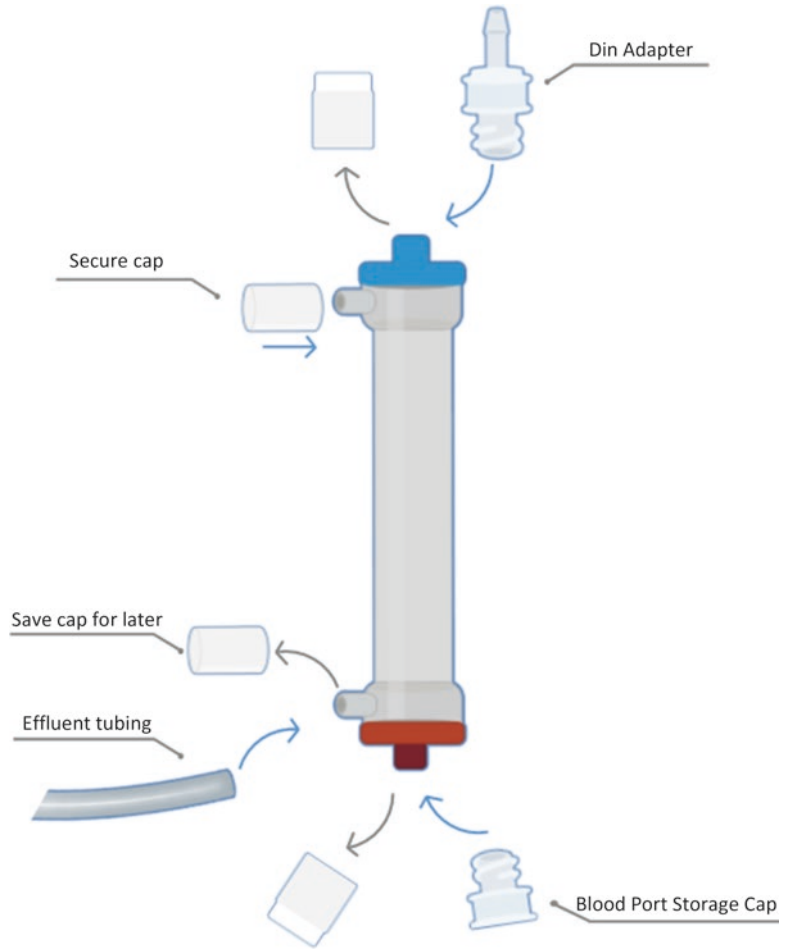
5. Push L/S 36 tubing onto the DIN adapter and secure with a SNP-8 clamp. The length of influent tubing needed will be determined by the distance to the water source.

6. Close off the side port closest to the influent port by pushing the cap until it clicks.

7. Screw in a blood port cap to the end port opposite the influent port.

8. Remove the cap from the effluent port (furthest from the influent port) and save. Push the flow totalizer tubing onto the effluent port, making sure that the arrow on the flow totalizer points away from the ultrafilter. No tubing clamp is needed.

9. Record the initial flow totalizer meter reading or reset to zero.

10. Place the effluent tubing in an empty 20 L cubitainer or equivalent container, downstream of the water being collected, or near a floor drain. The effluent water will be free of microbial contamination so it can be released into the environment as conditions allow (*see* **Note 4**).

11. Non-pressurized water sources (Fig. 1): Place the influent tubing into the water source (or water that has been collected in a 20 L cubitainer). Ensure that the end of the tubing will stay below the surface of the water (*see* **Note 5**). Feed the influent tubing through the pump head and close the pump head using the lever. Plug in the "battery" power cord into the pump and the other end into the battery (*see* **Note 6**). The Geopump comes with alligator clips so that any external battery that is 12–18 V DC @ 70 watts or 90–260 V AC @ 47–65 Hz can be used. Place the battery in a location where it will not get wet. Position the toggle switch on the pump for the appropriate direction of flow. Ensure the speed dial is set to zero and turn on the pump. Gradually increase the speed to the maximum setting (*see* **Note 7**).

12. Pressurized water sources (Fig. 2): wipe the faucet with an alcohol wipe or other sanitizer. Turn on the faucet and purge the water for 2–3 min. Turn off the faucet before setting up the ultrafilter. If sampling from a standard garden faucet (hose bib), screw on a FGHT adapter. Push influent tubing onto the adapter and secure with a SNP-12 clamp. If sampling from a non-standard faucet, push 4–6 inches of I/P 89 tubing over the faucet head and secure with a SNP-28 clamp. Use a reducing connector to connect the I/P 89 and L/S 36 influent tubing, and secure both sides with SNP-19 and SNP-8 clamps, respectively. Turn on the faucet and gradually increase the flow until the desired flow rate is achieved (3–5 L/min) (*see* **Note 8**).

13. If the flow totalizer is not reset to zero, calculate the desired flow totalizer end reading by adding the volume of water collected to the starting reading (*see* **Note 9**).



**Fig. 1** DEUF assembly for non-pressurized water sources



**Fig. 2** DEUF assembly for pressurized water sources

14. During filtration, visually inspect the flow rate from the effluent tubing. Dramatic changes in flow rate will indicate filter clogging, which can be due to water quality or entrapment of an object in the influent tubing.

15. Prepare the sodium thiosulfate solution if a chlorine residual is present (or suspected) in the water source. If water was not already added to the sodium thiosulfate bottle during preparation, fill the bottle to the 500 mL mark using the ultrafilter effluent. Shake to dissolve and set aside.

16. Once the desired volume of water has been filtered, turn off the pump or faucet.

17. For water sources with a chlorine residual, follow the chlorine quenching steps below. For non-chlorinated water sources, proceed to **step 20**.

18. Non-pressurized water sources: place the influent tubing into the sodium thiosulfate solution. Turn on the pump to draw the sodium thiosulfate solution into the ultrafilter. Continue until the sodium thiosulfate solution has been drawn through most of the influent tubing, but do not allow air to be pumped into the ultrafilter. Turn off the pump and release lever on the pump head.

19. Pressurized water sources: remove the influent tubing from the faucet and cut the tubing so that only 2–3 inches remain attached to the ultrafilter. Remove the plunger from the 60 mL syringe. Insert the tip of the syringe into the influent tubing and secure with an SNP-8 clamp. Pour 60 mL of the sodium thiosulfate solution into the syringe and insert the plunger to push the solution through the ultrafilter. Remove the SNP-8 clamp and take the syringe out of the tubing. Remove the plunger from the syringe. Pulling out the plunger before removing the syringe from the tubing will create negative pressure and draw liquid out of the ultrafilter. Repeat sodium thiosulfate injection (as described above) one more time.

20. Unscrew the DIN adapter with influent tubing. Screw a blood port cap into the influent port.

21. Place influent tubing and clamps into a waste bag for used supplies. Return to the lab for disposal or decontamination.

22. Remove the effluent tubing and re-cap the effluent port using the cap supplied with the ultrafilter. The effluent tubing and flow totalizer can be reused for subsequent samples.

23. Place the sealed ultrafilter in a cooler for shipment or transport to the laboratory. Use ice packs or bags of ice to keep the cooler chilled. The ideal temperature range is 2–8 °C.

*3.3 Backflushing and Secondary Concentration*

1. Prepare the backflush solution.

2. Assemble the system as shown in Fig. 3 (*see* **Note 10**): set the pump on the lab jack, raise the lab jack so that the pump head will be at the same height as the side port of the ultrafilter where the backflush will enter. Clamp ultrafilter vertically into support base so that end ports are at the top and bottom and side ports are pointed in the direction of the pump. Ensure the ultrafilter is high enough that a 1 L bottle can be placed under the end port. Ensure a blood port storage cap is screwed into the top port. Remove the cap from the highest side port. Ensure the remaining side port cap is secure.

3. Cut a 10–12″ length of L/S 36 tubing.

4. Push the tubing onto the open side port and secure with an SNP-12 or SNP-14 clamp.

5. Feed the tubing through the pump head.

6. Remove the cotton plug from a 10 mL serological pipette using a pair of forceps. Break off the conical tip of the plastic pipette (*see* **Note 11**).
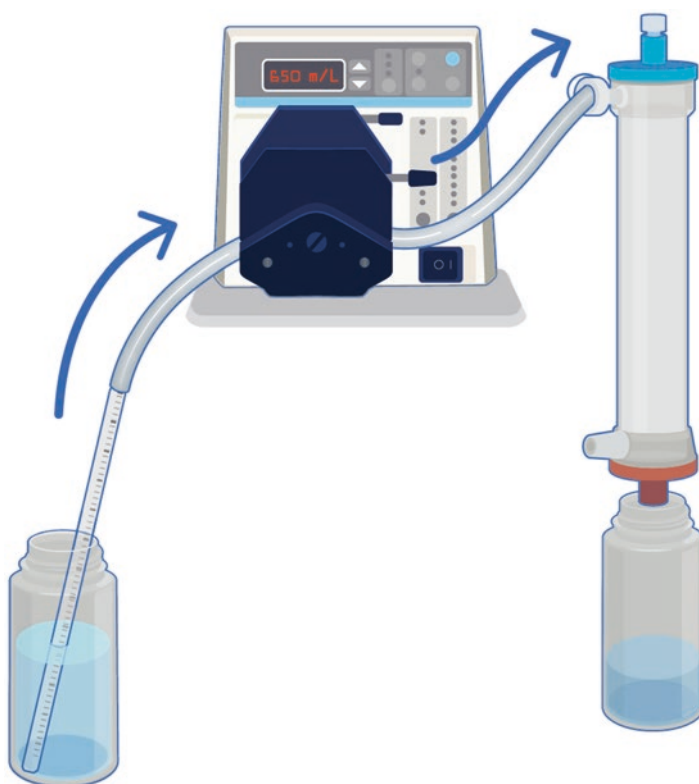


**Fig. 3** DEUF assembly for backflushing and recovery of captured sample concentrate

7. Push the influent tubing onto the top of the pipette and place the pipette into the backflush solution.

8. Close the pump head and ensure the direction flow is correct. Set the pump speed to 650 mL/min (*see* **Note 12**).

9. Remove the blood port storage cap from the lower end port and place a sterile 1 L bottle under the port.

10. Turn on the pump and pump the entire backflush solution through the ultrafilter.

11. Turn off the pump when no backflush solution remains in the botte and the flow out of the ultrafilter slows to a trickle. Do not continue pumping once the flow of liquid from the ultrafilter has stopped. Doing so will cause pressure to build up and increase the risk of the side port cap to burst off.

12. Open the pump head slowly to release the pressure in the influent tubing.

13. Remove the tubing clamp and save for subsequent ultrafilters.

14. Release the ultrafilter from the support base and cap the bottle containing the sample concentrate.

15. Unscrew the blood port caps and save for decontamination.

16. Measure and record the backflush concentrate volume, if necessary.

17. Centrifuge the backflush concentrate at $1500–2000 \times g$ for 15 min according to USEPA Method 1623.1 (*see* **Note 13**).

18. Carefully remove the supernatant using a 50 mL pipette, leaving 1–5 mL of supernatant above the pellet.

19. Measure and record the packed pellet volume and total concentrate volume.

20. Proceed to Subheadings 3.4–3.5 for *Cryptosporidium* detection by immunofluorescence assay microscopy or Subheadings 3.6–3.7 for *Cryptosporidium* detection by real-time PCR. If both detection methods are utilized, split the centrifuge concentrate for processing by each method (*see* **Note 14**).

***3.4 Immuno-magnetic Separation (IMS, Per USEPA Method 1623.1)***

1. If the packed pellet volume is ≤0.5 mL, the entire sample concentrate, or up to 10 mL, can be processed by IMS. If the packed pellet volume is >0.5 mL, process no more than the equivalent of 0.5 mL. If desired, the sample concentrate can be split into subsamples in order to process the entire volume. If the packed pellet volume is ≤0.5 mL, but the total sample concentrate volume is >10 mL, the sample concentrate can be split into subsamples in order to process the entire volume, if desired.

2. Prepare a 1× dilution of SL-A buffer from the provided 10× buffer stock using DI water. For each sample processed, 1.5 mL of 1× SL-A buffer will be required.

3. Add the sample concentrate to an L-10 tube. Bring the volume up to 10 mL with DI water if processing <10 mL sample concentrate.

4. Add 1 mL each of the 10× SL-A and SL-B buffers to the L-10 tube.

5. Vortex the Dynabeads for 10 s and add 100 μL to the L-10 tube.

6. Place the L-10 tube on the rotary mixer and rotate at 18 rpm for 1 h at room temperature.

7. Place the L-10 tube in the MPC-6, ensuring the flat side of the tube is flush against the magnet.

8. Starting in an upright position, gently rock the tube by hand through 180° for 2 min, with approximately 1 tilt per second.

9. Remove the cap from the L-10 tube and pour the supernatant into a collection beaker. Allow more supernatant to settle and remove with a 1 mL or 2 mL serological pipette.

10. Remove the L-10 tube from the MPC-6 and resuspend the sample in 0.5 mL of 1× SL-A buffer by repeatedly releasing the liquid down the flat side of the tube. Do not vortex.

11. Transfer the solution to a 1.5 mL microcentrifuge tube. Repeat rinsing procedure two more times.

12. Place the microcentrifuge tube in the MPC-S with the magnet in place.

13. Starting in an upright position, gently rock the tube by hand through 180° for 1 min, with approximately 1 tilt per second.

14. Aspirate the supernatant from the tube using a 1000 μL micropipettor.

15. Add 1 mL of 0.01 M PBS to the tube, taking care not to disturb the beads at the back of the tube.

16. Remove the magnet from the MPC-S and gently resuspend the beads.

17. Replace the magnetic strip in the MPC-S.

18. Starting in an upright position, gently rock the tube by hand through 180° for 1 min, with approximately 1 tilt per second.

19. Aspirate the supernatant from the tube using a 1000 μL micropipettor.

20. Let the tube stand undisturbed for 1 min, then aspirate any remaining liquid from the tube.

21. Remove the magnet from the MPC-S and add 50 µL of 0.1 N HCl to the tube. Vortex on high for 50 s.

22. Allow the tube to stand undisturbed in an upright position for 10 min at room temperature.

23. Vortex on high for 30 s.

24. Tap the sample into the base of the tube and place in the MPC-S.

25. Replace the magnetic strip in the MPC-S and allow the tube to stand undisturbed for at least 10 s.

26. Add 5 µL NaOH to two sample wells on a two-well adhesive microscopy slide, or 10 µL to one well if the volume from the two dissociation steps will be combined to the same well.

27. While the tube is still in the MPC-S, transfer the sample volume from the tube to a microscopy slide well.

28. Repeat the dissociation process (Subheading 3.4, **steps 21–27**); add the sample volume to a second sample well or combine with the volume from the first dissociation.

29. Proceed directly to fluorescent antibody microscopy or store the slides up to 24 h in a closed container at 4 °C.

*3.5 Fluorescent Antibody (FA) Microscopy*

1. Place the Fixing Buffer from the EasyStain kit into a beaker of ice water, hold at 4 °C until use.

2. Dry the sample suspension on the two-well adhesive microscope slides on a slide warmer or heat block (≤60 °C, usually ≤10 min). Alternatively, the slides can be dried inside a 37 °C incubator.

3. Add 50 µL EasyStain reagent to the well.

4. Incubate at room temperature for 30 min or in a box containing a moist tissue at 37 °C for 15 min (*see* **Note 15**).

5. Place the slide onto a paper towel and tilt the slide to absorb the stain from the well.

6. Add 300 µL ice-cold Fixing Buffer to the well, ensuring the entire well is covered. Let stand for 2 min.

7. Place the slide onto a Kimwipe or paper towel and tilt the slide to absorb the Fixing Buffer from the well.

8. Add one drop of Mounting Medium to the well and apply the coverslip (*see* **Note 16**).

9. Apply clear nail polish to the edges of the coverslip to seal the coverslip to the slide.

10. Examine the slide under 200×–400× magnification using a fluorescent microscope with a FITC filter. *Cryptosporidium* oocysts will appear as ~5 µm circles with a bright green fluorescent oocyst wall.

*3.6 Nucleic Acid Extraction*

1. Prepare 70% ethanol by combining 35 mL of 200-proof ethanol and 15 mL of NF water in a 50 mL conical tube. Store the solution at room temperature.

2. Prepare the extraction beads: submerge the 0.2 mm and 0.5 mm beads separately in 0.1 N HCl in a 250 mL glass beaker and mix for 30 s. Let it sit for 10 min, then decant the HCl. Rinse the beads by adding enough deionized or DI water to submerge the beads. Mix for 30 s and decant the DI water. Repeat the DI rinsing procedure four more times. Transfer the beads to separate clean 400 mL glass beakers. Bake the beads in a laboratory oven at 200 °C for 30 min (or until completely dry) and allow to cool to room temperature. Transfer the beads to a container with a cap, such as a 50 mL conical tube, and store at room temperature.

3. Cut the cap off a 0.5 mL microcentrifuge tube and secure with a twist tie onto the end of a double-ended spatula.

4. Scoop one capful each of the 0.2 mm and 0.5 mm acid-wash beads into a bead beating tube.

5. Add 750 μL water sample concentrate to the prepared beat beating tube (*see* **Note 17**). Add 750 μL UNEX lysis buffer and 75 μL Proteinase K, vortex to mix.

6. Allow the sample to sit at room temperature for at least 15 min for Proteinase K activity.

7. Place the bead beating tube securely in the bead beater. Balance with a similarly weighted bead beating tube if processing one sample.

8. Beat the sample at 6.0 m/s for 60 s.

9. Centrifuge the bead beating tube at $10,000 \times g$ for 30 s.

10. Prepare the silica column by placing into a provided 2 mL collection tube.

11. Transfer 700 μL of supernatant from the bead beating tube into a prepared silica column.

12. Centrifuge the silica column at $10,000 \times g$ for 1 min.

13. Pour the filtrate from the collection tube into an autoclave waste container, place the silica column back into the collection tube.

14. Repeat **steps 11–13** to process the remaining volume, being careful to avoid the beads and any pellet material while removing the supernatant.

15. Add 500 μL of 100% ethanol to the silica column.

16. Centrifuge the silica column at $10,000 \times g$ for 1 min.

17. Pour the filtrate from the collection tube into an autoclave waste container, place the silica column back into the collection tube.

18. Add 500 µL of 70% ethanol to the silica column.

19. Centrifuge the silica column at $10,000 \times g$ for 1 min.

20. Pour the filtrate from the collection tube into an autoclave waste container, place the silica column back into the collection tube.

21. Centrifuge the silica column at $10,000 \times g$ for 1 min. This is a dry spin to remove any residual ethanol.

22. Discard the collection tube and place the silica column into a labeled 1.5 mL microcentrifuge tube.

23. Add 80 µL TE buffer to the silica column.

24. Centrifuge the silica column at $9000 \times g$ for 1 min.

25. Insert a OneStep PCR inhibitor removal column into a provided collection tube.

26. Add 600 µL of the Prep-Solution and centrifuge at $8000 \times g$ for 3 min.

27. Transfer the column to a labeled 1.5 mL microcentrifuge tube.

28. Transfer the eluted TE buffer from the silica column to the OneStep column.

29. Centrifuge the OneStep column at $8000 \times g$ for 1 min (*see* **Note 18**).

30. Discard the OneStep column and store the nucleic acid eluate at −20 or − 80 °C until PCR analysis.

*3.7  Real-Time PCR*

1. Thaw frozen reagents in a laminar flow or PCR-compatible cabinet.

2. Prepare the master mixture solution by adding the PCR reagents to a 1.5 mL microcentrifuge tube (Table 1). Mix the solution inverting the tube several times or briefly vortexing.

3. Add 45 µL of the master mixture into each reaction tube in a 96-well plate or 8-tube strip.

4. Add 5 µL template DNA to the appropriate reaction tubes. Triplicate reaction tubes are recommended for environmental samples (*see* **Note 19**).

5. Add 5 µL NF water as a no-template control to the appropriate reaction tubes. Additional no-template controls using the internal control blank can also be analyzed.

6. Add 2–5 µL positive control, depending on the concentration and desired Ct value. If adding less than 5 µL, add additional NF water to bring the total reaction volume to 50 µL.

7. Briefly spin tube strips or 96-well plates to move liquid to the bottom of the tubes.

**Table 1**
*Cryptosporidium* **real-time PCR master mixture recipe**

| Reagent | Initial concentration | Volume per 50 μL reaction (μL) | Final concentration |
|---|---|---|---|
| Environmental Master Mix 2.0 | 2× | 25 | 1× |
| Forward primer | 10 μM | 1.25 | 250 nM |
| Reverse primer | 10 μM | 1.25 | 250 nM |
| Probe | 4 μM | 1.25 | 100 nM |
| BSA/gp32 | 10 mg/mL BSA; 500 μg/mL gp32 | 2.5 | 0.5 mg/mL BSA;25 μg/mL gp32 |
| Internal control mix | 10× | 5 | 1× |
| Internal control DNA | 50× | 1 | 1× |
| NF water | NA | 7.75 | NA |

8. Run samples with the following cycling conditions: 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 55 °C for 1 min. Fluorescence acquisition is done during the 55 °C step (*see* **Note 20**).

9. Analyze amplification curves visually to obtain detect/non-detect status of each sample. Calculate Ct values by manually or automatically setting the threshold level for the run. When comparing Ct values between real-time PCR runs, ensure the threshold level is the same for each run.

*3.8 Decontamination and Disposal*

1. Discard the ultrafilter and side port caps in an autoclave waste container.

2. Remove tubing clamps, faucet adapters, reducing connectors, and DIN adapters from influent sample collection tubing. Discard the tubing in an autoclave waste container.

3. Inspect and clean the effluent tubing and flow totalizer. The setup can remain assembled for future use. Discard tubing that has become cracked or worn. Store in a cool dry place.

4. Place reusable plastic DEUF components into a solution of 10% bleach for ≥1 h. Rinse thoroughly with DI water and air dry.

5. Discard centrifuge supernatant in a beaker containing 10% (v/v) sodium hypochlorite and dispose of according to the laboratory's procedures.

6. UV sterilize laminar flow or PCR-compatible cabinet and wipe down pipettes and equipment with RNase AWAY.

## 4   Notes

1. Catalog numbers are included for reference; substitutions can be made, provided the substitute item has the same specifications and equivalent performance as the suggested item.

2. In order to more easily pipette the Antifoam solution, cut off the tip of a 1000 µL pipette tip to create a larger opening.

3. NaPP requires considerable shaking to dissolve and a wrist shaker is ideal for this purpose. Alternatively, the stock solution can be prepared in advance to allow time to dissolve or the volume can be scaled up to 100 mL and the solution can be stirred in a beaker on a magnetic stir plate.

4. For sampling non-flowing bodies of water, the ultrafilter effluent should be directed away from the location where the influent tubing is drawing water.

5. It is important that the tubing stays submerged during filtration. A bit of air in the line will not cause a problem, but drawing in too much air will cause pumping to fail. When this occurs, the pump will not draw any water, even when the tubing is re-submerged. Turn off the pump and open the pump head to release the pressure. Pumping can resume once the tubing is re-submerged in the water. For natural water sources (e.g., lakes, rivers), it can be helpful to loosely attach the tubing to something in the water using a zip-tie. If that is not possible, a rock or other object can be gently placed over the tubing to secure it, provided the tip of the tubing can be positioned such that it will not draw in sediment.

6. Charge pump batteries 24 h before use. Non-United States outlets require a step-down converter with an outlet adapter.

7. If a Geotech pump (or equivalent) is not used, ensure the filtrate rate does not exceed 5 L/min.

8. Certain power sources may not provide enough power to achieve a flow rate of $\geq 3.8$ L/min. When this is the case, the pump should be run at full speed to achieve the highest flow rate possible. The flow totalizer will provide a less accurate (but still useful) estimate of the volume passed through the ultrafilter as the flow rate decreases. It may also be useful to purge any air from the totalizer at the beginning of the sample collection by briefly orienting the totalizer so the outlet is pointed "up" and visually noting the disappearance or absence of bubbles exiting the totalizer.

9. If you do not have a flow totalizer, you can manually calculate the flow rate using a graduated cylinder or 1 L bottle and a stopwatch.

10. There is no directionality to the ultrafilter. The end ports are color coded for hemodialysis, but there is no difference in functionality for water sampling.

11. The 10 mL pipette gives more control during the backflushing, but it is not necessary. If opting out using of the pipette, make the tubing long enough to place the end directly into the backflush bottle.

12. If the pump used for backflushing does not have a display for the speed setting, the desired pumping rate of 650 mL/min can be determined by pumping water through a length of tubing and measuring the flow rate. This setting can be marked on the pump for future reference. If the Geotech pump is used for backflushing, it requires going above the 650 mL/min setting to achieve pumping. Once pumping is initiated, the dial should immediately be turned back to the 650 mL/min setting.

13. A centrifugation speed up to $4000 \times g$ can be used if there are other analytes in the sample that require higher $g$ forces to pellet.

14. For potable or groundwater samples (i.e., small concentrate volume), the pellet can be split 50:50 to nucleic acid extraction and IMS. For surface water samples (i.e., larger concentrate volume), up to 750 μL can be used for nucleic acid extraction and a portion of the remaining volume can be used for IMS.

15. An empty micropipette tip box makes a suitable storage container for slides. For staining, remove the pipette tip tray and place a folded Kimwipe or paper towel in the bottom of the box. Mist or squirt DI water to moisten the towel. Replace the pipette tip tray to hold the slide. After the slide is stained, remove the moist towel. If the pipette tip box lid is not opaque, wrap the lid with aluminum foil. Store the stained slide in the dark box until microscopy is performed.

16. It is important to minimize the formation of bubbles when placing the coverslip, as they will interfere with examination under the microscope. It is helpful to connect the coverslip with the mounting medium at an angle. Place the long edge coverslip along one end of the slide and lower down at an angle until it is resting on top. Alternatively, lower the slide down onto the coverslip at an angle.

17. For low-quality water samples, or when sample volume is limited, less than 750 μL can be extracted. Add an equal volume of UNEX lysis buffer and 10% Proteinase K (10% of sample volume, not total volume after UNEX buffer is added).

18. Some water concentrates may produce a nucleic acid extract that is not completely recovered from the OneStep column after 1 min. Subsequent spins (1 min at a time) can be performed to recover a larger volume.

19. Because PCR inhibitors are sometimes present, it may be useful to also test template samples diluted 1:10.

20. Cycling times will vary depending on master mix and real-time thermocycler used. Follow the manufacturer's instructions for the appropriate cycling times.

## Acknowledgments

## References

1. Mull B, Hill VR (2012) Recovery of diverse microbes in high turbidity surface water samples using dead-end ultrafiltration. J Microbiol Methods 91(3):429–433. https://doi.org/10.1016/j.mimet.2012.10.001

2. Smith CM, Hill VR (2009) Dead-end hollow-fiber ultrafiltration for recovery of diverse microbes from water. Appl Env Microbiol 75(16):5284–5289. https://doi.org/10.1128/AEM.00456-09

3. Raphael BH, Lautenschlager M, Kahler A, Pai S, Parks BA, Kalb SR, Maslanka SE, Shah S, Magnuson M, Hill VR (2012) Ultrafiltration improves ELISA and Endopep MS analysis of botulinum neurotoxin type A in drinking water. J Microbiol Methods 90(3):267–272. https://doi.org/10.1016/j.mimet.2012.05.020

4. USEPA (2012) Method 1623.1: *Cryptosporidium* and *Giardia* in water by filtration/IMS/FA. Office of Water, Washington, D.C

5. Hill V, Narayanan J, Gallen R, Ferdinand K, Cromeans T, Vinje J (2015) Development of a nucleic acid extraction procedure for simultaneous recovery of DNA and RNA from diverse microbes in water. Pathogens 4:335–354

6. Jothikumar N, da Silva AJ, Moura I, Qvarnstrom Y, Hill VR (2008) Detection and differentiation of *Cryptosporidium hominis* and *Cryptosporidium parvum* by dual TaqMan assays. J Med Microbiol 57(9):1099–1105. https://doi.org/10.1099/jmm.0.2008/001461-0

# Chapter 4

# *Cryptosporidium* Oocyst Purification Using Discontinuous Gradient Centrifugation

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## Abstract

Many laboratory studies in cryptosporidial research require a source of purified oocysts. Sources can include experimentally infected laboratory animals or from samples collected from naturally infected animals and from clinical cases of human cryptosporidiosis. Purification of oocysts can be accomplished with readily available laboratory equipment including tabletop centrifuges and microcentrifuges. Following purification, oocysts can be stored in antibiotic-supplemented buffers or in 2.5% aqueous potassium dichromate for over 6 months. Ultimately, oocyst viability and infectivity decline to less than 10% after 1 year, so if isolates are expected to be maintained, serial passage in a suitable host at ≤6-month intervals is recommended. Oocysts purified as described in this chapter are suitable for animal infection studies, cell culture studies, and a wide range of molecular biological studies, environmental studies, drug testing, and disinfection studies.

**Key words** *Cryptosporidium*, Oocyst, Purification, Isolation, Sucrose, Sheather's solution, Cesium chloride, Centrifugation, Gradient

## 1 Introduction

The method proposed here describes a bulk stool processing method for cryptosporidial oocyst isolation and purification using discontinuous sucrose and cesium chloride gradients (*see* Fig. 1). The method is scalable, having been adapted for small-scale purification of individual mouse stool samples [1], as well as bulk stool processing from experimentally infected bovine calves [2, 3]. Using essentially the same conditions and reagents at the proportions described below, most stool samples can be processed with success. A known caveat is that clinical human stools can vary significantly in composition, affecting the efficiency of the purification process. Oocysts purified using these gradient techniques are suitable for immediate use or further purification and/or sorting using flow cytometry-based methods as well as immunomagnetic bead separation. This method does not include a "defatting" step using an
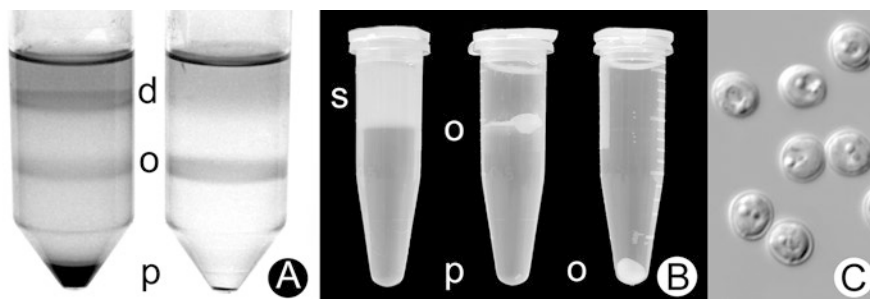
**Fig. 1** Panels (a) and (b) illustrate the progressive gradient techniques used to purify *C. parvum* oocysts. Panel (a) shows primary (*left*) and secondary (*right*) discontinuous sucrose gradients after centrifugation with light debris (d) at the interface between the sample and the STP 1:4 layer, the oocysts (o) at the interface between the STP 1:4 and STP 1:2 layers, and the pellet (p) of dense debris. Panel (b) shows examples of the cesium chloride step gradients: the first tube (left) shows the sucrose gradient purified oocyst sample (s) layered over the cesium chloride column before centrifugation; the second tube (*middle*) shows the same tube after centrifugation with the oocysts (o) at the interface between the sample and the cesium chloride and a small pellet (p) of dense debris at the bottom; the third tube (*right*) shows the purified pellet of oocysts (o) after the saline wash step. Panel C shows a differential interference contrast (DIC) view of representative *C. parvum* oocysts purified using the sucrose and cesium chloride gradient techniques

organic solvent (e.g., ethyl acetate, similar to methods for stool sedimentation before O&P evaluation), although some laboratories include it. If organic solvents are used, the useful shelf life of the oocysts may be shortened. If oocysts are to be sanitized before experimental use (e.g. "bleach" treated), we recommend delaying this step until just before use as the treatment is likely to shorten the shelf-life of the oocysts. Also, consider "sanitized" oocysts to be partially compromised and may not be suitable for disinfection studies as the sanitizing step is likely to alter the oocyst wall and make oocysts more susceptible to the experimental disinfectant treatment.

## 2    Materials

1. Large glass or plastic funnels.
2. Stainless steel sieves: size 60 and 20 mesh.
3. Stainless steel or dishwasher safe buckets (sized to match sieve diameter).
4. Stainless steel forceps.
5. Small plastic pan.
6. Plastic spatulas.
7. Ring-stand and holder (if using funnel).
8. Small wash bottles: saline, deionized water.
9. Paper towels.

10. Polypropylene bottles (1 and 4 L).

11. Repeater pipet.

12. 250 mL polycarbonate centrifuge bottles (transparent).

13. 200 mL polypropylene conical centrifuge bottles (translucent).

14. 50 mL polypropylene conical centrifuge tubes (transparent).

15. 5, 10, 50, and 100 mL disposable pipets.

16. 3 mL disposable transfer pipet.

17. Micropipettes and tips.

18. Deionized water ($dH_2O$).

19. Phosphate buffered saline (PBS): 0.025 M, pH 7.2.

20. Saline: 0.85% NaCl w/v aqueous.

21. Saline supplemented with 0.1% Tween 80 (Saline/Tween).

22. Antibiotic mix: e.g., Penicillin, Streptomycin, Fungizone, prepared at 1× working concentration in PBS.

23. Potassium dichromate ($K_2Cr_2O_7$) solutions: 2.5% and 5.0% w/v in deionized water.

24. Tween 80 or Tween 20.

25. Sheather's sucrose solution: 500 g sucrose, 320 mL $H_2O$, 9 mL aqueous phenol (85%).

26. Sheather's/Tween/PBS (STP) 1:2 solution (1.103 g/L density): 300 mL Sheather's solution, 600 mL PBS, 9 mL Tween 80 (*see* **Note 1**).

27. STP 1:4 solution (1.064 g/L density): 200 mL Sheather's solution, 800 mL PBS, 9 mL Tween 80 (*see* **Note 1**).

28. Antifoam Y-30 emulsion.

29. Bobbitt valves.

30. Aspirator bottles (1 L) with tubulation (bottom hose outlet) fitted with Bobbitt valve and shortened pipet (use silicone tubing) (*see* Fig. 2 and **Note 6**).

31. Vacuum aspirator flask (4 L) with vacuum flask trap (typically 1 L or 500 mL) outfitted with disposable Pasteur pipet inserted in silicone tubing connected to glass tubing in rubber stopper of aspirator flask.

32. Hydrophobic in-line filter for vacuum aspirator to protect vacuum system from contamination.

33. Carboy (~20 L) with spigot and Bobbitt valve; filled with deionized water ($dH_2O$) (*see* Fig. 2).

34. Hot water bath set at ~55 °C (if decontaminating dishwasher not available).
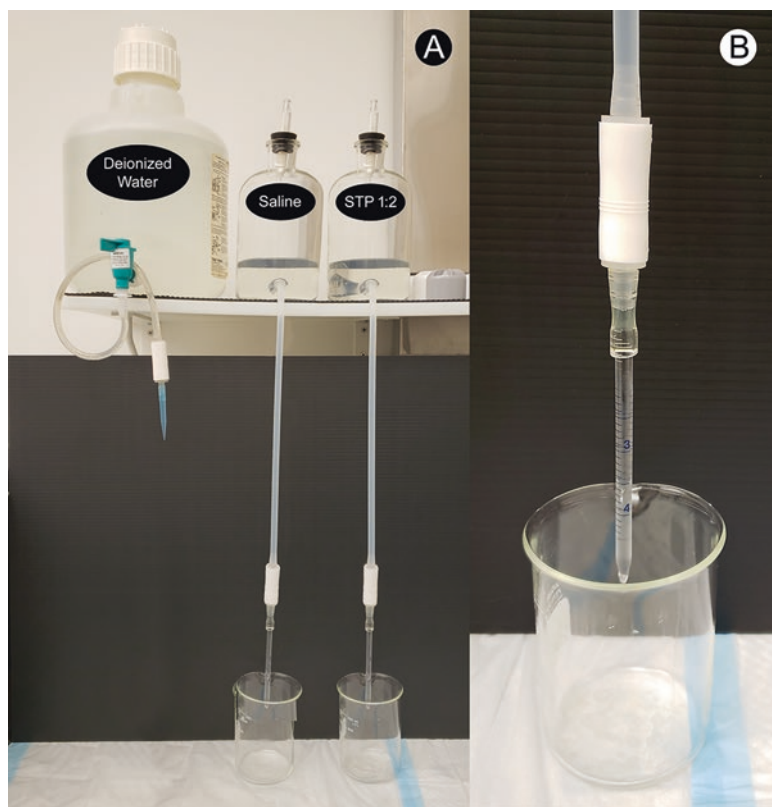
**Fig. 2** Assembled reagents used in purification of cryptosporidial oocysts. Reservoirs are located on shelf above the workbench, so reagents can be delivered to the bottles and tubes at bench height. Solid black background included to provide contrast behind tubing and Bobbitt valves, but in use, the workbench should be ventilated (ideally a laminar flow workbench or equivalent). Carboy on left is filled with deionized water and is used for sample dilution and washing of gradient components from samples. Note that Bobbitt valve can be fitted with a disposable 1 mL micropipette tip for small volume use (cesium chloride gradients). Two aspirator bottles with tubulations (bottom hose outlets) are fitted with Bobbitt valves and shortened pipets (use silicone tubing). Saline is used for washing gradient components from samples and for sample suspension and storage. The STP 1:2 reservoir is used to underlay the STP 1:4 volume in gradient bottles or tubes

35. Five gallon or larger tank for heat disinfection of waste (or equivalent).

36. Stir plate and stir bar (large, suitable for the 5-gallon tank used to disinfect waste).

37. Immersion heater or heat source suitable for use with disinfection tank.

38. Heat controller for immersion heater (typically includes a temperature sensor immersed in the disinfection tank with fail-safe circuitry if overheating is detected).

39. Timer for heating circuit (each system will need to be calibrated to achieve the target temperature and to hold it for the necessary time to achieve inactivation (i.e. ~55 °C for ≥1 h).

40. 5–15 gallon polypropylene barrels (or otherwise suitable) for disposal of heat-treated chemical waste.

41. Disposable counter pads (absorbent with plastic-backed, waterproof shell).

42. Cesium chloride solution (sp. gr. 1.15): 21.8 g CsCl, 103.3 mL dH$_2$O.

43. Tabletop centrifuge (swing bucket rotor, refrigerated, must reach ~3200 × $g$).

44. Microcentrifuge (refrigerated, must reach ~13,200 rpm or 16,000 × $g$).

45. Appropriate personal protective equipment (gloves, lab coat, safety glasses, etc.).

46. Biosafety level 2 workbench (laminar flow system recommended).

# 3   Methods

*See* Fig. 3 for schematic flowchart of purification strategy.

**3.1  Safety Recommendations When Working with Fecal Samples Containing Oocysts**

1. Gloves, safety glasses, and a lab coat are to be worn at all times. Consider wearing a full-face splash shield if working on open counter.

2. When working with fluids and tissues containing oocysts, dispensing bottles with undiluted commercial hydrogen peroxide (identified on bottle as 3% hydrogen peroxide or "10 vol" hydrogen peroxide) should be available in case of spills.

3. All instruments that come in contact with oocyst-contaminated materials should be placed in a stainless-steel pan to be autoclaved or sanitized in the appropriate cycle in the laboratory dishwasher. Instruments that cannot be autoclaved may be immersed in a heated water bath (~55 °C) for 60 min or placed in 3% hydrogen peroxide for a period of 30 min (minimum to 2 h maximum) before washing. Wash with a commercial disinfecting detergent before rinsing with tap and finally deionized water.

4. All disposable labware that comes into contact with oocyst-contaminated tissues or fluids should be placed in autoclave bags, and autoclaved as per local guidelines prior to disposal.

5. In case of spills, use absorbent disposable paper towels to remove contaminating material and transfer to autoclave bag. Flood contaminated areas with 3% hydrogen peroxide for a
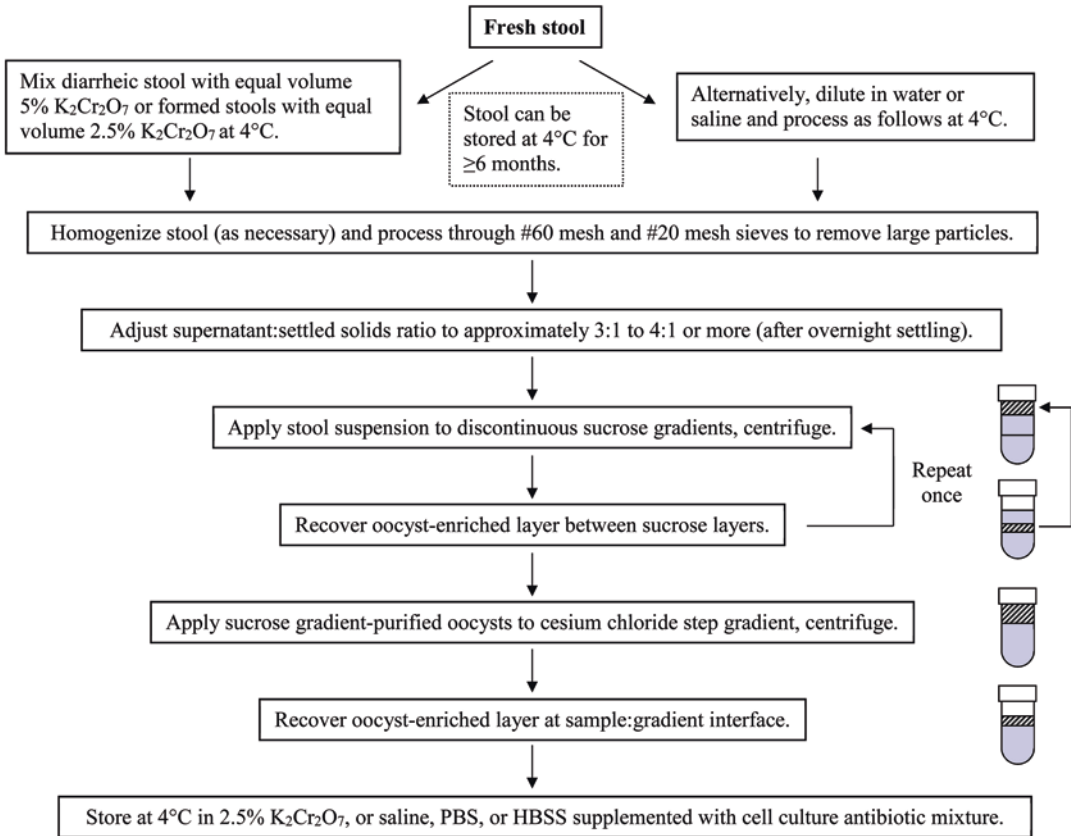
**Fig. 3** Schematic flowchart summarizing the purification strategy for *Cryptosporidium* oocysts useful in laboratory studies

minimum of 30 min (add additional hydrogen peroxide as necessary to prevent drying). Excess hydrogen peroxide and contaminating fluid can be absorbed with disposable paper towels and transferred to an autoclave bag. Allow surfaces to thoroughly dry before use.

*3.2 Sieving Stool Samples (See* **Note 2***)*

1. Cover workspace with plastic disposable counter pad (plastic backed pad) to protect counter. Assemble the sieving apparatus by inserting the #20 sieve (850 μm mesh) into a small (~1 gallon) stainless steel bucket ("primary sieve") and the #60 (250 μm mesh) sieve on a second stainless steel bucket ("secondary sieve"). Place the bucket in the large plastic tray (if used) to catch spills (*see* Fig. 4).

2. Pour the "raw" stool suspension/mixture through the screen into the bucket. Stop pouring when the liquid stops flowing freely through screen or bucket is approximately 3/4 "full."

**Fig. 4** Example of stainless steel buckets paired with stainless steel sieves for sieving stool samples. Note the use of forceps to create a break at the interface between the buckets and the sieves (prevents vapor seal/lock during sieving, otherwise sample spattering can occur when lifting the sieves). If buckets have pouring spouts, the forceps are not needed. Disposable plastic spatula for assisting sieving of stool samples (along with water or saline spray bottles)

3. Use a plastic scraper and deionized water sprayed from a wash bottle to gently push small particulate matter through the sieve. Large matter is removed with the spatula and discarded into the hazardous waste container.

4. If the sieve becomes clogged with solid material, replace with a clean sieve. Continue use until finished or pause while sieves are cleaned in dishwasher.

5. Continue sieving the feces until the day's collection has been processed through the #20 sieve. Transfer the sieve to the sanitizing dishwasher for processing/cleaning.

6. Rinse out the original feces bottle with a small amount of saline and set aside (do NOT pour bleach inside; these bottles will be cleaned and decontaminated in the dishwasher).

7. Pour the #20 sieved feces through the #60 sieve into the bucket. Stop pouring when the liquid stops flowing freely through screen or bucket is approximately 3/4 "full."

8. When the feces stop flowing freely through the #60 sieve, use the plastic spatula and saline sprayed from the wash bottle to gently push small particulate material through. Large matter is removed with the spatula and discarded into the biohazard waste container.

9. If sieve becomes clogged with solid material, repeat cleaning procedure as in **step 3**.

10. Continue sieving the feces until the day's collection has been processed through the #60 sieve. Transfer the sieve to the dishwasher (*see* **step 4**).

11. If the sieved fecal sample is to be condensed immediately, go to the next section (Subheading 3.3). If the sample is to be stored temporarily, transfer the sieved feces to a large bottle (typically one gallon wide-mouth polypropylene bottles), label as "sieved" and identify the sample appropriately (e.g., *C. parvum* isolate, calf ID, and date post-infection). Store bottle(s) at 4 °C in the laboratory refrigerator.

12. Stainless steel buckets are cleaned and sanitized in the laboratory dishwasher.

13. Insert spatulas, scrapers, and other tools in the laboratory dishwasher for cleaning and disinfection.

14. If contaminated, discard counter pad(s) into biohazard waste container. Replace with new disposable pad(s).

15. If countertop soiled, wipe counter with paper towels to remove bulk waste, spray/flood the cleaned surface with 3% hydrogen peroxide, allow peroxide to stand for ≥30 min (do not allow to dry, reapply peroxide as necessary). Dry, rinse with tap water, dry.

*3.3 Condensing Sieved Stool Samples*

1. If previously stored, mix bottle(s) of raw, sieved feces to suspend fecal sediment.

2. Pour approximately 700–750 mL into each 750 mL centrifuge bottle and centrifuge at a ~4000 rpm ($3200 \times g$) in refrigerated (if available) table-top centrifuge (with swing buckets) for 10 min at 4 °C.

3. Aspirate supernatant into vacuum flask or pour/decant supernatant into waste tank. If a large volume of sieved stool remains to be condensed, add sieved stool suspension to the bottles (on top of the pellet(s)) and centrifuge as before. Aspirate/pour/decant supernatant as before. Depending on pellet volumes, pool pellets into one or more centrifuge bottles and dilute to 750 mL with deionized water.

4. Centrifuge as before for 10 min. Aspirate or decant supernatant. Pool and disperse all pellets from 1 day's collection (or individual sample) into a single 1-L bottle and dilute to approximately 800 mL with 2.5% potassium dichromate or 0.85% saline. Label bottle with appropriate identifying details as before, including sieved status. Store sieved and condensed feces at 4 °C (*see* **Note 3**).

5. Disinfect pooled liquid waste using heat (*see* Subheading 3.7).

*3.4 Primary Oocyst Purification Using Discontinuous Sucrose Gradients (See* **Notes** 4 *and* 5*)*

1. Four 250 mL bottles equal one primary run (compared to original tube method employing 50 mL tubes [3]). Use four 250 mL transparent polycarbonate bottles per gradient centrifugation run.

2. Dispense 80 mL of 1:4 STP into each 250 mL bottle using a graduated cylinder. If not already marked, mark the bottle with permanent marker to indicate the top (meniscus) of the 1:4 layer.

3. Carefully dispense 80 mL of 1:2 STP under the 1:4 layer using the Bobbitt valve/pipet connected to the 1:2 STP reservoir (Fig. 2). The Bobbitt valve allows precise flow control when underlaying the gradient solutions so a sharp interface with minimal mixing of the STP solutions can be produced. If not already marked, mark the bottle to indicate the "new" top (meniscus) of the 1:4 layer overlaying the 1:2 layer.

4. Mix (or vortex) fecal sample(s) well. Using a 50 mL disposable pipet and an automatic pipet aid, carefully transfer/overlay 40 mL of the fecal sample(s) onto prepared gradients in each of four bottles. This step will require care to avoid mixing the sample into the 1:4 STP layer.

5. Centrifuge bottles at a setting of $1000 \times g$ (~2050 rpm) in a refrigerated, tabletop centrifuge for 25 min at 4 °C.

6. Using a vacuum flask assembly (with antifoam), carefully aspirate the upper sample layer (orange if dichromate, clear if saline) down to approximately halfway into the 1:4 layer.

7. Collect the oocyst-enriched layer (1:4/1:2 interface) by pipetting (100 mL disposable pipet) the supernatant of each bottle down to about $1/2$ cm from the pellet (~95–100 mL). Transfer/dispense the liquid into one 200 mL conical polypropylene bottle (1 conical bottle for each 250 mL gradient bottle). Repeat for each bottle. (Set gradient bottles aside: at the end of purification run(s), residual supernatant and pellets will be aspirated from original bottles into the waste flask.) Place empty original gradient bottles into the dishwasher (or hot water bath) for disinfection/cleaning.

8. Fill conical bottles containing oocyst-enriched fractions to the ~190–200 mL mark (shoulder of bottle) with $dH_2O$. Balance bottles in centrifuge carrier adapters (add $dH_2O$ or saline as necessary), cap tightly and mix.

9. Centrifuge bottles at a setting of $1500 \times g$ (minimum) to $3000 \times g$ (latter preferred) for 10 min at 4 °C.

10. Aspirate supernatants into waste flask and transfer pellets from two conical bottles into one 50 mL conical centrifuge tube. Repeat for the second pair of conical bottles.

11. Fill each tube to the 45–50 mL mark with $dH_2O$, mix thoroughly and centrifuge for 10 min using a setting of $1500 \times g$ (minimum) to $3000 \times g$ (latter preferred) in a refrigerated table-top centrifuge at 4 °C.

12. Aspirate supernatants, add a small volume (~5 mL) of saline or 2.5% $K_2Cr_2O_7$, thoroughly disperse/resuspend pellets using a disposable transfer pipet, dilute to 40 mL with 0.85% saline or 2.5% potassium dichromate. If processing calf stool, consider labeling each tube with these data: source ID/day post-infection/primary run ID/current date/initials and the primary symbol. For example:

$$6IA7 \quad C \quad 1° \quad 8/15/18 \quad XXX$$

6 = Passage number for isolate.
IA = Iowa isolate designation.
7 = Day post-infection that identifies this subsample.
C = Tube contents represent primary run C.
1° = Tube contents represent a product of a primary gradient purification.
8/15/18 = Current date (date of gradient run).
XXX = Initials of technician who performed gradient run.

13. Store labeled oocyst preparations at 4 °C.

14. Enumerate oocyst concentration using hemocytometer (*see* Subheading 3.6). Record tube ID and oocyst concentration in the logbook. (Oocyst numbers are recorded as **NUMBER** $\times 10^6$/mL.)

15. Disinfect pooled liquid waste using heat (*see* Subheading 3.7).

*3.5 Secondary Oocyst Purification Using Discontinuous Sucrose Gradients (See* Notes 4 *and* 5)

1. Select four tubes of primary sucrose gradient purified oocysts for secondary purification. Be careful to note sample ID and primary gradient run date on tube. If stored in 2.5% $K_2Cr_2O_7$, mix tubes thoroughly and centrifuge for 10 min at a setting $3000 \times g$ in the refrigerated tabletop centrifuge. Aspirate supernatants thoroughly disperse/resuspend pellets into a small volume (~ 5 mL) of saline using a disposable transfer pipet (3 mL). Dilute oocyst suspensions to 40 mL with Saline/Tween.

2. Dispense 80 mL of 1:4 STP into each 250 mL bottle using a graduated cylinder. If not already marked, mark the bottle with permanent marker to indicate the top (meniscus) of the 1:4 layer.

3. Carefully dispense 80 mL of 1:2 STP under the 1:4 layer using the Bobbitt valve/pipet connected to the 1:2 STP reservoir (Fig. 2). If not already marked, mark the bottle to indicate the "new" top (meniscus) of the 1:4 layer overlaying the 1:2 layer.

4. Overlay the sucrose gradients each with 40 mL oocyst suspension (one tube/bottle, and note each bottle accordingly). Tracking bottles can be simplified if using bottle adapters with labels.

5. Centrifuge bottles at a setting of $1000 \times g$ (2050 rpm) in the refrigerated, tabletop centrifuge for 25 min at 4 °C.

6. Using a vacuum flask assembly, aspirate the upper sample layer (typically clear if suspended in saline) down to approximately half way into the 1:4 layer.

7. Collect the oocyst-enriched layer (1,4/1,2 interface) by pipetting (100 mL pipet) the supernatant of each bottle down to about 1/2 cm from the pellet (~95–100 mL). Transfer/dispense the liquid into one 175 mL conical polypropylene bottle (one conical bottle for each 250 mL gradient bottle). Repeat for each bottle. Aspirate pellets from original bottles into waste flask. Place original gradient bottles into the dishwasher for disinfection/cleaning.

8. Fill bottles containing oocyst-enriched fractions to the ~190–200 mL mark (shoulder of bottle) with $dH_2O$. Balance bottles in centrifuge carrier adapters (add $dH_2O$ or saline as necessary), cap tightly and mix.

9. Centrifuge bottles at a setting of $1500 \times g$ to $3000 \times g$ (latter preferred) for 10 min at 4 °C.

10. Aspirate supernatants and transfer pellets from two conical bottles into one 50 mL polypropylene tube. Repeat for the second pair of conical bottles.

11. Fill each tube to the 45 mL mark with $dH_2O$ or saline, mix thoroughly, and centrifuge at a setting of $1500 \times g$ to $3000 \times g$ (latter preferred) for 10 min at 4 °C.

12. Aspirate supernatants, add a small volume (~5 mL) of saline or 2.5% $K_2Cr_2O_7$, thoroughly disperse/resuspend pellets using a disposable transfer pipet, dilute to 40 mL with 0.85% saline or 2.5% $K_2Cr_2O_7$. Each tube will now be labeled with these data: calf ID/day post-infection/primary run source ID/current date/initials and the secondary symbol. For example:

$$6IA7 \ \ C/D \ \ 2° \ \ 8/15/18 \ \ XXX$$

6 = Passage number for isolate.

IA = Iowa isolate designation.

7 = Day post-infection that identifies this subsample.

C/D = Tube contents represent pooled material from primary runs C & D.

2° = Tube contents represent a product of a secondary gradient purification.

8/15/18 = Current date (date of gradient run).

XXX = Initials of technician who performed gradient run.

13. Store labeled oocyst preparations at 4 °C.

14. Enumerate oocyst concentration using hemocytometer (*see* Subheading 3.6). Record tube ID and oocyst concentration in the logbook. (Oocyst numbers are recorded as **NUMBER** $\times 10^6$/mL.)

15. Disinfect pooled liquid waste using heat (*see* Subheading 3.7).

*3.6   Tertiary Oocyst Purification Using Discontinuous Cesium Chloride Gradients (See* Notes 4 *and* 5)

1. Select four tubes of secondary sucrose gradient purified oocysts for cesium chloride purification. Be careful to note sample ID and secondary Sheather's run date on tube. Mix tubes thoroughly and centrifuge for 10 min at a setting of $1500 \times g$ (minimum) to $3000 \times g$ (latter preferred) in the table top centrifuge. If oocysts were stored in 2.5% potassium dichromate, wash out dichromate with saline or PBS using centrifugation (do not apply dichromate to top of CsCl gradient, otherwise dichromate crystals may form). If oocysts were stored in saline or a similar buffer, aspirate supernatant and resuspend each pellet into 3 mL of saline.

2. Prepare and label six 1.7 mL clear siliconized (low binding) graduated microcentrifuge tubes for each tube of secondary oocyst preparations.

3. Dispense 1 mL of CsCl gradient solution into each 1.7 mL siliconized microcentrifuge tube. (This is simplified by using a "repeater" pipette.)

4. Carefully overlay each CsCl gradient with 0.5 mL of the saline/oocyst suspension, and close the cap tightly.

5. Centrifuge the tubes at ~13,200 rpm ($16,000 \times g$) for 3 min at 4 °C using the high-speed microcentrifuge. If available, use minimal to mild braking during deceleration.

6. Carefully remove the microcentrifuge tubes and collect the oocyst-containing layer at the interface between the sample and the CsCl layer: e.g., using a micropipette, draw 1.0 mL from the top of the tube (collecting the original 0.5 mL saline and 0.5 mL of the CsCl layer).

7. Transfer each 1.0 mL oocyst suspension into a clean 1.7 microcentrifuge tube.

8. Dilute each tube to ~1.5 mL with saline or deionized $H_2O$ and centrifuge the tubes at ~13,200 rpm ($16,000 \times g$) for 3 min at 4 °C using the microcentrifuge.

9. Aspirate and discard supernatants using vacuum aspirator assembly. Dispense 0.5 mL of saline into the first tube of each set of six tubes. Carefully resuspend pellets with only 0.5 mL of saline or deionized $H_2O$ and pool each set of six microcentrifuge tube suspensions into one microcentrifuge tube. Rinse each set of tubes with another 0.5 mL of saline or deionized

H$_2$O and pool with the first 0.5 mL suspension. There should be slightly more than 1 mL of suspension in each of four tubes. Dilute suspensions to 1.5 mL mark with saline or deionized H$_2$O.

10. Centrifuge the tubes at ~13,200 rpm (16,000 × $g$) for 3 min at 4 °C using the microcentrifuge.

11. Aspirate and discard supernatant. If there are any orange dichromate crystals, resuspend the pellets in 1.0 mL of saline and repeat centrifugation. Wash the pellets carefully until crystals are gone.

12. Resuspend each pellet to 1.0 mL with saline or PBS supplemented with antibiotics mix or use 2.5% potassium dichromate. Each tube will now be labeled with these data: sample ID/day post-infection/secondary run source ID/current date/initials and the "tertiary" symbol. For example:

    6IA7  C / D  3°  8 / 15 / 18  XXX

    6 = Passage number for isolate.
    IA = Iowa isolate designation.
    7 = Day post-infection that identifies this subsample.
    C/D = Tube contents represent pooled material from primary runs C & D.
    3° = Tube contents represent a product of a secondary gradient purification.
    8/15/18 = Current date (date of gradient run).
    XXX = Initials of technician who performed gradient run.

13. Store labeled oocyst preparations at 4 °C.

14. Enumerate oocyst concentration using hemocytometer (*see* Subheading 3.6). Record tube ID and oocyst concentration in the logbook. (Oocyst numbers are recorded as **NUMBER** × 10$^8$/mL.)

15. Disinfect pooled liquid waste using heat (*see* Subheading 3.7).

*3.7 Heat Disinfection of Liquid Waste Contaminated with Oocysts (See Note 6)*

1. Pour supernatant/pellet waste from the centrifuge bottles or vacuum aspirator waste flask into the bulk waste container.

2. Pool waste until the volume reaches the minimum for heat inactivation or has been supplemented with tap water to reach the minimum volume (e.g., 5 gallons—the minimum volume that covers the working surface of the immersion heating element).

3. Assemble the Teflon-coated heating rod (or equivalent heat source), temperature controller set at ~55 °C, magnetic stir plate (or equivalent), appropriate container top, and power timer for the waste tank. *Be sure sample is mixed during heat inactivation to assure uniform heat throughout the waste volume*.

4. Disinfect the supernatant/pellet suspension with appropriate heat treatment. For example, a five-gallon tank outfitted with a 500 watt immersion heater takes approximately 3 h to inactivate 5 gallons of waste. That is, the time it takes for the liquid to reach the target temperature (~55 °C) and remains there for at least 1 h. Inactivated waste can be transferred after the heating element has been off for at least 30 min (to allow element to cool). After cooling, transfer the disinfected waste into an appropriate disposal container (e.g., 5–15 gallon polypropylene drum).

5. Dispose of waste appropriately. Waste with potassium dichromate or cesium chloride will require hazardous chemical waste disposal.

*3.8   Enumerating Oocysts Using the Hemocytometer (See **Fig. 5**)*

1. Make appropriate dilution of oocyst sample in saline or deionized water (e.g., undilute to 1/5 dilution of primary gradient purified oocysts, 1/10 to 1/25 dilution of secondary gradient purified oocysts, or 1/50 to 1/100 dilution of tertiary gradient purified oocysts). Dilution may be adjusted up or down as necessary.

2. Place 30 mm × 40 mm coverslip over the center position of the hemocytometer. Carefully pipet diluted oocyst suspension into the counting chamber. Do not overfill. Allow cell suspension to settle (~ 30 s to 1 min).

3. Use phase contrast or bright field settings. Locate the center portion of the ruled area of the counting chamber with the 10× objective and adjust focus.

4. Count the number of oocysts using the 40× objective within the center square of the grid area's nine large squares (Fig. 5, *grid system center*). Count the oocysts in or touching the top and left borders of each square. Do not count oocysts touching the right or bottom borders of each square. Typically, count the number of oocysts in five small squares within the center large square (*see* Fig. 5, *grid square 5 × 5 in center*). A minimum of 77 oocysts should be counted within the five squares to give an acceptable approximation of the number of cells/mL. For cell counts <77, use a lower dilution of oocyst suspension or count all 25 squares (adjust formula below as necessary).

5. Calculate the number of oocysts/mL using the following formula:

$$\left(\#\,\text{of oocysts counted in 5 small squares} \times 5\right) \times r \times 10^4 = \#\,\text{oocysts}\,/\,\text{mL}.$$

where
Dilution factor of oocyst suspension = $r$ (inverse of dilution, e.g., inverse of 1/5 = 5).
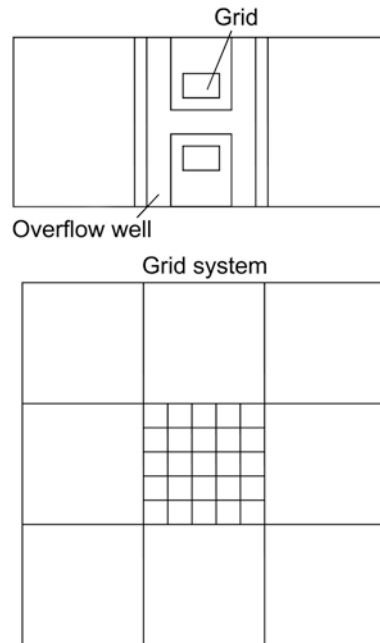Dilution factor of hemocytometer = $10^4$.

**Fig. 5** Schematic illustration of hemocytometer and hemocytometer grid system

6. Discard disposable cover glass into "Sharps" bottle, rinse hemocytometer with 70% alcohol and tap water, wipe dry with disposable "wipe" or air dry, or immerse in hot water bath (55 °C for 1 h). Alternatively, immerse hemocytometer in 3% hydrogen peroxide for ≥30 min, rinse with deionized water and dry.

# 4    Notes

1. Using a positive displacement pipet or syringe, add Tween 80 gradually with constant stirring until dissolved. Store at room temperature in sealed bottles.

2. Typical pattern is to sieve a portion of the raw stool through successive sieves (~3+ L per centrifuge run) and fill centrifuge bottles for condensing. Repeat until all stool has been sieved and condensed. If sieves fit tightly on buckets (no air space breaks, i.e., pouring spout), add forceps/tweezers or other spacer to achieve an air gap (*see* Fig. 4), otherwise sieve may form a liquid seal during sieving and cause spattering/contamination of the workspace when lifted to break the seal.

3. Rule of thumb regarding suspension of fecal pellet: wait several hours or overnight and check the ratio of settled solids to total volume. The settled solids should represent approximately 1/4 to 1/5 the total volume of 2.5% potassium dichromate or

0.85% saline. Adjust volume as necessary to achieve target ratio. Recovery efficiencies improve with added dilution, but require more gradient runs to complete purification.

4. Add a few drops of Antifoam Y-30 to the 4 L vacuum aspirator flask before starting a gradient purification run to prevent excessive foaming during the vacuum aspiration steps. For bulk purification runs, a typical sequence of three consecutive 4-bottle runs will generate just less than 4 L of waste which will fit in the aspirator flask. If antifoam is not used, foaming may cause a portion of the waste to be drawn into the small, vacuum trap flask between the aspirator flask and the vacuum supply. Be sure to have a hydrophobic in-line filter installed after the trap and before the vacuum valve to prevent contamination of the vacuum supply. Consider using a disposable 50 mL tube as temporary "docking station" for disposable Pasteur pipets attached to the 4 L vacuum aspirator flask via silicone tubing. The 50 mL tube and pipet can be changed with each sample or succession of gradient runs. Carefully balance bottles in the centrifuge bucket adapters to assure the centrifuge runs will not be interrupted by an "out-of-balance" error or imbalance that causes mixing of bottle contents (prepared gradients) during acceleration/deceleration.

5. While we track each gradient run to monitor purification performance, the method described can be simplified by pooling oocyst pellets together at the end of each gradient process (i.e., all of the primary sucrose gradient pellets can be pooled together after all of the stool material is processed). This way, only one count will need to be made before proceeding to the next gradient technique. Some laboratories may wish to keep oocysts separate based on the day shed post-infection. Alternatively, oocysts from an entire experimental shedding period may be pooled together into a single "lot" for subsequent studies. We have not found significant differences among oocyst generated between days 5 and 12 post-infection from experimentally infected bovine calves.

6. For safety reasons, heating systems should have overheating circuitry to shut off power in case of overheating. Use of a timer with a manual "ON" feature and automatic "OFF" feature will reduce the risk that heating cycles accidentally run too long if left unattended. Heating cycles can be timed routinely to accomplish the inactivation within a window of time for a given container/volume/heat source. A temperature of 55 °C and time of 1 h is used to minimize the risk of handling large volumes of hot liquid waste (e.g., autoclaving) while also assuring cryptosporidial oocysts are inactivated. While Pasteurization temperatures are recognized to inactivate oocysts, published data and our own quality control data support the use of the target temperature for disinfection [4–6].

## Acknowledgments

## References

1. Arrowood MJ, Hurd MR, Mead JR (1995) A new method for evaluating experimental cryptosporidial parasite loads using immuno-fluorescent flow cytometry. J Parasitol 81(3): 404–409

2. Arrowood MJ, Donaldson K (1996) Improved purification methods for calf-derived *Cryptosporidium parvum* oocysts using discontinuous sucrose and cesium chloride gradients. J Eukaryot Microbiol 43(5):89

3. Arrowood MJ, Sterling CR (1987) Isolation of *Cryptosporidium* oocysts and sporozoites using discontinuous sucrose and isopycnic Percoll gradients. J Parasitol 73:314–319

4. Anderson BC (1985) Moist heat inactivation of *Cryptosporidium* sp. Am J Public Health 75:1433–1434

5. Fujino T, Matsui T, Kobayashi F, Haruki K, Yoshino Y, Kajima J, Tsuji M (2002) The effect of heating against *Cryptosporidium* oocysts. J Vet Med Sci 64(3):199–200

6. Harp JA, Fayer R, Pesch BA, Jackson GJ (1996) Effect of pasteurization on infectivity of *Cryptosporidium parvum* oocysts in water and milk. Appl Environ Microbiol 62(8): 2866–2868

# Chapter 5

# Measuring *Cryptosporidium* Serologic Responses by Multiplex Bead Assay

## Jeffrey W. Priest and Delynn M. Moss

## Abstract

For more than 35 years, various assay formats have been used to detect *Cryptosporidium*-specific antibodies in human and animal sera. *Cryptosporidium parvum* 17- and 27-kDa antigens, identified from invasive sporozoites, have been used in serologic antibody assays to identify individuals infected in outbreaks of diarrheal disease caused by this protozoan parasite and to monitor exposures in communities. During infection, immunoglobulin (Ig) A, IgM, and IgG responses are elicited by these immunodominant antigens, and the parasite-specific Ig responses diminish following the resolution of infection. Using the recombinant forms of the 17- and 27-kDa *C. parvum* antigens and the relatively recently developed multiplex bead assay (MBA), data from serologic antibody responses can be economically and efficiently acquired, especially when the *Cryptosporidium* assays are integrated with assays for antibody responses to antigens from other pathogens monitored in community-wide or nation-wide serosurveys. Here we describe the coupling of the *C. parvum* recombinant antigens to carboxylated polystyrene beads, the data acquisition and analysis of IgG antibodies bound to the coupled beads, and the quality control methods required for data validation using the Luminex/MBA system.

**Key words** *Cryptosporidium*, Multiplex bead assay, Immunoglobulin G responses, 17-kDa antigen, 27-kDa antigen

## 1 Introduction

In many parts of the world, the diarrheal disease burden contributed by enteric parasites such as *Cryptosporidium* spp. is substantial, yet information on the prevalence and distribution of these diseases is limited because of the significant costs associated with stool collection and immunoassay- or polymerase chain reaction (PCR) assay-based detection methods [1, 2]. Serologic antibody detection assays for *Cryptosporidium* spp. have been used for more than 35 years (reviewed in [3]), and the identification of the immunodominant 17- and 27-kDa antigens led to improved enzyme-linked immunosorbent assay (ELISA) and enzyme-linked immunoelectrotransfer blot (EITB) assay techniques. These assays were used to confirm *Cryptosporidium* spp. as the causative agent

of diarrheal disease outbreaks that occurred aboard a U S Coast Guard cutter in 1993 (caused by onboard contaminated water) [4] and at a 1997 holiday party (caused by the consumption of contaminated food) [5, 6]. Further, these methods, despite their considerable costs and required expertise, have been useful in the monitoring of *Cryptosporidium* spp. exposures using cross-sectional samples from the communities [7–11], in the identification of *Cryptosporidium* spp. infections using longitudinal samples from a volunteer oocyst-feeding study [12], from a cohort of US HIV patients [13], and from outbreaks or hyperendemic communities [14–17]. Identification of *Cryptosporidium* spp. infections using longitudinal or paired samples is possible because the immunoglobulin (Ig) responses to these antigens initially increase during infection and then decrease after infection resolution. For a very short window of time, IgA primarily reacts to the 17-kDa antigen, and IgM primarily reacts to the 27-kDa antigen for a somewhat longer window of time [4, 12, 18]. Compared to the IgM and IgA responses, IgG responses to both the 17- and 27-kDa antigens are long-lived, and the IgG response to the 27-kDa remains elevated for a longer period of time than the IgG response to the 17-kDa antigen [4, 5, 12, 18, 19]. Approximately, 21% of the US population between 6 and 49 years of age are positive for IgG antibodies to both antigens [20].

For serology using the more economical multiplex bead assay (MBA), we have found that antibody assays using the recombinant forms of the 17-kDa (Cp17) and 27-kDa (Cp23) *C. parvum* antigens can be integrated with antibody assays using antigens from other enteric pathogens such as bacteria, protozoans, and viruses in addition to antigens from pathogens that cause neglected tropical, vector-borne, and vaccine-preventable diseases [5, 21–27]. Because the MBA conveniently allows the simultaneous acquisition of data from multiple sets of antigen-coupled beads (up to 100 in the system described here and up to 500 in another Luminex system), integrated information collection is possible to define the geographic distribution of disease exposures, the impacts of targeted interventions on disease, or the identification of risk factors associated with infection by specific pathogens [5, 28–38]. Using the MBA, IgG responses can be quantified from dried blood spots (DBS), serum/plasma specimens, and oral fluids (described elsewhere [34]). The signal generated from the bound r-phycoerythrin reporter molecule is directly proportional to the amount of IgG bound to the coupled antigen, and the MBA responses can be converted to International Unit values when appropriate reference standards are available [32, 39, 40]. The MBA is flexible not only in the variety of antigens that can be utilized, but also in that data acquisition can utilize magnetic or nonmagnetic beads.

Here, we describe the coupling of the recombinant Cp17 and Cp23 *C. parvum* antigens to magnetic and nonmagnetic beads,

the MBA data acquisition of human IgG attached to the antigen-coupled beads from serum specimens and DBS, quality control, MBA cut-off determination for "positive/negative" responses, and data analysis using Bio-Plex Manager 6.1 software (Bio-Rad, Hercules, CA) installed on a Bio-Plex 200 reader (Bio-Rad). Our experience has shown that adherence to this described protocol can result in consistently low inter-plate coefficients of variation (%CV).

## 2  Materials

Here, we describe a coupling consisting of $12.5 \times 10^6$ beads (1 mL of beads), and this coupling protocol can be applied to $6.25 \times 10^6$ beads (0.5 mL) with reagent volumes adjusted proportionally. We have coupled beads in the range of 25 μL to 4 mL (*see* **Note 1**). Unless otherwise indicated, chemical reagents should be analytical grade or better. Ultrapure (Type 1) water should be used for buffer preparation. Except where indicated, all buffers should be filtered (0.2 μm preferred) both to remove particulates that might interfere with the multiplex assay and to limit the potential for bacterial or fungal contamination. Sodium azide (0.02%) is commonly used as a preservative, and local regulations should be followed when disposing of sodium azide-containing waste. Unless indicated, all procedures, reagents, and solutions are used at room temperature.

*2.1  Bead Coupling*

1. Spectrally classified, 1–100, carboxylated polystyrene microspheres, either magnetic or nonmagnetic (Luminex Corp, Austin, TX). Use a different bead classification for each antigen (*see* **Note 2**).

2. Sonicator (*see* **Note 3**).

3. Aluminum foil, parafilm, rotator, and vortex mixer.

4. Microcentrifuge with $16,000 \times g$ capacity.

5. Snap-cap conical polypropylene tubes 1.5 mL with low binding affinity for polystyrene beads.

6. Sodium phosphate bead activation buffer (0.1 M pH 6.2): Dissolve 3 g sodium phosphate monobasic (anhydrous) in 200 mL ultrapure water. Use 8 M NaOH to adjust pH to 6.2. Adjust to a final volume of 250 mL with ultrapure water and filter. Store at room temperature.

7. *N*-hydroxysulfosuccinimide (Sulfo-NHS) reagent: 50 mg/mL Sulfo-NHS in 0.1 M sodium phosphate bead activation buffer (pH 6.2). Make fresh for each coupling.

8. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) reagent: (Calbiochem, Woburn, MA). 50 mg/mL in 0.1 M

sodium phosphate bead activation buffer (pH 6.2). Make fresh for each coupling (*see* **Note 4**).

9. Antigen coupling buffer containing 0.05 M 2-(*N*-morpholino) ethanesulfonic acid (MES) and 0.85% NaCl, pH 5.0: add 2.44 g of MES and 02.13 g of NaCl to 200 mL ultrapure water. Adjust pH to 5.0 with 8 M NaOH, add ultrapure water to a final volume of 250 mL, and filter. Store at room temperature.

10. Phosphate-buffered saline (PBS) containing 10 mM $Na_2HPO_4$ at pH 7.2 and 0.85% (w/v) NaCl. Filter and store at room temperature.

11. Purified recombinant *Schistosoma japonicum* glutathione-*S*-transferase (GST) control protein and purified recombinant *C. parvum* 17- and 27-kDa antigens linked with GST (Cp17-GST and Cp23-GST) (Fig. 1). Expression and purification of the proteins has been described [5, 41, 42] (*see* **Note 5**).

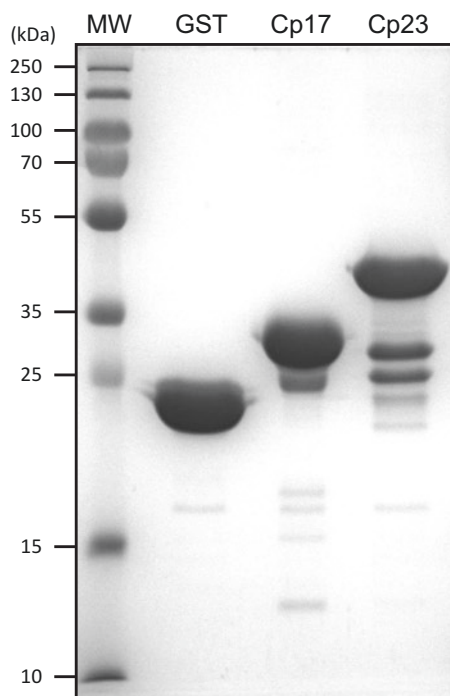12. Bicinchoninic acid (BCA) protein assay (*see* **Note 6**).



**Fig. 1** Antigen purity confirmed by SDS polyacrylamide gel electrophoresis. Purified GST, Cp17-GST (Cp17), and Cp23-GST (Cp23) proteins were resolved by SDS polyacrylamide gel electrophoresis on a 12% gel under reducing conditions using the discontinuous method of Laemmli [49]. Each lane contained 10 μg of total protein by the BCA method, and the gel was stained with Coomassie brilliant blue R-250. Molecular weights for PageRuler Plus pre-stained standards are indicated in kDa on the left

13. Bovine serum albumin (BSA), Cohn fraction V, ≥96% purity.

14. Blocking solution for antigen coupling reactions containing 1% BSA (w/v) Cohn fraction V, ≥96% purity: add 0.5 g BSA to 50 mL PBS. Mix until fully dissolved and filter. Use immediately and then discard.

15. Sodium azide, 20% (w/v): the contents of a 5 g bottle of $NaN_3$ can be dissolved in a final volume of 20 mL of ultrapure water without the need for weighing or other manipulation (*see* **Note 7**).

16. Bead storage buffer containing 1% BSA, 0.05% Tween 20, and 0.02% $NaN_3$ with protease inhibitors (made fresh for each coupling): add 10 g of BSA to 1 L PBS for a 1% (w/v) final concentration. Stir to fully dissolve, add 0.5 mL Tween 20, add 1 mL 20% $NaN_3$ for a 0.02% final concentration, and filter. Protease inhibitors must be added for long-term storage of antigen-coupled beads: dissolve 200 μg 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) /mL of bead storage buffer. This is a serine protease inhibitor. To this solution, add 1 μg leupeptin/mL (a cysteine, serine, and threonine protease inhibitor), add 1 μg pepstatin A/mL (an aspartic protease inhibitor), and add 200 μg EDTA/mL from a 0.5 M solution at pH 8.0 (a metalloprotease inhibitor) (*see* **Note 8**).

17. Hemocytometer and 1.5 mL polypropylene tubes with screw cap and *O*-ring for counting and storage of coupled beads.

18. Appropriate size magnet (immunomagnetic bead appropriate) for 1.5 mL tubes.

*2.2 Generation of Escherichia coli Extract*

1. HB101 strain *E. coli* cells containing pGEX 4 T2 plasmid with no insert.

2. Shaking incubator at 37 °C.

3. Standard LB culture medium [43].

4. Ampicillin and carbenicillin antibiotics.

5. Centrifuge with rotor capable large volume (250 mL) runs at $6000 \times g$.

6. Isopropylthio-β-D-galactoside (IPTG) at 0.1 M: dissolve 24 mg in 1 mL ultrapure water.

7. Phenymethylsulfonyl fluoride (PMSF) at 0.1 M in isopropanol: dissolve 17.4 mg in 1 mL isopropanol (*see* **Note 9**).

8. Microprobe type sonicator (Model W-225, Heat Systems, Plainview, NY).

9. Protein assay reagent (method of Bradford [44]) and appropriate protein standard.

| | |
|---|---|
| ***2.3   DBS Elution and Serum Dilution*** | 1. 0.5 mL tubes with screw caps and *O*-rings appropriate for microcentrifuge (*see* **Note 10**). |

2. DBS elution buffer containing 0.3% Tween 20 and 0.05% sodium azide in PBS [45]: add 3 mL of Tween 20 and 2.5 mL 20% $NaN_3$ to 1 L of filtered PBS.

3. Rocking platform in 4 °C area.

4. Polyvinyl alcohol (PVA), 87–90% hydrolyzed, average molecular weight 30,000–70,000.

5. Polyvinylpyrrolidone (PVP), average molecular weight 360,000.

6. Casein 1.0% (w/v) solution in PBS: PBS, casein solid (technical grade).

7. Serum dilution buffer (Buffer B) for SeroMap (nonmagnetic) bead assays containing 0.5% PVA, 0.8% PVP, 0.5% Casein, 0.3% Tween 20, and 0.02% $NaN_3$ in PBS: while vigorously stirring, slowly add 2.5 g PVA, 4 g of PVP, and 2.5 g of casein to 500 mL PBS in a 1 L glass bottle. Add solids slowly so that large clumps are not allowed to form. Add 1.5 mL Tween 20 and 0.5 mL 20% $NaN_3$. Stir the solution overnight at room temperature. Place the solution in a 60 °C water bath for 2 h, remove, and continue to stir until the solution returns to room temperature. Although the solution may appear slightly turbid, no precipitates should be visible. Buffer B is stored at 4 °C and can be used for 6 months or more. Prior to use, crude, unclarified *E. coli* extract is added for a final 3 μg/mL concentration (*see* **Note 11**).

8. Serum dilution buffer (Buffer C) for magnetic bead assays containing 0.5% BSA, 0.5% PVA, 0.8% PVP, 0.1% casein, 0.3% Tween 20, and 0.02% $NaN_3$: add PVA, PVP, Tween 20, and sodium azide to 450 mL PBS as described in **step 7** (above). Add 2.5 g BSA and 50 mL 1.0% casein in PBS (Thermo Fisher Scientific, Inc., Waltham, MA). Stir at room temperature. The solution will be slightly turbid. Do not filter. Buffer C is stored at 4 °C and can be used for 6 months or more. Prior to use, crude *E. coli* extract is added for a final 3 μg/mL concentration.

9. Microcentrifuge with $16,000 \times g$ capacity.

10. High-capacity conical bottom 96-well plates (0.45 mL capacity/well).

11. Film adhesive for high-capacity 96-well plates and for 96-well plates containing magnetic beads.

| | |
|---|---|
| ***2.4   Multiplex Bead Assay*** | 1. Multichannel pipettes and reagent reservoirs. |

2. For nonmagnetic beads, use Millipore MultiScreen filter-bottom 96-well assay plates (Millipore, Burlington, MA,). The

bottom of these plates are sealed with 1.2 μm hydrophilic polyvinylidene fluoride (PVDF) membranes that prevent the passage of 5.6 μm beads. For magnetic beads, use Bio-Rad 96-well plates (Bio-Rad).

3. Shaker for 96-well plates with sufficient rotation to keep beads in suspension without splashing.

4. Vacuum manifold for washing nonmagnetic beads (Millipore) using filter-bottom plates. Various plate washers for magnetic and nonmagnetic beads are available. See respective manuals for setup and adjustment (*see* **Note 12**).

5. Bead and reagent dilution buffer (Buffer A) containing 0.5% BSA, 0.05% Tween 20, and 0.02% $NaN_3$ in PBS: add 5 g of BSA to 1 L PBS for a 0.5% (w/v) final concentration. Stir to fully dissolve, add 0.5 mL Tween 20, add 1 mL 20% sodium azide, and filter. Store at 4 °C.

6. PBS-Tween 20 (PBST) wash buffer: add 0.5 mL Tween-20 to 1 L PBS for a 0.05% (v/v) final concentration, and stir to fully dissolve. Filter as needed. Store the solution at room temperature, and discard unused portion after 1 week (*see* **Note 13**).

7. Biotinylated mouse antihuman IgG (0.5 mg/mL, clone H2, Southern Biotech, Birmingham, AL) and biotinylated mouse antihuman $IgG_4$ (0.5 mg/mL, clone HP6025; Southern Biotech) reagent: add 10.4 μL anti-IgG and 8.3 μL anti-$IgG_4$ to 5.2 mL of Buffer A immediately prior to use. Each 50 μL assay well will contain 50 ng of anti-IgG and 40 ng of anti-$IgG_4$ [37]. Do not change antibody lots during the course of a study (*see* **Note 14**).

8. Streptavidin-coupled *r*-phycoerythrin (SAPE) reagent (Life Technologies, Carlsbad, CA) at 1 mg/mL: add 26 μl SAPE to 5.2 mL of Buffer A immediately prior to use. Add 50 μL per well (250 ng SAPE/well) [37]. Do not change SAPE lots during the course of a study (*see* **Note 15**).

9. Polyclonal anti-GST antibody (GE Healthcare) and biotinylated rabbit anti-goat IgG antibody (Thermo Fisher Scientific Inc.) for the determination of bead coupling efficiency on GST- and GST-antigen-linked beads. Monoclonal anti-GST antibody is not advised; antigen linked to GST can sterically hinder the monospecific GST epitope.

*2.5 Data Acquisition*

1. Luminex reader with Bio-Plex Manager 6.1 software (Bio-Rad) that has been properly calibrated and validated according to the manufacturer instructions.

2. Bio-Plex System Validation and Calibration kits (Bio-Rad).

3. Deionized or ultrapure water, 70% ethanol, and 10% household bleach solutions.

## 3  Methods

### 3.1  Coupling of Antigens to Carboxylated Beads

1. If using nonmagnetic SeroMap beads that can only be purchased in 4.0 mL vials ($50 \times 10^6$ beads), do not disturb settled beads in vial while removing and saving 1.0 mL supernatant (to allow room for proper mixing). At more than medium speed, vortex the 3.0 mL volume for 15 s. Sonicate for 30 s (*see* **Note 3**). Set a micropipette to about 0.7 mL and withdraw and expel (triturate) multiple times at various locations at the bottom of the 4.0 mL vial to further mix the contents. Return the 1.0 mL of supernatant back to the 3.0 mL of mixed SeroMap beads, gently mix the 4.0 mL bead suspension to make homogenous, and transfer 1.0 mL containing $12.5 \times 10^6$ beads to a 1.5 mL snap-cap polypropylene tube. If using the last of a vial of beads, transfer the vortexed and sonicated volume to a 1.5 mL snap-cap tube, wash the empty vial 2 times with 125 μL sodium phosphate activation buffer, and add to the tube (*see* **Note 16**).

2. Microcentrifuge at $16,000 \times g$ for 5 min.

3. The nonmagnetic bead pellet may be difficult to see or may be spread along the tube wall. For maximum bead recovery, tubes may be reoriented in the microcentrifuge (turned 180°) and spun again to further pellet the beads. Use a micropipette set at 200 μL and carefully remove and discard all supernatant. If clouds of nonmagnetic beads are seen releasing from the tube wall, stop, and pulse spin at top speed for 30 s. Repeat until all supernatant is removed. For magnetic beads, an appropriate magnet for 1.5 mL tubes conveniently allows supernatant removal without the need for pulse spins.

4. Add 400 μL sodium phosphate activation buffer, vortex for 10 s and sonicate for 10 s.

5. Microcentrifuge as in **step 2**. Remove and discard supernatant as in **step 3**.

6. Add 800 μL sodium phosphate activation buffer, vortex for 10 s, and sonicate for 30 s.

7. Add 100 μL of 50 mg/mL Sulfo-NHS solution and 100 μL of 50 mg/mL EDAC solution. Mix, seal tube cap with parafilm, protect from light with aluminum foil, and rotate tubes gently for 20 min. Make sure that bead suspensions are moving in the tubes.

8. Microcentrifuge as in **step 2** and remove supernatant as in **step 3**.

9. Add 400 μL of MES/NaCl antigen coupling buffer to each tube. Vortex to suspend beads (no sonication).

10. Microcentrifuge as in **step 2** and remove supernatant as in **step 3**.

11. Suspend washed and activated beads in 0.5 mL of MES/NaCl antigen coupling buffer *minus* the volume required for antigen. Our experience has shown that 6.8 μg Cp17-GST, 12.5 μg Cp23-GST, and 15.0 μg GST are sufficient for coupling to $12.5 \times 10^6$ nonmagnetic beads. For magnetic beads add 15% more antigen (*see* **Note 17**). Add the required antigen volume, mix, seal tube cap with parafilm, protect from light with aluminum foil, and rotate tubes gently for 2 h. Make sure bead suspensions are moving in the tubes.

12. Microcentrifuge as in **step 2** and remove supernatant as in **step 3**. At this point, the coupled beads have less affinity for the tube wall, thus, supernatants from nonmagnetic beads are more easily removed.

13. Suspend beads in 500 μL PBS, microcentrifuge as in **step 2**, and remove supernatant as in **step 3**.

14. Prepare fresh 1% BSA/PBS blocking buffer 30 min before completion of antigen coupling in **step 9**. Suspend beads in 500 μL blocking buffer, seal tube cap with parafilm, protect from light with aluminum foil, and rotate tubes gently for 30 min (*see* **Note 18**).

15. Microcentrifuge as in **step 2** and remove supernatant as in **step 3**.

16. Suspend beads in 1.2 mL of bead storage buffer with protease inhibitors.

17. Determine bead concentration by hemocytometer.

18. Transfer bead suspensions to 1.5 mL *O*-ring screw cap tubes, protect from light with aluminum sleeve, label, and store at 4 °C. Never freeze. Although we have not conducted an exhaustive study, we have used beads 1–2 years after coupling with good results.

*3.2 Generation of Escherichia coli Extract*

1. Inoculate a 5 mL tube of LB medium containing 50 μg/mL carbenicillin with an *E. coli* strain (we use HB101 strain from Promega) containing the pGEX 4T2 plasmid (Stratagene).

2. Grow overnight at 225 RPM and 37 °C.

3. Use the 5 mL culture to inoculate 500 mL of LB medium and add 50 mg of ampicillin (100 μg/mL).

4. Grow culture at 225 RPM and 37 °C until the optical density at 600 nm is 0.6 (about 3 h).

5. Add IPTG to 0.1 mM final concentration, and grow for an additional 2 h.

6. Collect cells by centrifugation.

7. Suspend cells in 10 mL PBS at 4 °C with 10 mM EDTA, 1 mM PMSF, and 0.2% $NaN_3$ in a 50 mL polypropylene conical tube.

8. Sonicate for 2 min using a microtip at setting #6. Keep solution cool during sonication by submerging the bottom of the tube in an ice water bath. Adequate mixing during the sonication process will ensure complete cell disruption.

9. Subject to at least three freeze thaw cycles using a dry ice-ethanol bath and a water bath at 37 °C.

10. Determine the total protein concentration using the Bio-Rad microassay procedure (method of Bradford [44]) and an appropriate protein standard.

11. Aliquot and freeze at −20 °C.

*3.3 DBS Elution and Serum Sample Dilution*

1. In a 0.5 mL screw cap tube with an O-ring, add 1.0 μL of serum or plasma to 400 μL of Buffer B (nonmagnetic beads) or Buffer C (magnetic beads) supplemented with 3 μg/mL *E. coli* extract for a 1:400 final serum dilution. Rinse the pipette tip several times in the buffer to ensure complete transfer (*see* **Note 19**).

2. Dried blood spots must be eluted prior to dilution. Add a DBS containing 10 μL of whole blood to a 0.5 mL screw cap tube with an O-ring (*see* **Note 20**).

3. Add 200 μL of elution buffer for a 1:40 serum concentration, and incubate on a rocker platform overnight at 4 °C (*see* **Note 21**).

4. Vortex briefly and microcentrifuge to collect all eluate at the bottom of the tube. It is not necessary to remove the filter paper.

5. In another tube, perform a further 1:10 dilution by adding 50 μL of DBS eluate to 450 μL of Buffer B (SeroMap) or Buffer C (Magnetic beads) supplemented with 3 μg/mL *E. coli* extract for a 1:400 final serum dilution. Rinse the pipette tip several times in the buffer to ensure complete transfer (*see* **Note 19**).

6. Serum/plasma and DBS dilutions are typically stored overnight at 4 °C prior to use (*see* **Note 22**).

7. For nonmagnetic bead assays, dilutions are centrifuged for 10 min at $16,000 \times g$ to pellet any particulate matter that might interfere with the Luminex run. For magnetic bead assays, centrifugation is not required when using magnetic separation for the assay washing steps.

8. Add 140 μL of each dilution to individual wells of a conical bottom 96-well plate (0.45 mL capacity/well) according to the proposed assay template and assuming duplicates (software provides up to 10 replicates in vertical or horizontal orientation)

in adjacent columns. Normally, we run in duplicates in the horizontal (on the same row) (50 µl each), thus, 48 wells are needed for a 96-well. This "assay loading plate" can be sealed and stored at 4 °C for future use if needed.

9. Each multiplex assay must include a set of blank wells loaded with Buffer B (nonmagnetic) or Buffer C (magnetic) only (no serum antibodies) in addition to appropriate positive and negative controls (*see* **Note 23**).

*3.4   Multiplex Bead Assay*

Multiplex bead assays are conducted at room temperature. When dispensing buffers containing Tween detergent (including beads and diluted sera), reverse pipetting techniques must be used. Good reverse pipetting skills are critical for inter-plate assay reproducibility and for low coefficients of variation (%CV) for intra-plate duplicate wells. Operations involving diluted sera (such as loading of the multiplex plate) should be conducted in a biosafety cabinet while wearing appropriate personal protective equipment.

1. Decide on the total number of wells to be used and allow for a few extra. A single 96-well plate will require a final volume of 5.2 mL of Buffer A per operation (assuming 50 µL/well).

2. For each classification of coupled bead, determine the volume of beads required to achieve 1100–1500 beads per well. Small differences in the numbers of beads added to the assay have little impact on the response (Fig. 2) (*see* **Note 24**).

3. Vortex the antigen-coupled beads for 15–20 s, and add the calculated volume into a reservoir for multichannel pipettes containing the appropriate volume of Buffer A (*see* **Note 25**).

4. With a multichannel pipette, make the bead suspension homogenous in the reservoir, and add 50 µL of the bead suspension to each well of the appropriate 96-well plate described in Subheading 2.4, **step 2**. Use an 8- or 12-multichannel pipette and reverse pipetting techniques. If using a 96-well filter-bottom plate, pre-wet all wells with 100 µL of PBST wash solution, remove by vacuum filtration, and blot the bottom of the filter plate with large Kimwipe prior to loading the beads.

5. Wash the beads two times with at least 100 µL wash PBST wash solution/well using the method appropriate for the bead and plate type used (either the vacuum manifold system for nonmagnetic beads or the magnetic plate washer described in Subheading 2.4, **step 4** and **Note 12**). Use a 12-multichannel pipette and reverse pipetting techniques for PBST washes.

6. Transfer 50 µL of each DBS or serum/plasma dilution from the assay loading plate described in Subheading 3.3, **step 8** to the plate containing washed beads. An 8-multichannel pipette
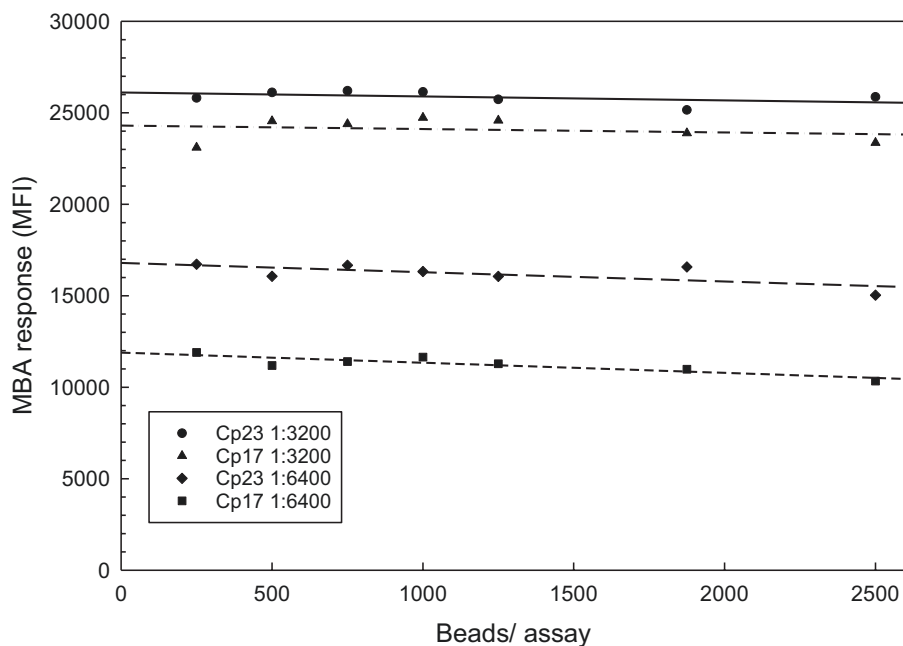
**Fig. 2** Assay response values are minimally impacted by the number of beads used in the assay. MBA assays were run in duplicate using the standard assay protocol except that the numbers of beads per assay well were varied as indicated. A strong positive control serum was used as the primary antibody either at 1:3200 dilution (top two lines) or at 1:6400 dilution (bottom two lines). Cp17-GST MBA responses (no background subtracted) are indicated by triangles and squares, respectively, while Cp23-GST responses (no background subtracted) are indicated by circles and diamonds, respectively. At 250 beads/assay the BioPlex instrument was not able to count 50 beads per classification in a 2 min run. At 1000 beads/assay, a run time of approximately 60 seconds was required to count 100 beads per classification

and reverse pipetting techniques should be used to load duplicate samples into adjacent columns of the plate (*see* **Note 26**).

7. For plates with magnetic beads, cover with film adhesive. Do not use film adhesive on plates with nonmagnetic beads. Cover plate with a lid, secure on a shaker, and cover with aluminum foil. Increase speed slowly to an intensity to keep beads in suspension without splashing for 90 min (Fig. 3) (*see* **Note 27**).

8. Remove the serum dilutions using the appropriate wash method for the bead type as described in **step 5** (*see* **Note 28**).

9. Wash the beads three times with at least 100 μL wash PBST wash solution/well as described in **step 5**.

10. Add 50 μL/well of Buffer A containing the two biotinylated secondary antibodies. Use an 8- or 12-multichannel pipette and reverse pipetting techniques.

11. Incubate as described in **step 7** for 45 min.

12. Remove the solution using the appropriate wash method for the bead type as described in **step 5**.
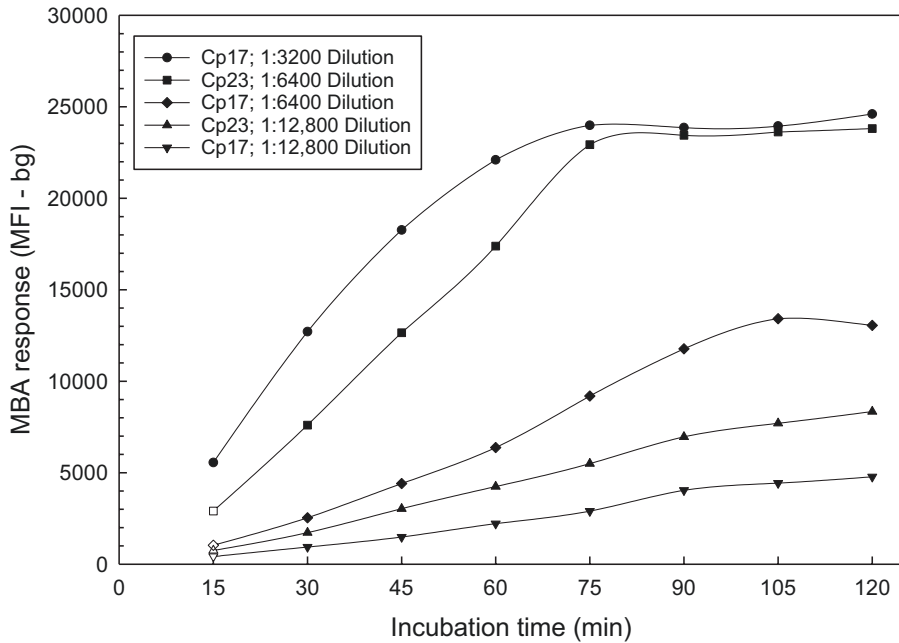
**Fig. 3** Assay response values increase with increasing primary antibody incubation time. MBA assays were run in duplicate using the standard assay protocol except that the incubation time with diluted serum was varied between 15 and 120 min. A strong positive control serum was used as the primary antibody at dilutions between 1:3200 and 1:12,800

13. Wash as described in **step 9**.

14. Add 50 μL well of Buffer A containing SAPE. Use an 8- or 12-multichannel pipette and reverse pipetting techniques.

15. Incubate as described in **step 7** for 30 min.

16. Remove the solution using the appropriate wash method for the bead type as described in **step 5**.

17. Wash as described in **step 9**.

18. For a final extended wash, add 50 μL of Buffer A per well. Use an 8- or 12-multichannel pipette and reverse pipetting techniques.

19. Incubate as described in **step 7** for 30 min.

20. Remove the solution using the appropriate wash method for the bead type as described in **step 5**.

21. Wash the plate once with at least 100 μL wash PBST wash solution/well as described in **step 9**.

22. Add 100–125 μL of PBS per well using an 8- or 12-multichannel pipette. Prepare the plate for shaking as described in **step 5** and shake for 30 s. The plate is ready for immediate data acquisition.

*3.5  Data Acquisition*

1. Power up the instrument, perform prime and startup, and create a protocol to count 50 or 100 beads per classification. After 30 min of laser warm up, perform calibration. Perform validation, if prompted by the Bio-Plex Manager software. Complete these tasks at least 30 min before data acquisition (*see* **Note 29**).

2. Insert plate, and acquire data using high voltage for the reporter molecule detector.

3. Data should be recorded as the average of the median fluorescent intensity *minus* background (MFI-bg) for the duplicate well responses (*see* **Note 30**).

4. An external standard curve can be incorporated for either or both of the Cp17-GST and the Cp23-GST antigens provided the bead classification for the standards are the same as the unknowns.

5. After acquisition, shut down the instrument with water and bleach treatment as instructed in the manual.

*3.6  Cut-off Assignment and Data Analysis*

Our typical cut-off values for the Cp17-GST and Cp23-GST assays are in the 200–900 and 500–1800 MFI-bg ranges, respectively, but the specific values for a particular lot of beads are impacted by many variables including the quality of the antigen, coupling efficiency, and the reagents used in MBA. Cut-off values also change after long-term storage of coupled beads (*see* **Note 31**).

1. Identify a cut-off by Receiver Operating Characteristic (ROC) curve analysis using a serum sample set characterized with the "gold standard" large format EITB (western blot) assay [4, 12, 19]. From the results shown in Fig. 4, the cut-offs for the Cp17-GST and Cp23-GST MBA were 390 (sensitivity, 93%; specificity 90%) and 865 (sensitivity, 96%; specificity 97%), respectively. Note that extremely low-intensity bands may not be visible to the human naked eye.

2. Alternatively, identify a low-response serum panel that can be used for a mean *plus* 3 standard deviation (SD) cut-off determination (*see* **Note 32**).

3. Once cut-offs have been established, use a standard curve to translate the MFI-bg values to an arbitrary unit value. Prepare a standard curve by twofold serial dilution of a strong IgG-positive serum specimen or sample pool with sufficient volume for future use. For our standard curve serum pool, we make twofold serial dilutions starting at 1:400 and assign that dilution a value of 6400 arbitrary units (Fig. 5). The MFI-bg cut-off values are converted to arbitrary unit values based on a linear regression plot of log transformed data (*see* **Note 33**).

4. Cut-off values need to be reestablished when new lots of beads are coupled or when beads have been stored for several months.
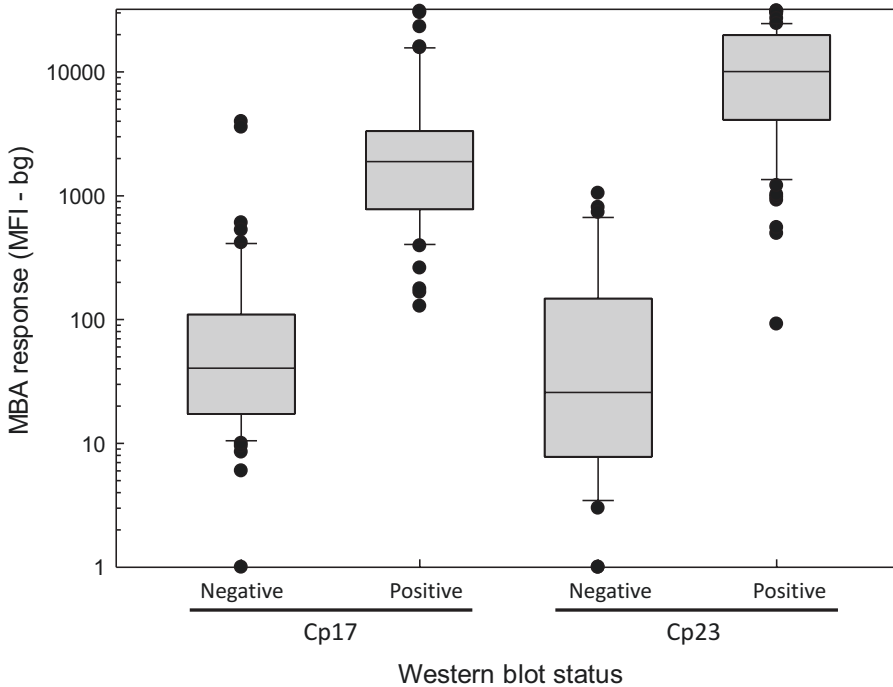
**Fig. 4** Cp17-GST and Cp23-GST MBA responses for serum samples characterized by large format western blot [26, 41]. A panel of 108 sera that were previously characterized by large format western blot were assayed using the standard MBA protocol. By blot, the panel contained 51 negatives and 57 positives for antibodies to the 17-kDa antigen and 31 negatives and 77 positives for antibodies against the 27-kDa antigen. Boxes include 25th to 75th percentile, while bars indicate tenth and 90th percentiles. Outliers are indicated by closed circles

Prepare a twofold serial dilution curve using the same serum or sample pool described in **step 3** and determine the MFI-bg responses by MBA. We often run the standard curve in the middle of a study, especially if the study lasts 2–3 months. Use the previously established arbitrary unit cut-off values to determine the equivalent MFI-bg values for the bead sets being used (*see* **Note 34**).

*3.7 Quality Control Considerations*

1. Coupling efficiency can be monitored using tenfold serial dilutions of goat anti-GST polyclonal antibody (beginning at 1:1000) in Buffer A as the primary antibody (no *E. coli* extract should be added). Develop using biotinylated rabbit anti-goat IgG (1:1000 in Buffer A) as the secondary antibody. Incubation times and other reagents are identical to the standard MBA protocol (*see* **Note 35**).

2. If the responses for Cp17-GST and Cp23-GST assays for a particular sample are both positive and have %CVs >15%, that sample should be retested. %CVs for MFI-bg values below the cut-off values are unreliable and can be ignored (*see* **Note 36**).
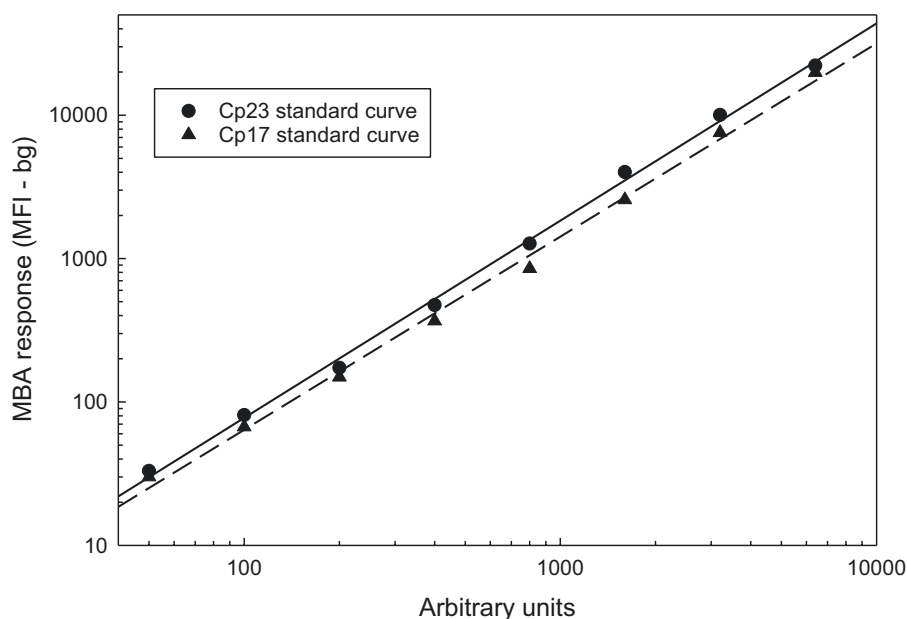
**Fig. 5** Development of an in-house standard curve for MBA assay standardization. A serum pool was created that gave good responses between dilutions of 1:400 and 1:51,200. The 1:400 dilution was assigned an arbitrary unit value of 6400. A standard curve was generated using eight twofold serial dilutions beginning at 1:400, and the standard MBA was run using both Cp17-GST and Cp23-GST beads. On this curve, a cut-off of 390 MFI-bg units for the Cp17-GST assay corresponds to 383 arbitrary units, and a cut-off of 865 MFI-bg units for the Cp23-GST assay corresponds to 578 arbitrary units

3. Responses to the control GST bead should be consistently at or below 100 MFI-bg units, especially when using *E. coli* extract for pre-absorption. High GST only responders (>1000 MFI-bg) are extremely rare [5, 26, 25].

4. Each plate we run includes a negative control and a collection of positive controls. The positive controls are diluted to provide low, medium, and high range MFI-bg values so that assay performance can be monitored over the entire response range. For four Cp17-GST assay positive controls used in a 10-week study (shown in Fig. 6; closed symbols), %CVs were <6%. If the positive control values from a particular plate contribute to a significant increase in the overall study control value %CVs, consider rerunning the plate. Alternatively, control rules can be developed similar to those described by Westgard et al. so that the assay performance can be monitored [46] (*see* **Note 37**).

# 4    Notes

1. If a coupling scale other than 0.5–1.0 mL is required, contact authors for procedure. Outside this range, bead suspension
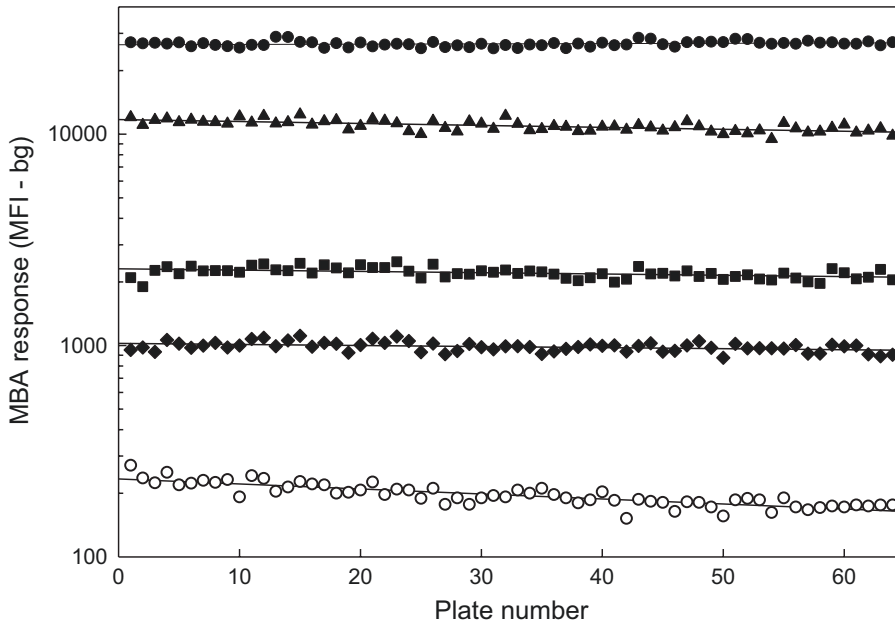
**Fig. 6** Control value stability for Cp17-GST assays over the course of a 10-week MBA study. A 10-week MBA study was performed using a total of 64 plates. Four positive control sera (circles, triangles, squares, and diamonds) and one negative control (open circles) were included on each plate run during the study. MFI-bg values for the Cp17-GST assay are plotted. For the overall study, the %CVs for the positive controls ranged from 3% to 6%, and the %CV for the negative control was 12%

procedures, centrifugations, and tubes are different than described here.

2. Polystyrene beads can be purchased in spectral classifications, 1–100, for both magnetic (MagPlex, Luminex Corp.) and nonmagnetic (SeroMap, Luminex Corp.) beads. Classifications are the same for both magnetic and nonmagnetic beads, and the classifications are based on 100 ratios of two fluorochromes, a red and infrared, that are incorporated in the polystyrene and excited by a 635 nm red laser [47]. The SeroMap beads use proprietary technology to reduce nonspecific serum antibody binding; however, we have not observed any distinction between the nonmagnetic beads (MicroPlex, Luminex Corp.) and the nonmagnetic SeroMap beads described above in serology assays (unpublished observation). The MicroPlex and MagPlex beads can be purchased in 1.0 mL volumes, while the minimum SeroMap purchase is 4.0 mL. With the use of applicable size magnets, magnetic beads are easily manipulated and more efficient in post-coupling recovery than nonmagnetic beads.

3. Do not use a strong sonicator that might disrupt beads. A water-bath sonicator of about 0.75 gallons or less with a frequency rating not more than 40 kHz is sufficient.

4. The choice of good quality EDAC is likely the single largest factor related to low background antibody responses, and EDAC from various commercial sources can contribute to unexpectedly high background reactivity. Mass spectrometry was not able to distinguish between low background and high background EDAC (unpublished observation). Thus, this reagent should be screened by test coupling GST to beads using the candidate EDAC, and IgG responses to the GST-coupled beads should be determined using a dozen or so human sera. If the mean IgG signal from these sera to the GST-coupled beads is more than the "positive/negative" cut-off, then consider using EDAC from another source. One should identify a superior lot and purchase in bulk for future work. EDAC is moisture sensitive. We typically divide a 25 g bottle into small tubes containing approximately 100 mg each in a dry nitrogen environment and store the aliquots at −20 °C in the presence of desiccant and warmed to room temperature before use. Once opened, a 100 mg tube can be used for 1–2 months.

5. We use the pGEX expression system (Stratagene, La Jolla, CA) to generate GST fusion proteins for our assays. Cp17 and Cp23 are small proteins (79 and 112 amino acids, respectively), and the GST component provides additional Lys residues for coupling to activated beads in regions that are not vital for antibody binding. Proteins should be in PBS buffer, and should be essentially free of contaminating bacterial proteins. Proteins in tris(hydroxymethyl)-aminomethane (Tris) buffer cannot be used for coupling due to the presence of a free primary amine, and Tris-containing protein solutions should be extensively dialyzed against PBS prior to bead coupling.

6. Recombinant protein concentrations are determined using the bicinchoninic acid (BCA) microassay. The BCA microassay and the Coomassie blue-based Bradford microassay are often discordant as the Cp23 protein contains no hydrophobic amino acids.

7. Consult the Safety Data Sheet prior to use as sodium azide is extremely toxic and forms explosive compounds when in contact with metals. Purchase only the amount of material necessary for the expected work. Perform all manipulations of concentrated (20% w/v) $NaN_3$ solution in a chemical fume hood with proper personal protective equipment including double nitrile gloves. Hazardous waste must be collected for proper disposal.

8. Make 1 mg/mL stock solutions of leupeptin and Pepstatin A in DMSO or water. Aliquot in convenient volumes and store at

−20 to −70 °C. Commercially prepared protease inhibitor cocktails may also be used.

9. PMSF is toxic and should be handled with care using proper personal protective equipment.

10. Screw cap tubes with *O*-rings and a sealed microcentrifuge rotor are recommended when working with potentially infectious human materials to minimize exposures from potentially infectious aerosols.

11. Although not required, a solution that appears essentially clear can sometimes be filtered using a 0.45 μm filter. PVA and PVP suppress binding of serum antibodies to the polystyrene beads [48]. After long-term storage, precipitates (likely casein) will form, but the quality of the buffer is not adversely impacted. Precipitates will not interfere with the multiplex assay so long as serum dilutions are properly centrifuged prior to use in the multiplex assays.

12. To minimize bead loss, the vacuum system must be adjusted so that 5–10 seconds are required to evacuate 100 μL from the plate wells. The evacuated plate should not stick to the manifold but should release easily. There are a number of wash systems for magnetic beads, ranging from hand-held magnets (Luminex Corp) to sophisticated wash systems from BioTek (BioTek, Winooski, VT). However, not all filter-bottom plates (Millipore) are compatible with the magnetic bead plate washer system from BioTek.

13. Monitor closely and discard if any cloudiness or precipitates appear as this may be an indicator of contamination.

14. Monoclonal secondary antibody reagents are preferable to polyclonal antibody reagents for background minimization. The monoclonal anti-IgG from clone H2 does not react well with $IgG_4$, thus, a dedicated monoclonal anti-$IgG_4$ antibody is included in the assay. The extent of biotinylation is not closely monitored during manufacture, so lot-to-lot variability may impact response values.

15. Product specifications call for the ratio of phycoerythrin absorbance at $\lambda_{max}$/absorbance at 280 nm to be between 3.0 and 4.0. We typically use SAPE having a ratio ≥3.6.

16. The uncoupled beads are hydrophobic and tend to aggregate and stick to tube surfaces if vortexed excessively. This behavior may reduce post-bead coupling recovery thereby reducing the effective bead concentration. Brief sonication of beads prior to use is recommended by the manufacturer, but sonication is not absolutely required.

17. For magnetic beads, add 15% more antigen than that used on nonmagnetic beads. The magnetic beads are 6.5 μm in diameter

and have about 30% more surface area than the nonmagnetic beads at 5.6 μm diameter.

18. The BSA will couple to any sites that remain activated but unoccupied by antigen.

19. *E. coli* extract is added to dilution Buffer B and dilution Buffer C solutions to absorb any *E. coli* antibodies that might react with residual *E. coli* proteins that were not completely removed from the antigen purification process. *E. coli* extract can also block nonspecific antibody binding to beads coupled with other (non-*Cryptosporidium*) parasite antigens [37].

20. Other elution/dilution protocols are possible. For instance, DBS can be eluted directly into 500 μL of Buffer B or Buffer C and then diluted an additional 1:4 for a final 1:400 serum concentration.

21. DBS used in our lab are circular filter paper extensions (TropBio Pty Ltd., Townsville, Australia) designed to absorb 10 μL whole blood. DBS must be allowed to dry completely at room temperature and must be stored at −20 °C and protected from moisture with desiccant [45]. A 50% hematocrit is assumed. DBS elutions are stable at 4 °C for long-term storage. Do not freeze.

22. Dilutions are stable at 4 °C for long-term storage. Do not freeze.

23. Negative controls ensure that no unexpected background is present, while positive controls help to ensure consistency between plates (Subheading 3.7, **step 4**).

24. For data acquisition, we commonly use 1500 beads/classification/well. If the bead numbers have been estimated correctly, the instrument should require 30–45 s to acquire data from 100 beads/classification/well. If the run time is too slow or too fast, adjust by increasing or decreasing with a few hundred beads/well of the appropriate classification. Under ideal conditions, all adjustments should be finalized prior to beginning a study.

25. Care should be taken to ensure that bead stocks are completely suspended prior to use. Do not allow the beads to sit for an extended period of time prior to use as they will settle out again.

26. Plate loading should require 5 min or less.

27. Do not cover filter-bottom plates with a sealing film as this will force solution through the filter. The incubation times for primary and secondary antibodies described in this protocol are designed to balance the time necessary to achieve sufficient antibody binding with the need to run multiple plates in 1 day. Recommended incubation times should be rigorously followed to ensure maximum reproducibility.

28. For this step and for all subsequent wash steps, filtrate should be considered as potentially infectious and must be collected into an appropriate container for proper decontamination and disposal.

29. Become familiar with the instrument functions such as startup, needle adjustment, calibration, validation, protocol creation, plate formatting, data analysis, and shutdown. All are described in the software/hardware user manual.

30. The Bio-Plex software determines the median channel with signal (1–32,766) from the reporter molecule, the median fluorescence intensity (MFI). The software can also show the histogram. The software then calculates the average mean MFI from the multiple wells and subtracts the background MFI mean (bg) from wells containing no primary antibody. We routinely use duplicates and report MFI-bg as data. The signal from the reporter molecule is directly proportional to the amount of IgG attached to the antigen-coupled bead. We have observed no difference in MFI determination between data collected by counting 50 or 100 beads per classification.

31. Cut-off determination for *Cryptosporidium*-specific serologic antibody assays is challenging because unexposed populations likely do not exist even in the US and because many are unaware of their infection history due to the often nondescript nature of the diarrheal symptoms and the possibility of asymptomatic infection. Antibody positivity is strongly associated with increasing age [20], therefore, samples from young children are better candidates for negative serostatus than those from adults. In addition, cut-off values are specific for a particular lot of coupled beads, and they must be reassigned for each new set of coupled beads. There are no international reference standards for *Cryptosporidium* serology.

32. Identify candidate specimens from young children (1–5 years of age) from communities with good quality public water systems who are likely to be negative for antibodies to *Cryptosporidium* spp. Screen samples by MBA using the Cp17-GST and Cp23-GST antigens and eliminate specimens with obvious strong responses >5000 MFI-bg. From the remaining responses, calculate the mean *plus* 3 SD as the cut-off.

33. If multiple points are on the upper plateau of the curve and outside the linear range, more dilution points will be required to extend the line below the calculated cut-off value. Store the standard serum in aliquots at −80 °C.

34. Alternatively, the standard curve can be used as an external reference standard for the multiplex assays of an entire study, and the Bio-Plex software can be used to convert MFI-bg values into arbitrary unit values. This method only works when

the same bead classification is used for both the study and the reference standard.

35. Responses should be 29,000–31,000 MFI-bg units at a 1:1000 anti-GST dilution.

36. Close monitoring of the duplicate well %CVs provides an excellent indication of assay performance. A very high %CV can indicate loss of beads or loss of PBS from one of the filter bottom test wells. High %CV values for multiple samples can indicate degradation of that particular bead or an instrumentation issue such as a clogged needle.

37. Control samples should be diluted at the beginning of a study and included in each plate run. With proper storage at 4 °C, control values should remain stable for 2–3 months with only minimal loss of signal. Our preferred target %CV for positive control values for a 2–3 month study is 15% or less.

## Acknowledgments

## References

1. Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, Panchalingam S, Wu Y, Sow SO, Sur D, Breiman RF, Faruque AS, Zaidi AK, Saha D, Alonso PL, Tamboura B, Sanogo D, Onwuchekwa U, Manna B, Ramamurthy T, Kanungo S, Ochieng JB, Omore R, Oundo JO, Hossain A, Das SK, Ahmed S, Qureshi S, Quadri F, Adegbola RA, Antonio M, Hossain MJ, Akinsola A, Mandomando I, Nhampossa T, Acacio S, Biswas K, O'Reilly CE, Mintz ED, Berkeley LY, Muhsen K, Sommerfelt H, Robins-Browne RM, Levine MM (2013) Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. Lancet 382(9888):209–222. https://doi.org/10.1016/S0140-6736(13)60844-2

2. Sow SO, Muhsen K, Nasrin D, Blackwelder WC, Wu Y, Farag TH, Panchalingam S, Sur D, Zaidi AK, Faruque AS, Saha D, Adegbola R, Alonso PL, Breiman RF, Bassat Q, Tamboura B, Sanogo D, Onwuchekwa U, Manna B, Ramamurthy T, Kanungo S, Ahmed S, Qureshi S, Quadri F, Hossain A, Das SK, Antonio M, Hossain MJ, Mandomando I, Nhampossa T, Acacio S, Omore R, Oundo JO, Ochieng JB, Mintz ED, O'Reilly CE, Berkeley LY, Livio S, Tennant SM, Sommerfelt H, Nataro JP, Ziv-Baran T, Robins-Browne RM, Mishcherkin V, Zhang J, Liu J, Houpt ER, Kotloff KL, Levine MM (2016) The burden of *Cryptosporidium* diarrheal disease among children < 24 months of age in moderate/high mortality regions of sub-Saharan Africa and South Asia, utilizing data from the global enteric Multicenter study (GEMS). PLoS Negl Trop Dis 10(5):e0004729.

https://doi.org/10.1371/journal.pntd.0004729

3. Casemore D (2006) Towards a US national estimate of the risk of endemic waterborne disease—sero-epidemiologic studies. J Water Health 4(Suppl 2):121–163

4. Moss DM, Bennett SN, Arrowood MJ, Wahlquist SP, Lammie PJ (1998) Enzyme-linked immunoelectrotransfer blot analysis of a cryptosporidiosis outbreak on a United States Coast Guard cutter. Am J Trop Med Hyg 58(1):110–118

5. Moss DM, Montgomery JM, Newland SV, Priest JW, Lammie PJ (2004) Detection of *Cryptosporidium* antibodies in sera and oral fluids using multiplex bead assay. J Parasitol 90(2):397–404. https://doi.org/10.1645/GE-3267

6. Centers for Disease Control and Prevention (1998) Foodborne outbreak of cryptosporidiosis—Spokane, Washington, 1997. MMWR Morb Mortal Wkly Rep 47(27):565–567

7. Ong CS, Li AS, Priest JW, Copes R, Khan M, Fyfe MW, Marion SA, Roberts JM, Lammie PJ, Isaac-Renton JL (2005) Enzyme immunoassay of *Cryptosporidium*-specific immunoglobulin G antibodies to assess longitudinal infection trends in six communities in British Columbia, Canada. Am J Trop Med Hyg 73(2):288–295

8. Frost FJ, de la Cruz A, Moss DM, Curry M, Calderon RL (1998) Comparisons of ELISA and Western blot assays for detection of *Cryptosporidium* antibody. Epidemiol Infect 121(1):205–211

9. Frost FJ, Muller T, Craun GF, Calderon RL, Roefer PA (2001) Paired city *Cryptosporidium* serosurvey in the southwest USA. Epidemiol Infect 126(2):301–307

10. Frost FJ, Kunde TR, Muller TB, Craun GF, Katz LM, Hibbard AJ, Calderon RL (2003) Serological responses to *Cryptosporidium* antigens among users of surface- vs. ground-water sources. Epidemiol Infect 131(3):1131–1138

11. Steinberg EB, Mendoza CE, Glass R, Arana B, Lopez MB, Mejia M, Gold BD, Priest JW, Bibb W, Monroe SS, Bern C, Bell BP, Hoekstra RM, Klein R, Mintz ED, Luby S (2004) Prevalence of infection with waterborne pathogens: a sero-epidemiologic study in children 6-36 months old in San Juan Sacatepequez, Guatemala. Am J Trop Med Hyg 70(1):83–88

12. Moss DM, Chappell CL, Okhuysen PC, DuPont HL, Arrowood MJ, Hightower AW, Lammie PJ (1998) The antibody response to 27-, 17-, and 15-kDa *Cryptosporidium* antigens following experimental infection in humans. J Infect Dis 178(3):827–833

13. Sandhu SK, Priest JW, Lammie PJ, Hubbard A, Colford JM Jr, Eisenberg JN (2006) The natural history of antibody responses to *Cryptosporidium* parasites in men at high risk of HIV infection. J Infect Dis 194(10):1428–1437. https://doi.org/10.1086/508194

14. Priest JW, Bern C, Xiao L, Roberts JM, Kwon JP, Lescano AG, Checkley W, Cabrera L, Moss DM, Arrowood MJ, Sterling CR, Gilman RH, Lammie PJ (2006) Longitudinal analysis of *Cryptosporidium* species-specific immunoglobulin G antibody responses in Peruvian children. Clin Vaccine Immunol 13(1):123–131. https://doi.org/10.1128/CVI.13.1.123-131.2006

15. Priest JW, Bern C, Roberts JM, Kwon JP, Lescano AG, Checkley W, Cabrera L, Moss DM, Arrowood MJ, Sterling CR, Gilman RH, Lammie PJ (2005) Changes in serum immunoglobulin G levels as a marker for *Cryptosporidium* sp. infection in Peruvian children. J Clin Microbiol 43(10):5298–5300. https://doi.org/10.1128/JCM.43.10.5298-5300.2005

16. Frost FJ, Calderon RL, Muller TB, Curry M, Rodman JS, Moss DM, de la Cruz A (1998) A two-year follow-up survey of antibody to *Cryptosporidium* in Jackson County, Oregon following an outbreak of waterborne disease. Epidemiol Infect 121(1):213–217

17. Crump JA, Mendoza CE, Priest JW, Glass RI, Monroe SS, Dauphin LA, Bibb WF, Lopez MB, Alvarez M, Mintz ED, Luby SP (2007) Comparing serologic response against enteric pathogens with reported diarrhea to assess the impact of improved household drinking water quality. Am J Trop Med Hyg 77(1):136–141

18. Moss DM, Bennett SN, Arrowood MJ, Hurd MR, Lammie PJ, Wahlquist SP, Addiss DG (1994) Kinetic and isotypic analysis of specific immunoglobulins from crew members with cryptosporidiosis on a U.S. Coast Guard cutter. J Eukaryot Microbiol 41(5):52S–55S

19. Priest JW, Li A, Khan M, Arrowood MJ, Lammie PJ, Ong CS, Roberts JM, Isaac-Renton J (2001) Enzyme immunoassay detection of antigen-specific immunoglobulin g antibodies in longitudinal serum samples from patients with cryptosporidiosis. Clin Diagn Lab Immunol 8(2):415–423. https://doi.org/10.1128/CDLI.8.2.415-423.2001

20. Becker DJ, Oloya J, Ezeamama AE (2015) Household socioeconomic and demographic correlates of *Cryptosporidium* seropositivity in the United States. PLoS Negl Trop Dis 9(9):e0004080. https://doi.org/10.1371/journal.pntd.0004080

21. Hamlin KL, Moss DM, Priest JW, Roberts J, Kubofcik J, Gass K, Streit TG, Nutman TB,

Eberhard ML, Lammie PJ (2012) Longitudinal monitoring of the development of antifilarial antibodies and acquisition of *Wuchereria bancrofti* in a highly endemic area of Haiti. PLoS Negl Trop Dis 6(12):e1941. https://doi.org/10.1371/journal.pntd.0001941

22. Priest JW, Moss DM, Arnold BF, Hamlin K, Jones CC, Lammie PJ (2015) Seroepidemiology of *Toxoplasma* in a coastal region of Haiti: multiplex bead assay detection of immunoglobulin G antibodies that recognize the SAG2A antigen. Epidemiol Infect 143(3):618–630. https://doi.org/10.1017/S0950268814001216

23. Arnold BF, Priest JW, Hamlin KL, Moss DM, Colford JM Jr, Lammie PJ (2014) Serological measures of malaria transmission in Haiti: comparison of longitudinal and cross-sectional methods. PLoS One 9(4):e93684. https://doi.org/10.1371/journal.pone.0093684

24. Arnold BF, van der Laan MJ, Hubbard AE, Steel C, Kubofcik J, Hamlin KL, Moss DM, Nutman TB, Priest JW, Lammie PJ (2017) Measuring changes in transmission of neglected tropical diseases, malaria, and enteric pathogens from quantitative antibody levels. PLoS Negl Trop Dis 11(5):e0005616. https://doi.org/10.1371/journal.pntd.0005616

25. Moss DM, Priest JW, Hamlin K, Derado G, Herbein J, Petri WA Jr, Lammie PJ (2014) Longitudinal evaluation of enteric protozoa in Haitian children by stool exam and multiplex serologic assay. Am J Trop Med Hyg 90(4):653–660. https://doi.org/10.4269/ajtmh.13-0545

26. Priest JW, Moss DM, Visvesvara GS, Jones CC, Li A, Isaac-Renton JL (2010) Multiplex assay detection of immunoglobulin G antibodies that recognize *Giardia intestinalis* and *Cryptosporidium parvum* antigens. Clin Vaccine Immunol 17(11):1695–1707. https://doi.org/10.1128/CVI.00160-10

27. Zambrano LD, Priest JW, Ivan E, Rusine J, Nagel C, Kirby M, Rosa G, Clasen TF (2017) Use of serologic responses against enteropathogens to assess the impact of a point-of-use water filter: a randomized controlled trial in Western Province, Rwanda. Am J Trop Med Hyg 97(3):876–887. https://doi.org/10.4269/ajtmh.16-1006

28. Moss DM, Chard AN, Trinies V, Doumbia S, Freeman MC, Lammie PJ (2017) Serological responses to filarial antigens in Malian children attending elementary schools. Am J Trop Med Hyg 96(1):229–232. https://doi.org/10.4269/ajtmh.16-0560

29. Poirier MJ, Moss DM, Feeser KR, Streit TG, Chang GJ, Whitney M, Russell BJ, Johnson BW, Basile AJ, Goodman CH, Barry AK, Lammie PJ (2016) Measuring Haitian children's exposure to chikungunya, dengue and malaria. Bull World Health Organ 94(11):817–825A. https://doi.org/10.2471/BLT.16.173252

30. Priest JW, Jenks MH, Moss DM, Mao B, Buth S, Wannemuehler K, Soeung SC, Lucchi NW, Udhayakumar V, Gregory CJ, Huy R, Muth S, Lammie PJ (2016) Integration of multiplex bead assays for parasitic diseases into a national, population-based serosurvey of women 15-39 years of age in Cambodia. PLoS Negl Trop Dis 10(5):e0004699. https://doi.org/10.1371/journal.pntd.0004699

31. Rogier E, Moss DM, Chard AN, Trinies V, Doumbia S, Freeman MC, Lammie PJ (2017) Evaluation of immunoglobulin G responses to *Plasmodium falciparum* and *Plasmodium vivax* in Malian school children using multiplex bead assay. Am J Trop Med Hyg 96(2):312–318. https://doi.org/10.4269/ajtmh.16-0476

32. Scobie HM, Mao B, Buth S, Wannemuehler KA, Sorensen C, Kannarath C, Jenks MH, Moss DM, Priest JW, Soeung SC, Deming MS, Lammie PJ, Gregory CJ (2016) Tetanus immunity among women aged 15 to 39 years in Cambodia: a national population-based serosurvey, 2012. Clin Vaccine Immunol 23(7):546–554. https://doi.org/10.1128/CVI.00052-16

33. Moss DM, Handali S, Chard AN, Trinies V, Bullard S, Wiegand RE, Doumbia S, Freeman MC, Lammie PJ (2018) Detection of immunoglobulin G antibodies to *Taenia solium* cysticercosis antigen glutathione-s-transferase-rT24H in Malian children using multiplex bead assay. Am J Trop Med Hyg. https://doi.org/10.4269/ajtmh.17-0310

34. Griffin SM, Chen IM, Fout GS, Wade TJ, Egorov AI (2011) Development of a multiplex microsphere immunoassay for the quantitation of salivary antibody responses to selected waterborne pathogens. J Immunol Methods 364(1–2):83–93. https://doi.org/10.1016/j.jim.2010.11.005

35. Plucinski MM, Candrinho B, Chambe G, Muchanga J, Muguande O, Matsinhe G, Mathe G, Rogier E, Doyle T, Zulliger R, Colborn J, Saifodine A, Lammie P, Priest JW (2018) Multiplex serology for impact evaluation of bed net distribution on burden of lymphatic filariasis and four species of human malaria in northern Mozambique. PLoS Negl Trop Dis 12(2):e0006278. https://doi.org/10.1371/journal.pntd.0006278

36. Lammie PJ, Moss DM, Brook Goodhew E, Hamlin K, Krolewiecki A, West SK, Priest JW (2012) Development of a new platform for neglected tropical disease surveillance. Int J Parasitol 42(9):797–800. https://doi.org/10.1016/j.ijpara.2012.07.002

37. Moss DM, Priest JW, Boyd A, Weinkopff T, Kucerova Z, Beach MJ, Lammie PJ (2011) Multiplex bead assay for serum samples from children in Haiti enrolled in a drug study for the treatment of lymphatic filariasis. Am J Trop Med Hyg 85(2):229–237. https://doi.org/10.4269/ajtmh.2011.11-0029

38. Fujii Y, Kaneko S, Nzou SM, Mwau M, Njenga SM, Tanigawa C, Kimotho J, Mwangi AW, Kiche I, Matsumoto S, Niki M, Osada-Oka M, Ichinose Y, Inoue M, Itoh M, Tachibana H, Ishii K, Tsuboi T, Yoshida LM, Mondal D, Haque R, Hamano S, Changoma M, Hoshi T, Kamo K, Karama M, Miura M, Hirayama K (2014) Serological surveillance development for tropical infectious diseases using simultaneous microsphere-based multiplex assays and finite mixture models. PLoS Negl Trop Dis 8(7):e3040. https://doi.org/10.1371/journal.pntd.0003040

39. van Gageldonk PG, von Hunolstein C, van der Klis FR, Berbers GA (2011) Improved specificity of a multiplex immunoassay for quantitation of anti-diphtheria toxin antibodies with the use of diphtheria toxoid. Clin Vaccine Immunol 18(7):1183–1186. https://doi.org/10.1128/CVI.05081-11

40. Smits GP, van Gageldonk PG, Schouls LM, van der Klis FR, Berbers GA (2012) Development of a bead-based multiplex immunoassay for simultaneous quantitative detection of IgG serum antibodies against measles, mumps, rubella, and varicella-zoster virus. Clin Vaccine Immunol 19(3):396–400. https://doi.org/10.1128/CVI.05537-11

41. Priest JW, Kwon JP, Moss DM, Roberts JM, Arrowood MJ, Dworkin MS, Juranek DD, Lammie PJ (1999) Detection by enzyme immunoassay of serum immunoglobulin G antibodies that recognize specific *Cryptosporidium parvum* antigens. J Clin Microbiol 37(5):1385–1392

42. Priest JW, Kwon JP, Arrowood MJ, Lammie PJ (2000) Cloning of the immunodominant 17-kDa antigen from *Cryptosporidium parvum*. Mol Biochem Parasitol 106(2):261–271

43. Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y

44. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

45. Corran PH, Cook J, Lynch C, Leenertse H, Manjurano A, Griffin J, Cox J, Abeku T, Bousema T, Ghani AC, Drakeley C, Riley E (2008) Dried blood spots as a source of anti-malarial antibodies for epidemiological studies. Malar J 7:195. https://doi.org/10.1186/1475-2875-7-195

46. Westgard JO, Barry PL, Hunt MR, Groth T (1981) A multi-rule Shewhart chart for quality control in clinical chemistry. Clin Chem 27(3):493–501

47. Earley MC, Vogt RF Jr, Shapiro HM, Mandy FF, Kellar KL, Bellisario R, Pass KA, Marti GE, Stewart CC, Hannon WH (2002) Report from a workshop on multianalyte microsphere assays. Cytometry 50(5):239–242. https://doi.org/10.1002/cyto.10140

48. Waterboer T, Sehr P, Pawlita M (2006) Suppression of non-specific binding in serological Luminex assays. J Immunol Methods 309(1–2):200–204. https://doi.org/10.1016/j.jim.2005.11.008

49. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227(5259):680–685

# Production and Purification of Functional *Cryptosporidium* Glycoproteins by Heterologous Expression in *Toxoplasma gondii*

## Iwona Driskell and Roberta M. O'Connor

## Abstract

Development of an effective vaccine against cryptosporidiosis is a medical and veterinary priority. However, many putative *Cryptosporidium* vaccine candidates such as surface and apical complex antigens are posttranslationally modified with O- and N-linked glycans. This presents a significant challenge to understanding the functions of these antigens and the immune responses to them. Isolation of large amounts of native antigen from *Cryptosporidium* oocysts is expensive and is only feasible for *C. parvum* antigens. Here, we describe a method of producing recombinant, functional *Cryptosporidium* glycoprotein antigens in *Toxoplasma gondii*. These functional recombinant proteins can be used to investigate the role of glycotopes in *Cryptosporidium* immune responses and parasite–host cell interactions.

**Key words** Glycoproteins, *Cryptosporidium*, *Toxoplasma*, Surface antigens, Glycotopes, Affinity purification

## 1 Introduction

Unlike other apicomplexans, many *Cryptosporidium* antigens are posttranslationally modified with N- and O-linked glycans [1–5] that are critical for antigen function [6] and immune recognition [7]. Thus, recombinant proteins generated in *E. coli* are often not adequate for investigation of the role of a particular antigen in *Cryptosporidium* host–parasite interactions, and isolation of a native antigen for studies is prohibitively expensive and often not feasible. While genetic manipulation of *Cryptosporidium* has recently been reported [8], and improved culture systems have been developed [9–11], production of *Cryptosporidium* transformants is still quite challenging. Moreover, since these transformants are produced in mice, parasites that have a deletion or modification of an antigen necessary for in vivo infection cannot be generated.

To address these challenges, we developed an expression system in *Toxoplasma gondii* that produces recombinant *Cryptosporidium* glycoproteins that can mimic the function of the native antigen [12, 13]. These recombinant glycoproteins can be used to explore the importance of glycotopes for the development of protective immune responses, or identify host receptors for antigens that are critical for parasite attachment and invasion. For example, we have demonstrated that expression of gp40 in *T. gondii* resulted in recombinant glycoproteins recognized by mAb 4E9, a glycotope-specific antibody that neutralizes infection in vitro [7, 13]. In another example of the utility of this system, we found that binding of purified *T. gondii* recombinant CpMuc4 to host cells mimicked that of native CpMuc4, whereas recombinant CpMuc4 produced in *E. coli* exhibited no specific recognition of host cells at all [6].

We describe here a protocol for the generation of recombinant glycoproteins in *T. gondii* and provide recommendations for comparison of the recombinant glycoproteins to the native antigens. We also provide a method for affinity purification of these antigens employing antipeptide antibodies that yields highly purified glycoproteins for experimentation. The methods for generation of *T. gondii* stable transfectants described here are the same as have been described previously by other authors [14]. For routine culturing of human foreskin fibroblast (HFF) cells and *T. gondii*, a recent publication provides an excellent detailed guide [15]. For all protocols listed here, it is important to maintain sufficient flasks and plates of HFF cells to propagate the parasites. The authors recommend that before beginning the protocols described here, scientists new to *Toxoplasma* research familiarize themselves with standard practices in *Toxoplasma* research [15].

## 2   Materials

**General safety concerns/notes:** *Toxoplasma gondii*, *Cryptosporidium parvum*, and HFF cells must be handled following biosafety level 2 (BSL-2) precautions and generation of recombinant *Toxoplasma* parasites must be registered with your institution's Institutional Biosafety Committee.

**Antipeptide antibodies considerations:** Before considering expressing a *Cryptosporidium* antigen in *Toxoplasma gondii*, specific antibodies are needed to identify, characterize, and purify the *Cryptosporidium* glycoprotein of interest. Antipeptide antibodies not only enable affinity purification strategies that result in very highly purified glycoproteins, but in our experience work well in immunofluorescence assays (IFAs) and western blots as well. Many companies produce antipeptide antibodies, but for purposes of the affinity purification strategy outlined here, be sure that the antibodies are raised to a single peptide. Additionally, to achieve a high

enough concentration of the antipeptide antibody for the purification to work, you will need the antipeptide antibody affinity purified from rabbit serum over a peptide column. Most companies will do this as part of the antibody production. Keep in mind that you are seeking to isolate glycoproteins, so, avoid using peptides that are predicted to be glycosylated.

*2.1 Transfections*

1. Cell scrapers.

2. 10 mL Syringes.

3. 25 gauge needles.

4. 4 mm gap cuvettes.

5. Hemocytometer.

6. Centrifuge.

7. Microcentrifuge tubes.

8. T25 flasks.

9. 15 mL centrifuge tubes.

10. Bio-Rad Gene Pulser II electroporator (Bio-Rad Laboratories Inc., Hercules, CA).

11. Parasites and cells: *Toxoplasma gondii*, RH strain, lacking the hypoxanthine–xanthine–guanine phosphoribosyl transferase gene (ΔHXGPRT) propagated in human foreskin fibroblast (HFF) cells (SCRC1041, ATCC) [14, 15].

12. *Toxoplasma* Expression vector: pHLEM vector with the *Cryptosporidium* glycoprotein of interest cloned into the expression site [12]. Expression is under the control of the GRA1 promoter and the stability of the transcript is improved by the inclusion of the GRA2 5′ UTR downstream (*see* **Note 1**). To enable selection of stable transfectants, the plasmid contains the *T. gondii* HXGPRT gene under the control of the *T. gondii* dihydrofolate reductase (DHFR) gene regulatory elements (*see* **Note 2**). You will need 50–100 μg of plasmid DNA per transfection.

13. Incomplete Cytomix: 120 mM KCl, 0.15 mM $CaCl_2$, 10 mM $K_2HPO_4/KH_2PO_4$, pH 7.6, 25 mM HEPES, pH 7.6, 2 mM EGTA, pH 7.6, 5 mM $MgCl_2$, pH all stock solutions with KOH. Filter sterilize and store at 4 °C.

14. Complete cytomix: To incomplete cytomix, add 2 mM ATP, pH 7.6 (a 1:50 dilution of a 100 mM stock), and 5 mM glutathione (GSH) (a 1:20 dilution of a 100 mM stock) and re-sterilize. Complete cytomix can be made fresh the day of the transfection or stored aliquoted at –20 °C.

15. Selection medium: Dulbecco's minimum essential medium (DMEM) containing 10% fetal calf serum, 25 mM HEPES,

2× L-glutamine, 1× penicillin/streptomycin, 50 μg/mL xanthine and 25 μg/mL of mycophenolic acid.

16. Selection flasks: T25 flasks (one per transfection) containing a confluent monolayer of HFF cells in selection medium.

*2.2 Immuno-fluorescence*

1. 22 mm square glass cover slips.
2. 6-well tissue culture plates.
3. Glass microscope slides.
4. Nail polish.
5. Phosphate-buffered saline (PBS), pH 7.2, 0.01 M (autoclaved or sterile filtered).
6. Blocking buffer: 2% v/v normal goat serum in PBS, pH 7.2.
7. Primary antibody solution: An anti-*T. gondii* SAG1 antibody (or other antibody to a *T. gondii* antigen that will identify all the parasites) and antibody to the *Cryptosporidium* antigen to be expressed, diluted to the appropriate concentrations in blocking buffer. The antibodies must be from different species. For example, if you have an anti-*Cryptosporidium* antigen–antibody raised in a rabbit, and this could be coupled with a mouse monoclonal anti-*T. gondii* SAG-1 antibody. You will need 0.5 mL of antibody solution per well of a 6-well plate.
8. Secondary antibody solution: Appropriate secondary antibodies, one labeled with a green fluorescent tag and the other with a red fluorescent tag diluted to the appropriate concentrations in blocking buffer. To continue with the example above, the anti-*Cryptosporidium* antigen–antibody would be detected with a Texas Red labeled goat anti-rabbit IgG and the anti-SAG1 with fluorescein-labeled goat anti-mouse IgG.
9. Ice-cold 100% methanol.
10. Slide mounting medium with fluorescence quenching inhibitor.

*2.3 Western Blots*

1. Dry ice.
2. Ethanol.
3. PVDF membranes.
4. *Cryptosporidium oocysts*, Iowa isolate: These can be purchased from Bunchgrass Farm, Deary Idaho (bunchgrassfarm@gmail.com) or from the *Cryptosporidium* Production Laboratory at University of Arizona (https://acbs.cals.arizona.edu/crypto).
5. Protease Inhibitor Cocktail Set III: OGS lysis buffer (PBS, pH 7.2 containing 1% w/v octyl glucoside), 1× protease inhibitor cocktail set III.

6. 6X SDS-PAGE sample buffer: 375 mM Tris-HCl, pH 6.8, 6% (w/v) SDS, 9% v/v β-mercaptoethanol, 0.03% w/v bromophenol blue. Combine and incubate at 65 °C until all components go into the solution, usually within about 30 min.

7. Biotinylated-HPA lectin.

8. N-acetyl-D-galactosamine.

9. PBST: 0.1% Tween 20 in PBS, pH 7.2.

10. HPA buffer: 0.01 M Tris, 0.15 M NaCl, pH 8.0.

11. Alkaline phosphatase staining kit.

12. 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate: BCIP/NBT alkaline phosphatase (AP) substrate.

**2.4 Cloning Stable Transfectants**

1. 96-well plates.

2. 6-well plates.

3. 22 mm square glass cover slips.

4. 3 μm polycarbonate track-etch membrane filters (to fit Swinnex filter holder).

5. Swinnex filter holders.

6. 25 gauge needles.

7. 10 mL Syringes.

8. Cell scrapers.

9. 15 mL tubes.

**2.5 Affinity Purification**

1. Sonicator with a microprobe that will fit in a 1.5 mL microcentrifuge tube.

2. Anti-*Cryptosporidium* peptide antibody (see above).

3. Cognate peptide.

4. Protein G magnetic beads (Dynabeads, Thermo Fisher Scientific Inc., Waltham, MA).

5. DynaMag-5 Magnet: magnetic tube holder for microcentrifuge tubes (Thermo Fisher Scientific Inc.).

6. Protein G magnetic bead wash solution: Phosphate buffer, pH 8.2 with 0.01% Tween 20.

7. Microcentrifuge filters: 10,000 or 30,000 MWCO (Microcon, Merck Millipore Ltd.).

## 3 Methods

**3.1 Transfections**

1. For every two transfections, set up one T25 flask of HFF cells, heavily infected with RH strain *T. gondii* ΔHXGPRT. Allow tachyzoites to exit spontaneously (about 48 h postinfection).

2. In the morning of the transfection, prepare the plasmid for transfection: Precipitate the plasmid (50–100 μg per transfection) with 1/10th volume 3 M K acetate and 2× volume 100% ethanol. Pellet the DNA at $16,000 \times g$ for 30 min. Remove the supernatant and wash the DNA pellet in 70% ethanol. Allow the pellet to air-dry and resuspend in complete cytomix to 50–100 μg per 100 μL.

3. Scrape flask with a cell scraper to suspend the tachyzoites, and run the suspension three times through a 25 gauge needle to break up any remaining HFF cells and separate clumps of tachyzoites.

4. Pellet the tachyzoites at $400 \times g$ for 15 min at 4 °C, resuspend in incomplete cytomix, and count tachyzoites with a hemocytometer.

5. Pellet the tachyzoites again and then resuspend the parasites in complete cytomix to at least $10^7$ tachyzoites per 800 μL. Note that one T25 flask should yield enough parasites for 2 transfections.

6. In a 4 mm gap cuvette, combine 800 μL of the parasites and 100 μL of prepared *Toxoplasma* expression plasmid (from **step 2**) both in complete cytomix (*see* **Note 3**). Pipette gently to mix.

7. Electroporate using a Bio-Rad Gene Pulser II set at 1.9 kV and 50 μF capacitance (*see* **Note 4**).

8. Leave electroporated tachyzoites at room temperature for 10 min in the cuvette, then use to infect one T25 selection flask with the contents of 1 cuvette.

9. Replicating transfected tachyzoites should be visible after about 1 week in culture. Before proceeding to analysis and cloning, expand the transfected cultures in selection flasks and freeze several vials of parasites as a back-up.

10. Maintain the population in culture under selection in preparation for cloning

### 3.2 Analysis of Transfected Parasites

Before starting the cloning process, we analyze the transfected population to see what percentage of parasites are expressing the *Cryptosporidium* glycoprotein (by IFA), if the glycoprotein is expressed intact, how the apparent molecular weight of the *Toxoplasma* recombinant compares to the *Cryptosporidium* native glycoprotein (by western blot), and if both recombinant and native are similarly glycosylated (by lectin blotting).

### 3.3 Immuno-fluorescence Assays (See Note 5)

1. Place sterile 22 mm square coverslips into the wells of a 6-well plate and seed the wells with HFF cells (*see* **Note 6**).

2. When the HFF monolayer is established, infected the cells with the transfected tachyzoites, using about $10^6$ tachyzoites from a freshly lysed T25.

3. 24 h postinfection (*see* **Note 7**) remove the media and gently rinse the monolayers with PBS. Remove the PBS and fix the cells in ice-cold methanol for 10 min.

4. Remove methanol and wash the cells three times with 3 mL of PBS per well per wash. Add blocking solution and incubate 1 h at room temperature

5. Remove blocking buffer from wells and replace with primary antibody solution. Incubate 1 h at room temperature (*see* **Note 5**).

6. Wash wells 3× with PBS, 5 min per wash.

7. Remove last PBS wash and add secondary antibody solution. Incubate for 1 h.

8. Wash wells 3× with PBS, 5 min per wash.

9. Set up one slide for each cover slip with a drop of mounting media (with anti-quenching reagent) in the center of each slide

10. Leave the PBS in the well. Pick up the coverslip gently with fine forceps and place it cell side down in the drop of mounting media.

11. Firmly press coverslip down to remove excess mounting media. Remove any excess liquid from around the coverslip with a vacuum.

12. Seal the edges of the cover slip with nail polish.

13. Examine under a wide field fluorescence microscope or confocal microscope. Count the number of *Cryptosporidium* antibody positive parasites versus the total number of *Toxoplasma* parasites to get an estimate of the percentage of successful transfectants. You will need to know the percent of parasite population expressing the *Cryptosporidium* glycoprotein in order to successfully clone the parasites (see cloning procedure below)

### 3.4 Western Blot Analysis

The samples to be resolved, blotted and detected by antibody and lectins include non-transfected *Toxoplasma* lysate (negative control), transfected *Toxoplasma* lysate, *Cryptosporidium* lysate (positive control), and if available, the antigen of interest expressed in *E. coli*. Run three sets of these samples for western blotting and lectin blotting (see below).

### 3.4.1 Preparation of Cryptosporidium Lysate (See Notes 8 and 9)

1. Pellet $10^8$ oocysts in a 15 mL conical tube at $4000 \times g$ for 30 min at 4 °C. While oocysts are being pelleted prepare PBS containing 1× protease inhibitors.

2. Resuspend oocysts in 500 μL PBS containing 1× protease inhibitors and transfer to a 1.5 mL microcentrifuge tube.

3. Rinse the 15 mL tube two more times with 250 μL of PBS with protease inhibitors and transfer each rinse to the 1.5 mL tube. Vortex each time as oocysts tend to stick to sides.

4. Pellet the oocysts in 1.5 mL microcentrifuge tube at $8000 \times g$ for 5 min at 4 °C. Remove PBS and resuspend oocysts in 100 μL of PBS with protease inhibitors.

5. Rapidly freeze and thaw the oocysts 20 times alternating between a dry ice ethanol bath and a 37 °C water bath.

6. Add 20 μL of 6× SDS-PAGE running buffer to the 1.5 mL microcentrifuge tube 100 ul of lysed oocysts. Mix gently by inverting to avoid foaming.

7. Boil lysed oocysts sample at 100 °C for 10 min in a heating block.

8. Centrifuge at $16,000 \times g$ for 10 min at room temperature.

9. Distribute five 10 μL aliquots of the supernatant into 1.5 mL microcentrifuge tubes and store at −80 °C.

10. Use 5–10 μL of the aliquots of the oocyst lysate prepared above per lane of the SDS-PAGE gel.

*3.4.2 Preparation of Toxoplasma Lysates*

1. Seed one T25 per transfection with HFF cells and allow the monolayer to become confluent.

2. Replace the medium with selection medium and infect the T25 flask with $2.5 \times 10^6$ of the transfected *Toxoplasma* tachyzoites (From Subheading 3.1, **step 10**).

3. 24 h postinfection (*see* **Note 7**), remove selection medium and rinse/wash cells one time with PBS using 5 mL of PBS per flask.

4. Aspirate PBS use to wash flask and add 1 mL of fresh PBS to the cells and scrape the cells to suspend them in solution.

5. Transfer the cell suspension to 1.5 mL microcentrifuge tube on ice.

6. Pellet the cells at $8000 \times g$ for 10 min 4 °C

7. Take off the supernatant and resuspend the cell pellet in 100 μL of cold OGS lysis buffer.

8. Vortex gently and incubate on ice for 20 min.

9. Centrifuge the sample at $16,000 \times g$ for 30 min in order to pellet insoluble fraction.

10. Distribute 10 μL aliquots of the supernatant into 1.5 mL microcentrifuge tubes and store at −80 °C.

11. Use 5–10 μL of the aliquots of *Toxoplasma* lysate prepared above per lane of the SDS-PAGE gel.

| | |
|---|---|
| *3.4.3 SDS-PAGE and Western Blotting* | 1. All samples should be prepared in SDS-PAGE sample buffer at a final concentration of 1× and boiled for 5 min before being loaded on the gel. |

*3.4.3 SDS-PAGE and Western Blotting*

1. All samples should be prepared in SDS-PAGE sample buffer at a final concentration of 1× and boiled for 5 min before being loaded on the gel.

2. Run three replicate sets of samples for detection with antibody and lectin. Each set of samples should include molecular weight standards, *Cryptosporidium* lysate, non-transfected *Toxoplasma* lysate, transfected *Toxoplasma* lysate, and E. *coli* expressed antigen if available.

3. Resolve lysates by SDS-PAGE and transfer to PVDF membranes following standard practices in your laboratory.

4. Detect one set of the samples by western blotting using your anti-*Cryptosporidium* antigen–antibody following the optimal protocol for that antibody. The glycoprotein produced by *Toxoplasma* should be similar in apparent molecular weight to the native glycoprotein antigen.

**3.5 Lectin Blotting with HPA Lectin**

To determine if the *T. gondii* transfectant has been glycosylated, detection of the antigens with an appropriate lectin should be conducted (*see* **Note 10**). Here we provide a protocol for detecting glycoproteins with HPA lectin (lectin from *Helix pomatia*). HPA detects terminal N-acetyl galactosamine residues, a common glycotope on *Cryptosporidium* antigens.

1. Block the remaining membranes from **step 3** above with PBST for 1 h at room temperature.

2. Rinse blots in HPA buffer.

3. Dilute biotin-conjugated HPA to 10 mg/mL in a sufficient amount of HPA buffer for one of the blots. For the other blot, dilute biotin-conjugated HPA to 10 mg/mL in HPA buffer containing 250 mM N-acetyl galactosamine, HPA's cognate sugar (*see* **Note 11**). This will need to be adjusted for your lectin.

4. Incubate blots with the lectin or lectin plus sugar for 1 h at room temperature.

5. Wash the blots four times, 5 min each with PBST.

6. Incubate the blot with avidin-biotin-alkaline phosphatase complex prepared following the manufacturer's directions.

7. Develop the blot with substrate BCIP/NBT alkaline phosphatase (AP) substrate kit.

**3.6 Isolating Stable Transfectants**

Once expression of the glycoprotein of interest in the *Toxoplasma* transfectants has been verified, clonal populations must be established before proceeding to large-scale cultures and purification of the glycoprotein antigens.

1. Seed a T25 flask with HFF cells and allow to grow to confluence.

2. Infect with transfected parasites as described above, and allow parasites to egress from the cells.

3. Calculate how many 96-well plates you will need for the cloning (*see* **Note 12**), and seed these with HFF cells.

4. Prepare the Swinnex filter holders by placing a 3 μm polycarbonate track-etch membrane filter in each and autoclaving.

5. When the parasites have egressed, scrape the flask to suspend all the parasites in solution and run the suspension through a 25 gauge needle to break up clumps of parasites.

6. Remove a plunger from a 10 mL syringe and attach a sterile Swinnex filter containing a 3 μm polycarbonate track-etch membrane to the syringe. Balance the syringe and filter over a 15 mL centrifuge tube.

7. Add the parasite suspension to the syringe and allow it to run through the filter by gravity. Only a few parasites are needed for the cloning, so there is no need to force the whole suspension through the filter.

8. Count the filtered parasites on a hemocytometer. Dilute to 1.25 parasites/mL medium.

9. Distribute 200 μL of parasite suspension into the wells of a 96-well plate in which HFFs have been grown to confluency.

10. Assuming that you will have about 5–10 clones per plate, seed HFFs into enough 6-well plates to propagate the clones. Also prepare the same number of 6-well plates with cover slips for IFA analysis of each clone (as described in **step 1** of IFA protocol)

11. Leave plates in incubator for 1 week undisturbed. At the end of the week, check each well for growth. Parasite growth will still be discreet enough that you will be able to tell if there is more than one colony in the well.

12. Mark the wells that have a single colony. When these parasites have replicated enough to have established a plaque encompassing about 1/10th to 1/5th of the well, pipet the media up and down in the well to dislodge the parasites.

13. Seed 100 μL of the cloned parasites from the 96-well plate into the well of a 6-well plate, and the remainder into the well of a 6-well plate containing a coverslip for IFA analysis.

14. Run IFAs 24 h postinfection to identify positive clones (*see* **Note 7**).

15. Continue to propagate the positive clones from the 6-well plates by infecting two T25 flasks of HFFs with the parasites

from the 6-well plate. Keep one flask for expansion of the parasites and use the other for western blot analysis.

16. Positive clones that express the full-length glycoproteins should be propagated and frozen stocks established as described [14, 15].

### 3.7 Affinity Purification of Glycoproteins

Now that clonal populations expressing the *Cryptosporidium* glycoprotein antigen of interest have been established, large-scale cultures can be grown for isolation of the recombinant glycoprotein. To confirm that the recombinant glycoprotein is a valid replacement for the native antigen, native antigen should be isolated in parallel for purposes of comparison in studies of the glycoproteins' role in host–parasite interactions.

### 3.7.1 Affinity Purification with Antipeptide Antibody (See Notes 8 and 9)

At all times during the preparation of lysates, samples must be kept on ice and all solutions kept at 4 °C.

### 3.7.2 Preparation of Cryptosporidium Lysate

1. Pellet $6 \times 10^8$ oocysts in a 15 mL tube at $4000 \times g$ for 30 min at 4 °C.

2. Resuspend oocysts in 200 μL PBS with protease inhibitors and transfer to a 2 mL microcentrifuge tube.

3. Rinse the 15 mL tube two more times with 200 μL of PBS with protease inhibitors and transfer each rinse to the 1.5 mL tube. Vortex each time as oocysts tend to stick to sides.

4. Using a sonicator with a microprobe, sonicate for 20–30 min on ice, using 2, 15 s pulses per minute, until more than 90% of the oocysts are lysed as determined by microscopic examination.

5. Centrifuge lysate for 30 min at $16,000 \times g$ in cold room. Transfer supernatant (it will be a bit cloudy) to a new 1.5 mL microcentrifuge tube.

6. Set aside 5 μL per lane for western blot analysis. The remainder will be used for affinity purification.

### 3.7.3 Preparation of Toxoplasma Lysates

1. Seed five T150 flasks with HFF cells and allow the monolayer to become confluent.

2. Infect each T150 flasks with $2 \times 10^7$ freshly lysed tachyzoites (Each T25 flask yields approximately $2 \times 10^7$ tachyzoites).

3. When the parasites have egressed from the host cells (*see* **Note 13**), scrape the flask to suspend the parasites, and transfer the parasite suspension into 50 mL conical centrifuge tubes.

4. Pellet the parasite suspension at $4000 \times g$ for 10 min 4 °C.

5. Remove the supernatant and resuspend the cell pellets in 2–5 mL of PBS.

6. Combine the pellets into a single 50 mL conical tube and pellet again at $4000 \times g$ for 10 min 4 °C.

7. Resuspend the parasites in 1 mL of PBS plus protease inhibitors.

8. Using a sonicator with a microprobe, sonicate for 5–10 min on ice, using two 15 s pulses per minute, until tachyzoites are lysed. Check for lysis by examining a drop under a microscope.

9. Centrifuge suspension at $16,000 \times g$ for 15 min to pellet insoluble material transfer the supernatant to a fresh tube.

10. Set aside 5 µL per lane for western blot analysis

*3.7.4 Affinity Purification (See Note 14)*

1. Add antipeptide antibody to the *Toxoplasma* and *Cryptosporidium* lysates to give a final concentration of 75 µg/mL of antibody.

2. Incubate overnight at 4 °C with gentle mixing, as on a rocking platform.

3. The next day, prepare the beads for affinity purification by placing 250 µL of protein G magnetic beads (*see* **Note 15**) into a 1.5 mL microcentrifuge tube and pull the beads to the side of the tube by insertion into the magnetic holder.

4. Remove the supernatant using a manual pipette, not a vacuum line, as the vacuum will pull the magnetic beads off the magnet.

5. Add 0.5 mL of phosphate buffer, pH 8.2 containing 0.01% Tween 20 to the tube containing beads.

6. Remove the tube from the magnetic holder, vortex beads in the buffer and return tube to the magnetic holder. Once beads have migrated to the side of the tube, again remove the buffer as above.

7. In the same manner, wash the beads two more times.

8. After removing the last wash, add the lysate–antibody mixture from **step 1** to the beads.

9. Incubate with rotation for 1–2 h at 4 °C.

10. Place the tube back on the magnetic holder, and collect the supernatant. This is equivalent to the "flow through" from an affinity column and should be saved for western blot analysis to determine the efficiency of the purification.

11. Add 600 µL of cognate peptide prepared at 10 mg/mL in PBS and incubate 15 min at room temperature with rotation.

12. Place the tube in magnetic holder and collect the supernatant (=eluate).

13. Repeat **steps 11** and **12** two more times, collecting the eluate each time into a separate tube.

14. Take a 5 μL samples from each elution for western blot analysis

15. Concentrate elutions in a 10,000 or 30,000 MWCO microcentrifuge tube filter down to a 20 μL volume.

16. The peptide can be separated from the eluted antigen by repeated ultrafiltration in the microcentrifuge tube filter (*see* **Note 16**). To remove peptide, dilute the concentrated elutions in PBS back-up to 0.5 mL and concentrate again in the microcentrifuge tube filter. Repeat dilution and concentration three times.

17. Evaluate the success of the purification by western blot. Run the pre-column sample (from Subheading 3.7, **step 6** of *Cryptosporidium* lysate preparation and Subheading 3.7, **step 10** of the *Toxoplasma* preparation, the "flow through" (**step 10**) and the elutions (**steps 11–13**) and probe with the anti-*Cryptosporidium* antigen–antibody (*see* **Note 17**).

Depending on the expression levels of the antigen of interest, this procedure should provide 25–100 μg of purified glycoprotein (*see* **Note 18**) that can be used for host cell binding assays, mass spectrometry to characterize glycotopes, or for investigations of the immune response.

## 4  Notes

1. Expression can also be improved by substituting the native signal sequence for a *Toxoplasma* signal sequence. For gp40/15 expression we used the native signal sequence, but discovered that the native gp40 glycoprotein's N-terminus was 10 amino acids further downstream than the N-terminus of the *Toxoplasma* recombinant gp40 [12]. To express CpMuc4 in *Toxoplasma*, we replaced the native signal sequence with the GRA1 signal sequence and the SAG1 signal sequence [6]. Both theseCpMuc4 constructs produced a higher level of CpMuc4 expression in *Toxoplasma* than constructs with the *Cryptosporidium* signal sequence.

2. There are many *Toxoplasma* expression vectors and other selection strategies available; the example we give here is the system that worked for the expression of *Cryptosporidium* surface antigens in our studies [6, 12]. If the *Cryptosporidium* gene to be expressed proves toxic to *Toxoplasma*, another approach would be to place the expression of the gene under a tetracycline inducible promoter [16].

3. Alternatively, electroporation can be carried out in a 2 mm gap cuvette, using $10^7$ parasites in 300 μL and 50–100 μg of plasmid in 100 μL.

4. If the electroporator you are using has a resistance setting, such as the ECM 630 (BTX, Holliston, MA), the suggested settings are 1.8 kV, 25 μF, and 200 Ω resistance.

5. All of the incubations can be done for longer periods of time, or overnight at 4 °C. If the primary antibodies have high background or give a low signal, sometimes, extending the incubation overnight at 4 °C lowers the background and improves the signal.

6. Sometimes, the HFF cells will not establish a monolayer on the glass coverslip. In this case, coat the coverslip with collagen (for example, rat tail collagen coating solution before seeding the cells. To run more samples in a smaller format, 12 mm diameter round coverslips will fit into the wells of a 24-well plate. Alternatively, the assay can be done in 8-well chamber slides, but recently, we have had difficulty establishing the HFF cells in the chamber slides, even when they have been coated with collagen.

7. We generally have good expression of the *Cryptosporidium* glycoprotein by 24 h, but if expression is low, a 48 h timepoint and free tachyzoites should be examined as well.

8. The recommendations given here for preparing the *Cryptosporidium* lysate assume that the antigen of interest will be present at low concentrations. Depending on the antigen you are working with, you may find that less oocysts are needed. In general, however, a highly concentrated lysate gives a cleaner, stronger signal.

9. The directions for generating lysates assume that the antigen to be purified is soluble. If it is not soluble, the addition of 1% octyl glucoside, or some other appropriate detergent to the lysis, wash, and elution buffers may be necessary.

10. EY laboratories (http://eylabs.com/) is an excellent resource for information on lectin specificity and reaction conditions, as well as a source for labeled and immobilized lectins.

11. To confirm the specificity of the lectin reactivity with antigens on the blot, a second identical blot is run in parallel in the presence of the sugar, the lectin is specific for.

12. You will have about 5–10 clones per 96-well plate, so figure out from the percent positive in the mixed population how many plates you will need (i.e., if 20% of the parasites are positive, 1 plate will yield about 1–2 positive clones, thus you would want to use five 96-well plates for the cloning). Some things are harder to clone than others and require more plates.

13. We have found the cleanest way to isolate glycoproteins is to start from free tachyzoites. However, if the antigen is more highly expressed during intracellular stages, better results may

be obtained by collecting the cultures before the parasites have egressed from the HFF cells.

14. This method also works for HA-tagged *Toxoplasma* recombinant glycoproteins using an anti-HA antibody and HA peptide to elute.

15. Dynabead magnetic beads are crucial for the success of the purification. Other brands, often cheaper, have larger beads that do not disperse well into solution, and only capture a fraction of the antigen–antibody complexes that Dynabeads are capable of capturing.

16. When solutions are concentrated in microcentrifuge tube filters, any solute smaller that the MWCO will flow through the filter and larger solutes will be trapped above in the concentrated solution. The peptide is small enough to go through the filter. For the best separation, use the largest MWCO, possible. For example, if the antigen is larger than 40 kDa, the 30 kDa cutoff can be used.

17. Ideally, most of the antigen should have been removed from the flow through by the antibody and beads, and 2–3 elutions with the cognate peptide removed most of the antigen from the antibody. Adjust the concentration of antibody and peptides, the length of the incubations and the temperature, and the number of elutions to optimize the purification.

18. To estimate the amount of antigen purified, we run the native and *T. gondii* recombinant antigen and known amounts of the *E. coli* expressed antigen on a western blot and use densitometry to determine the concentrations of the glycoproteins relative to the *E. coli* protein standard curve.

## References

1. Riggs MW, Stone AL, Yount PA, Langer RC, Arrowood MJ, Bentley DL (1997) Protective monoclonal antibody defines a circumsporozoite-like glycoprotein exoantigen of *Cryptosporidium parvum* sporozoites and merozoites. J Immunol 158(4):1787–1795

2. Cevallos AM, Zhang X, Waldor MK, Jaison S, Zhou X, Tzipori S, Neutra MR, Ward HD (2000) Molecular cloning and expression of a gene encoding *Cryptosporidium parvum* glycoproteins gp40 and gp15. Infect Immun 68(7):4108–4116

3. Perryman LE, Jasmer DP, Riggs MW, Bohnet SG, McGuire TC, Arrowood MJ (1996) A cloned gene of *Cryptosporidium parvum* encodes neutralization-sensitive epitopes. Mol Biochem Parasit 80(2):137–147

4. Barnes DA, Bonnin A, Huang JX, Gousset L, Wu J, Gut J, Doyle P, Dubremetz JF, Ward H, Petersen C (1998) A novel multi-domain mucin-like glycoprotein of *Cryptosporidium parvum* mediates invasion. Mol Biochem Parasit 96(1–2):93–110

5. Priest JW, Kwon JP, Arrowood MJ, Lammie PJ (2000) Cloning of the immunodominant 17-kDa antigen from *Cryptosporidium parvum*. Mol Biochem Parasit 106(2):261–271

6. Paluszynski J, Monahan Z, Williams M, Lai O, Morris C, Burns P, O'Connor R (2014) Biochemical and functional characterization of CpMuc4, a *Cryptosporidium* surface antigen that binds to host epithelial cells. Mol Biochem Parasit 193(2):114–121. https://doi.org/10.1016/j.molbiopara.2014.03.005

7. Cevallos AM, Bhat N, Verdon R, Hamer DH, Stein B, Tzipori S, Pereira ME, Keusch GT, Ward HD (2000) Mediation of *Cryptosporidium parvum* infection in vitro by mucin-like glycoproteins defined by a neutralizing monoclonal antibody. Infect Immun 68(9):5167–5175

8. Vinayak S, Pawlowic MC, Sateriale A, Brooks CF, Studstill CJ, Bar-Peled Y, Cipriano MJ, Striepen B (2015) Genetic modification of the diarrhoeal pathogen *Cryptosporidium parvum*. Nature 523(7561):477–480. https://doi.org/10.1038/nature14651

9. DeCicco RePass MA, Chen Y, Lin Y, Zhou W, Kaplan DL, Ward HD (2017) Novel bioengineered three-dimensional human intestinal model for long-term infection of *Cryptosporidium parvum*. Infect Immun 85(3). https://doi.org/10.1128/IAI.00731-16

10. Heo I, Dutta D, Schaefer DA, Iakobachvili N, Artegiani B, Sachs N, Boonekamp KE, Bowden G, Hendrickx APA, Willems RJL, Peters PJ, Riggs MW, O'Connor R, Clevers H (2018) Modelling *Cryptosporidium* infection in human small intestinal and lung organoids. Nature Microbiol 3(7):814–823. https://doi.org/10.1038/s41564-018-0177-8

11. Morada M, Lee S, Gunther-Cummins L, Weiss LM, Widmer G, Tzipori S, Yarlett N (2016) Continuous culture of *Cryptosporidium parvum* using hollow fiber technology. Int J Parasitol 46(1):21–29. https://doi.org/10.1016/j.ijpara.2015.07.006

12. O'Connor RM, Wanyiri JW, Wojczyk BS, Kim K, Ward H (2007) Stable expression of *Cryptosporidium parvum* glycoprotein gp40/15 in toxoplasma gondii. Mol Biochem Parasitol 152(2):149–158

13. O'Connor RM, Kim K, Khan F, Ward H (2003) Expression of *Cpgp40/15* in *Toxoplasma gondii*: a surrogate system for the study of *Cryptosporidium* glycoprotein antigens. Infect Immun 71:6027–6034

14. Roos DS, Donald RG, Morrissette NS, Moulton AL (1994) Molecular tools for genetic dissection of the protozoan parasite Toxoplasma gondii. Methods Cell Biol 45:27–63

15. Khan A, Grigg ME (2017) Toxoplasma gondii: laboratory maintenance and growth. Curr Protoc Microbiol 44:20C 21 21–20C 21 17. https://doi.org/10.1002/cpmc.26

16. Meissner M, Brecht S, Bujard H, Soldati D (2001) Modulation of myosin A expression by a newly established tetracycline repressor-based inducible system in Toxoplasma gondii. Nucleic Acids Res 29(22):E115

# Chapter 7

# *Cryptosporidium* Genotyping for Epidemiology Tracking

## Dawn M. Roellig and Lihua Xiao

## Abstract

*Cryptosporidium* genotyping has made significant contributions to the species structure and population genetics of *Cryptosporidium* spp. In addition, the standardized method has shown utility in epidemiologic investigations identifying case linkages and contamination sources that could not be determined with traditional epidemiologic tools. The standardized method for *Cryptosporidium* genotyping from stool specimens described here comprises multiple individual protocols to amplify and sequence regions of the SSU rRNA and gp60 genes of *Cryptosporidium*.

**Key words** Genotyping, Subtyping, Molecular epidemiology, PCR, PCR-RFLP, DNA sequencing

## 1 Introduction

Molecular methods have rewritten *Cryptosporidium* taxonomy; what was once considered a single species causing cryptosporidiosis in humans and other animals is now known to be a genera of nearly 40 species, many with multiple subtypes [1]. As all forms are morphologically indistinguishable by traditional diagnostics, molecular tools must be used to identify and distinguish the responsible etiologic agent in specimens.

The utility of these *Cryptosporidium* genotyping methods has expanded beyond population genetics and includes molecular epidemiology of cryptosporidiosis. As recently as 2002, the previously identified *C. parvum* human genotype and bovine genotype were renamed *C. hominis* and *C. parvum* following biological distinction between the two species [2]. *Cryptosporidium* specimens have been systematically collected and characterized since then to reveal *C. hominis* and *C. parvum* account for >95% of human cryptosporidiosis [3]. In addition to cryptosporidiosis surveillance, genotyping has been used in outbreak investigations to differentiate outbreaks within the same geographic locale, link seemingly sporadic cases, confirm foodborne and waterborne contamination
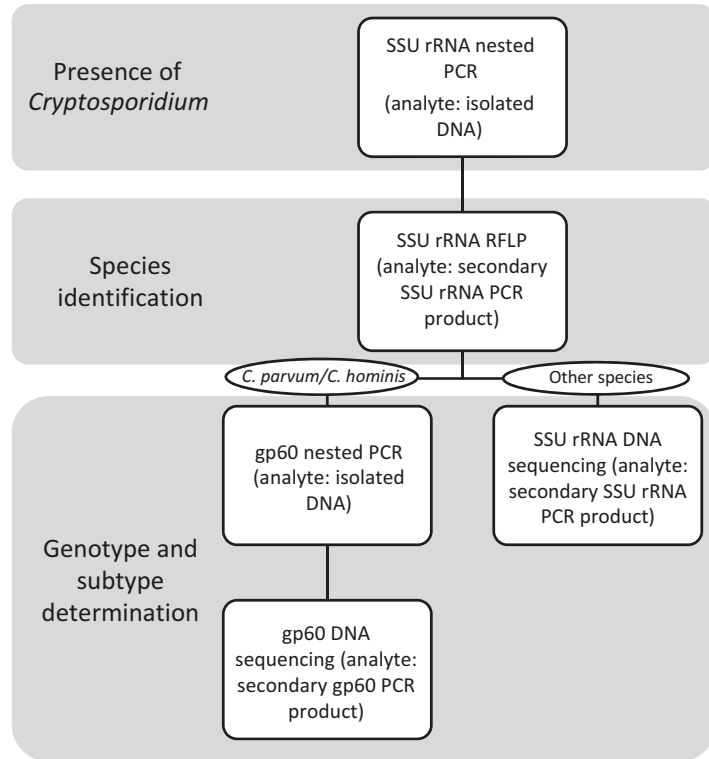
**Fig. 1** The standardized method for *Cryptosporidium* genotyping

sources in outbreaks, and identify rare species and/or subtypes in sporadic and outbreak cases [4–8].

Multiple individual protocols comprise the standardized method for *Cryptosporidium* genotyping (Fig. 1). A nested polymerase chain reaction (PCR) is used to amplify the SSU rRNA gene, a high copy number and conserved gene interspersed with highly polymorphic regions, in all species and genotypes of *Cryptosporidium* [9, 10]. Next, a restriction fragment length polymorphism reaction utilizing the SSU rRNA gene PCR product as the analyte yields unique banding patterns that can distinguish the predominant human pathogenic species, *C. parvum* and *C. hominis*, from other species and genotypes. Then, for specimens that are identified as either *C. parvum* or *C. hominis* by the SSU rRNA PCR-RFLP, amplification and subsequent DNA sequencing of the gp60 gene is used to determine the subtype based on sequence identity to the reference sequences and the number of trinucleotide repeats [10–12]. For those specimens that are not identified as *C. parvum* nor *C. hominis* in the SSU rRNA PCR-RFLP, DNA sequencing of the SSU rRNA amplicon is used to determine and/or confirm the species of *Cryptosporidium* based on sequence identity to reference sequences.

## 2    Materials

| | |
|---|---|
| *2.1    PCR Analysis (SSU rRNA or gp60)* | |

1. Vortex mixer.
2. UV PCR Workstation.
3. Microcentrifuge for 1.5 mL tubes and 0.2 mL tubes.
4. PCR thermal cycler (ABI GeneAmp® PCR System 9700, Thermo Fisher Scientific Inc., Waltham, MA).
5. 1.5 mL microcentrifuge tubes.
6. 0.2 mL PCR tubes.
7. Aerosol resistant pipette tips with filter: 10, 20, 100, 200, 1000 µL.
8. Primary SSU rRNA gene PCR primers:

    Forward (F1): 5′-TTC TAG AGC TAA TAC ATG CG-3′.

    Reverse (R1): 5′-CCC ATT TCC TTC GAA ACA GGA-3′

9. Secondary SSU rRNA gene PCR primers:

    Forward (F2): 5′-GGA AGG GTT GTA TTT ATT AGA TAA AG-3′.

    Reverse (R2): 5′-CTC ATA AGG TGC TGA AGG AGT A-3′

10. Primary PCR gp60 gene primers:

    Forward (F1): 5′-ATG AGA TTG TCG CTC ATT ATC G-3′.

    Reverse (R1): 5′-TTA CAA CAC GAA TAA GGC TGC-3′

11. Secondary PCR gp60 gene primers:

    Forward (F2): 5′-TCC GCT GTA TTC TCA GCC-3′.

    Reverse (R2): 5′-GGA AGG AAC GAT GTA TCT-3′

12. GeneAmp® 10× PCR Buffer I with 15 mM $MgCl_2$ (Applied Biosystems, Foster City, CA).
13. GeneAmp® 10× PCR Buffer II (Applied Biosystems).
14. 100 mM dNTP.
15. 25 mM $MgCl_2$.
16. 100 mg bovine serum albumin (BSA; molecular biology grade).
17. GoTaq® DNA polymerase, 5 U/µL (Promega, Madison, WI).
18. Distilled and deionized water.

| | |
|---|---|
| *2.2    RFLP Analysis of the SSU rRNA Gene* | |

1. Vortex mixer.
2. UV PCR Workstation.
3. Microcentrifuges for 1.5 mL tubes and 0.2 mL tubes.
4. ABI GeneAmp® PCR System 9700.
5. Horizontal gel electrophoresis system.

6. Electrophoresis power supply.

7. Camera photographic hood (documenting gels).

8. Balance.

9. Microwave.

10. Water bath.

11. 1.5 mL microcentrifuge tubes.

12. 0.2 mL PCR tubes.

13. Aerosol resistant pipette tips with filter: 10, 20, 100, 200, 1000 μL.

14. Nested PCR products.

15. Agarose LE (molecular biology grade).

16. 10× Tris borate EDTA (TBE).

17. 100 bp DNA ladder.

18. GelRed nucleic acid gel stain, 10,000× in water.

19. Gel loading dye (Bromophenol Blue).

20. Distilled and deionized water.

21. *Ssp*I restriction enzyme.

22. *Ase*I restriction enzyme.

*2.3  Sequence Analysis*

1. Vortex mixer.

2. UV PCR Workstation.

3. Microcentrifuges for 1.5 mL tubes and 0.2 mL tubes.

4. ABI GeneAmp® PCR System 9700 (Applied Biosystems).

5. Plate mixer.

6. Vacuum centrifuge/concentrator.

7. ABI 3130xl Genetic Analyzer (Applied Biosystems).

8. 1.5 mL microcentrifuge tubes.

9. 0.2 mL PCR tubes.

10. Aerosol resistant pipette tips with filter: 10, 20, 100, 200, 1000 μL.

11. 96-well vacuum manifold (for 96-well PCR plates): Millipore, MultiScreen HTS Vacuum Manifold (Thermo Fisher Scientific Inc.).

12. 96-well PCR filter plates: Millipore, MultiScreen HTS PCR Plate (Thermo Fisher Scientific Inc.).

13. Centrisep Spin Column (Princeton Separations, Adelphia, NJ).

14. MicroAmp Optical 96-well reaction plate (0.2 mL tubes) (Applied Biosystems).

15. 3100 Genetic Analyzer Plate Septa 96-well (Applied Biosystems).

16. Secondary SSU rRNA gene PCR primers:

    Forward (F2): 5′-GGA AGG GTT GTA TTT ATT AGA TAA AG-3′.

    Reverse (R2): 5′-CTC ATA AGG TGC TGA AGG AGT A-3′

17. Secondary PCR gp60 gene primers:

    Forward (F2): 5′-TCC GCT GTA TTC TCA GCC-3′.

    Reverse (R2): 5′-GGA AGG AAC GAT GTA TCT-3′

18. BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

19. Distilled and deionized water.

# 3   Methods

*3.1   PCR Analysis (SSU rRNA or gp60)*

1. Defrost PCR reagents at room temperature.

2. Prepare the PCR master mixture solution by adding all the PCR reagents in a 1.5 mL microcentrifuge tube as suggested in appropriate PCR target reaction (Tables 1 or 2; *see* **Notes 1** and **2**).

**Table 1**
**PCR master mixture recipe for SSU rRNA gene of *Cryptosporidium***

| SSU rRNA gene | | | | | |
|---|---|---|---|---|---|
| **Primary PCR master mixture** | | | **Secondary PCR master mixture** | | |
| Reagent | Per 50 μL reaction (μL) | Final concentration | Reagent | Per 50 μL reaction (μL) | Final concentration |
| Distilled water | 31.35 | | Distilled water | 29.85 | |
| dNTP (1.25 mM) | 4 | 100 μM | dNTP (1.25 mM) | 4 | 100 μM |
| 10× PCR buffer (no MgCl₂) | 5 | 1× | 10× PCR buffer (no MgCl₂) | 5 | 1× |
| MgCl₂ (25 mM) | 4 | 2 mM | MgCl₂ (25 mM) | 4 | 2 mM |
| Primer F1 (10 μM) | 1.25 | 250 nM | Primer F2 (10 μM) | 2.5 | 500 nM |
| Primer R1 (10 μM) | 1.25 | 250 nM | Primer R2 (10 μM) | 2.5 | 500 nM |
| BSA (10 mg/mL) | 2 | 400 ng/μL | GoTaq (5 U/μL) | 0.15 | 0.75 U |
| GoTaq (5 U/μL) | 0.15 | 0.75 U | – | – | – |

**Table 2**
**PCR master mixture recipe for gp60 gene of *Cryptosporidium***

| gp60 Gene | | | | | |
|---|---|---|---|---|---|
| **Primary PCR master mixture** | | | **Secondary PCR master mixture** | | |
| Reagent | Per 50 μL reaction (μL) | Final concentration | Reagent | Per 50 μL reaction (μL) | Final concentration |
| Distilled water | 31.35 | | Distilled water | 29.85 | |
| dNTP (1.25 mM) | 8 | 200 μM | dNTP (1.25 mM) | 8 | 200 μM |
| 10× PCR buffer (w/15 mM MgCl₂) | 5 | 1× | 10× PCR buffer (w/15 mM MgCl₂) | 5 | 1× |
| Primer F1 (10 μM) | 1.25 | 250 nM | Primer F2 (10 μM) | 2.5 | 500 nM |
| Primer R1 (10 μM) | 1.25 | 250 nM | Primer R2 (10 μM) | 2.5 | 500 nM |
| BSA (10 mg/mL) | 2 | 400 ng/μL | GoTaq (5U/μL) | 0.15 | 0.75 U |
| GoTaq (5 U/μL) | 0.15 | 0.75 U | – | – | – |

3. Mix well by vortexing briefly and centrifuge the tube for 5 s to remove any solution trapped in the tube cap.

4. Add 49 μL (for primary PCR) or 48 μL (for secondary PCR) of the PCR master mixture prepared above to each 0.2 mL PCR tube.

5. Add 1 μL UV-treated distilled water to the tube designated as the blank negative control, or add 2 μL PCR product of primary PCRnegative control to the secondary PCR tube designated as secondary PCR negative control.

6. Add 1 μL of the unknown DNA samples to the respective tubes with the master mixture for primary PCR, or add 2 μL of the primary PCR products to the respective tubes with the master mixture for secondary PCR (*see* **Notes 3** and **4**).

7. Add 1 μL positive control DNA to the tube designated as primary PCR positive control, or add 2 μL PCR product of primary PCR positive control to the secondary PCR tube designated as secondary PCR positive control. The positive controls should not be handled until this step to avoid cross-contamination.

**Table 3**
***Cryptosporidium* PCR conditions for genotyping**

| Gene | bp | Primers (10 μM) | 1′ program | 2′ program |
|------|----|-----------------| ---------- | ---------- |
| SSU rRNA (18S) | ~800 | Forward (F1): 5′-TTC TAG AGC TAA TAC ATG CG-3′<br>Reverse (R1): 5′-CCC ATT TCC TTC GAA ACA GGA-3′<br>Forward (F2): 5′-GGA AGG GTT GTA TTT ATT AGA TAA AG-3′<br>Reverse (R2): 5′-CTC ATA AGG TGC TGA AGG AGT A-3′ | *55*<br>94 °C 3 min<br>35 cycles:<br>*94 °C 45s*<br>*55 °C 45s*<br>*72 °C 60s*<br>72 °C 5 min | *58*<br>94 °C 3 min<br>35 cycles:<br>*94 °C 45s*<br>*58 °C 45s*<br>*72 °C 60s*<br>72 °C 5 min |
| gp60 | ~800–850 | Forward (F1): 5′-ATG AGA TTG TCG CTC ATT ATC G-3′<br>Reverse (R1): 5′-TTA CAA CAC GAA TAA GGC TGC-3′<br>Forward (F2): 5′-TCC GCT GTA TTC TCA GCC-3′<br>Reverse (R2): 5′-GGA AGG AAC GAT GTA TCT-3′ | *52*<br>94 °C 3 min<br>35 cycles:<br>*94 °C 45s*<br>*52 °C 45s*<br>*72 °C 60s*<br>72 °C 5 min | *52*<br>94 °C 3 min<br>35 cycles:<br>*94 °C 45s*<br>*52 °C 45s*<br>*72 °C 60s*<br>72 °C 5 min |

8. Mix sample tubes by centrifuging briefly.

9. Select the cycling program for the appropriate PCR reaction (Table 3). Start the thermal cycler.

10. Run the amplified products on a 1.5% agarose gel. For SSU rRNA PCR, a band of ~800 bp should be present to be considered POSITIVE for *Cryptosporidium* DNA. For gp60 PCR, samples with a band of 800–850 bp on the agarose gel are considered POSITIVE, indicating the presence of *Cryptosporidium parvum* or *C. hominis* DNA in the sample analyzed. If no band is visible, or if the band is of incorrect size, the sample is considered NEGATIVE. The negative controls must be negative. The positive controls must be positive. The test should be repeated if any of these quality control samples fail (*see* **Note** 5)**.**

*3.2  RFLP Analysis of the SSU rRNA Gene*

1. Prepare the RFLP master mixture solution for each restriction enzyme by adding all the reagents in the tube labeled for the PCR master mixture (Table 4).

2. Mix well by vortexing briefly and centrifuge the tube for 5 s to remove any solution trapped in the tube cap.

3. Add 30 μL of the RFLP master mixture prepared above to each 1.5 mL tube.

4. Add 10 μL of the secondary PCR products to each respective tube with the master mixture.

**Table 4**
**Restriction fragment length polymorphism assay master mixture recipe for *Cryptosporidium***

| RFLP master mixture | Per 40 μL reaction (μL) | Final concentration |
|---|---|---|
| Distilled water | 22 | – |
| 10× NEBuffer S*sp*I or *Ase*I | 4 | 1× |
| Enzyme *Ssp*I (5 U/μL) or *Ase*I (5 U/μL) | 4 | 20 U |

5. Mix by centrifuging briefly.

6. Incubate the 1.5 mL tubes in 37 °C water bath overnight (at least 12 h).

7. After incubation, centrifuge the tubes briefly to remove any solution trapped in the tube cap.

8. Samples should be run on a 2.0% agarose gel at 100 V for 60 min. *Ssp*I and *Ase*I can differentiate common *Cryptosporidium* species and genotypes by RFLP patterns (Fig. 2 and Table 5; *see* **Notes 6 and 7**).

*3.3 Sequence Analysis (SSU rRNA Gene or gp60 Gene)*

*3.3.1 Cleaning of Secondary PCR Products*

1. Use positive secondary PCR products of SSU rRNA or gp60 nested PCR in DNA sequencing.

2. Load each approximately 40 μL PCR reaction solution (after running the agarose gel) into an individual well in the 96-well PCR filter plate.

3. Place the 96-well PCR filter plate on top of the 96-well PCR filter plate vacuum manifold.

4. Apply vacuum at 25 in. Hg for 10 min or until wells have emptied (~5–12 min, wells will need more time to dry if pressure is <25 in. Hg).

5. Allow 30 extra seconds under vacuum after the wells appear empty to be sure all liquid has filtered. The filters will appear shiny after they are dry.

6. Remove the plate from the manifold, blot from underneath with paper towels, and add 50 μL distilled water into each well.

7. Mix samples on a plate mixer/rocker for 10 min.

8. Transfer sample from each well into its own individual 1.5 mL tube.

*3.3.2 DNA Sequencing Reaction*

1. Thaw sequencing reaction reagents at room temperature.

2. The cleaned PCR products will be sequenced using two sequencing primers (the respective forward and reverse secondary PCR), thus, there are two sequencing reactions for
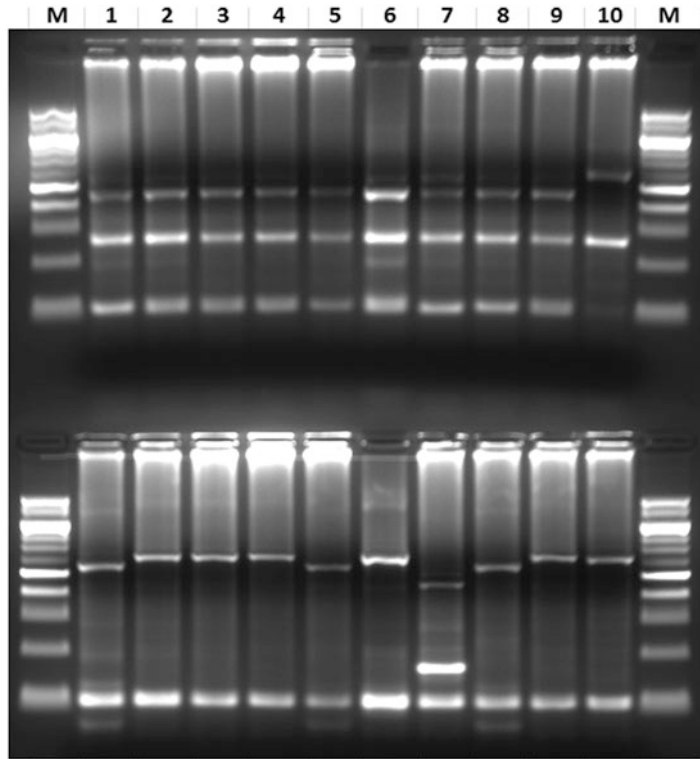
**Fig. 2** RFLP patterns of three major *Cryptosporidium* species in humans using restriction enzymes *Ssp*I (*upper panel*) and *Ase*I (lower panel). Lanes 1, 5, and 8: *C. hominis*; lanes 2, 3, 4, 6, and 9: *C. parvum*; lane 7: *C. meleagridis*; lane 10: *C. baileyi* (positive control); M: 100-bp molecular markers

each PCR product. For each sequencing reaction of 20 µL, prepare the following: Distilled water: 12.5 µL, BigDye Terminator Buffer: 4 µL, Primer (10 µM): 2 µL, BigDye Terminator: 1 µL, (Total: 19.5 µL).

3. Add 19.5 µL of the master mixture to each 0.2 mL PCR tube.

4. In the PCR template area, add 0.5 µL of cleaned PCR product to each respective PCR tube with master mixture solution.

5. Mix by centrifuging briefly.

6. Select and start the cycling program for DNA sequencing as follows: 25 cycles of denaturation at 96 °C, 10 s; annealing at 50 °C, 5 s; extension at 60 °C, 4 min; hold 4 °C until removed from the thermal cycler.

*3.3.3 Cleaning of Sequencing Reaction Products*

1. Rehydrate the Centrisep spin columns to be used: tap the dehydrated contents down, add 0.8 mL distilled water, cap the top, mix well by vortexing, remove bubbles by inverting and sharply tapping the column, and allow hydration for at least 30 min.

**Table 5**
**RFLP patterns by *Ssp* I and *Ase*I digestion in differentiation of common *Cryptosporidium* species and genotypes**

| *Cryptosporidium* | PCR product size (bp) | *Ssp*I products (bp) | *Ase*I products (bp) |
|---|---|---|---|
| *C. hominis* | 851 | **450, 267, 111**, 12, 11 | **561, 115**, 104, **71** |
| *C. cuniculus* | 849 | **473, 267, 109** | **559, 115**, 104, **71** |
| *C. parvum* | 848 | **450, 267, 108**, 12, 11 | **629, 115**, 104 |
| *C. wrairi* | 848 | **450, 267, 109**, 11, 11 | **629, 115**, 104 |
| *C. fayeri* | 851 | **441, 267, 109**, 34 | **632, 115**, 104 |
| Duck genotype | 835 | **432, 267, 102**, 34 | **616, 115**, 104 |
| *C. bovis* | 836 | **432, 267, 103**, 34 | **617, 115**, 104 |
| *C. ryanae* | 836 | **432, 267, 103**, 34 | **617, 115**, 104 |
| *C. xiaoi* | 836 | **413, 267, 103**, 34, 19 | **617, 115**, 104 |
| *C. canis* | 843 | **417, 267, 105**, 34, 20 | **624, 115**, 104 |
| *C. varanii* | 847 | **418, 267, 109**, 34, 19 | **628, 115**, 104 |
| Bear genotype | 847 | **418, 267, 106**, 34, 22 | **628, 115**, 104 |
| Skunk genotype | 852 | **418, 267, 110**, 34, 12, 11 | **460, 173, 115**, 104 |
| Horse genotype | 849 | **450, 267, 109**, 12, 11 | **497, 133, 115**, 104 |
| *C. meleagridis* | 847 | **450, 267, 108**, 11, 11 | **457, 171, 115**, 104 |
| *C. tyzzeri* | 852 | **450, 273, 112**, 12, 11 | **458, 175, 115**, 104 |
| Ferret genotype | 851 | **450, 267, 111**, 12, 11 | **458, 174, 115**, 104 |
| *C. baileyi* | 840 | **573, 267** | **621, 115**, 104 |
| Goose I | 835 | **534, 267**, 34 | **616, 115**, 104 |
| Goose II | 835 | **534, 267**, 34 | **616, 115**, 104 |
| Opossum II | 924 | **495, 429** | **668, 115**, 104, 37 |
| *C. muris* | 847 | **449, 398** | **732, 115** |
| *C. andersoni* | 846 | **449, 397** | **731, 115** |
| *C. serpentis* | 845 | **414, 383**, 34, 14 | **730, 115** |
| *C. ubiquitum* | 849 | **454, 384**, 11 | **461, 169, 115**, 104 |
| *C. suis* | 852 | **454, 378**, 11, 9 | **633, 115**, 104 |
| Muskrat I | 863 | **449, 380**, 34 | **608, 115**, 104, 36 |
| Fox genotype | 846 | **448, 377**, 21 | **627, 115**, 104 |
| *C. felis* | 878 | **426, 404**, 34, 14 | **659, 115**, 104 |

(continued)

**Table 5**
**(continued)**

| *Cryptosporidium* | PCR product size (bp) | *Ssp*I products (bp) | *Ase*I products (bp) |
|---|---|---|---|
| Muskrat II | 847 | **417, 375**, 34, 21 | **592, 115**, 104, 36 |
| *C. galli* | 843 | **843** | **728, 115** |
| *C. testudinis* | 845 | **811**, 34 | **730, 115** |
| Snake genotype | 838 | **804**, 34 | **619, 115**, 104 |

Note: visible bands are shown in bold

2. Remove the top cap and end stopper and place the column into a Wash Tube (2 mL).

3. Centrifuge at 750 × *g* for 2 min. Keep track of the position/orientation of column with respect to G forces, so next time, it is placed in the same orientation.

4. Transfer 20 μL of amplified sequencing reaction products on top of the gel of each column. Do not disturb the top of gel, and do not touch the sides of the column.

5. Place loaded column in Sample Collection Tube, with proper column orientation. Centrifuge 2 min at 750 × *g*.

6. Place the Sample Collection Tubes in the vacuum centrifuge to dry the DNA products. DNA products are ready to be run on an ABI 3130xl Genetic Analyzer or similar instrument.

*3.3.4 Sequence Analysis*

1. Read the chromatograms generated by the DNA sequencer using the ChromasPro software (www.technelysium.com.au/ChromasPro.html) or similar program, assemble the two sequences generated by two sequencing primers for each sample. Check the assembled contigs for sequence uncertainty and trim ambiguities on the 5′ and 3′ end.

2. Align the generated SSU rRNA or gp60 sequences with each other and reference sequences using the software Mega (http://megasoftware.net/) or similar software. Check the sequence alignment for sequencing accuracy.

3. Determine sequence designation based on sequence identity to reference sequences. Determine subtype designation based on sequence identity to reference sequences and the number of trinucleotide repeats (*see* **Note 8**).

## 4    Notes

1. All master mixtures should be made in preprocessing spaces that are free of contaminants and specimens; template should be added in a clean PCR workstation. Both precautions will reduce contamination.

2. All materials used in making the master mixture (all pipettes, the tray with 0.2 mL PCR tubes, the tubes labeled for primers and master mixture, and the tube with distilled water) should be exposed to UV light inside a UV Cross-linker for automatic cross-link. Do not expose the tubes containing reagents to the UV light.

3. PCR of all stool specimens should be run in duplicate.

4. Water or environmental specimens can be tested with the method, but success varies widely. A minimum of five replicates should be tested. The template volume can be adjusted up to 5 μL in primary PCR reactions. The volume of water added to the master mixture should be adjusted to accommodate the additional template to ensure the final reaction volume remains 50 μL.

5. Any instance where expected gp60 amplification is not successful, DNA sequencing of the SSU rRNA gene should be completed. For example, *C.* Chipmunk genotype I can infect humans. The SSU rRNA-RFLP banding pattern resembles *C. parvum* and gp60 will not amplify; ruling out or confirming *C. parvum* in such samples would require sequencing of the SSU rRNA product.

6. When results of *Ssp*I/*Ase*I digestion have shown the RFLP pattern of *C. andersoni*/*C. muris*, the enzyme *Dde*I can differentiate the two species.

7. The enzyme *Mbo*II is used in ruminant studies in conjunction with *Ssp*I to differentiate *C. parvum*, *C. bovis* and C. *ryanae* (deer-like genotype), and *Ase*I RFLP is not needed for these samples.

8. Subtypes of *C. parvum* and *C. hominis* at the gp60 locus are named based on their subtype family designations and the number of each type of trinucleotide repeats. For each subtype, the name starts with the subtype family designation (Ia, Ib, Id, Ie, If, Ig, etc. for *C. hominis* and IIa, IIb, IIc, IId, IIe, IIf, IIg, IIh, IIi, etc. for *C. parvum*) followed by the number of TCA (represented by the letter A), TCG (represented by the letter G), and TCT (represented by the letter T) repeats found (Table 6; Fig. 3). In the IIa subtype family, a few subtypes have two copies of the ACATCA sequence right after the trinucleotide repeats, which are represented by "R2" (R1 for most subtypes). The subtype family Ia has different copies of a 15-bp repetitive sequence 5′-AAA/G ACG GTG GTA AGG- 3′ (the

**Table 6**

**Trinucleotide repeats present in the *Cryptosporidium* gp60 gene and associated designation for subtype nomenclature**

| Trinucleotide repeat (5′ → 3′) | Subtype designation |
|---|---|
| TCA | A |
| TCG | G |
| TCT | T |
| ACATCA | R[a] |
| AAA/G ACG GTG GTA AGG | R[b] |
| C/AAG AA/GG GCA AAG AAG | R[c] |

[a]Only within *C. parvum* subtype family IIa
[b]Only within *C. hominis* subtype family Ia
[c]Only within *C. hominis* subtype family If
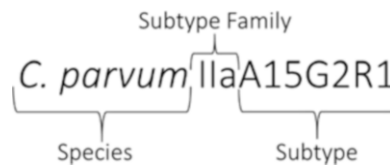


**Fig. 3** Example of *C. parvum* subtype

last copy is 13-bp) shortly after the trinucleotide repeats, which is represented by the letter "R." The subtype family IIc was previously known as Ic. It differs from other subtype families by having no variation in the number of trinucleotide repeats (all A5G3) and by having extensive sequence polymorphism at the 3′ end of the gene. Thus, subtypes in the family are differentiated from each other by the additional letters "a" to "g," such as IIcA5G3a (reference sequence AF164491), IIcA5G3b (reference sequence AF164501), IIcA5G3c, IIcA5G3d (reference sequence AF440621), IIcA5G3e, IIcA5G3f, and IIcA5G3g. Occasionally, a few subtypes in other families have the same trinucleotide repeat sequence, but differ from each other by one or two nucleotides in the sequence after the repeat region. They are differentiated from each other by the addition of lower case letters, such as IIdA18G1a, IIdA18G1b, IIdA18G1c, and IIdA18G1d.

# Acknowledgments

conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

## References

1. Ryan U, Fayer R, Xiao L (2014) *Cryptosporidium* species in humans and animals: current understanding and research needs. Parasitology 141(13):1667–1685. https://doi.org/10.1017/S0031182014001085

2. Morgan-Ryan UM, Fall A, Ward LA, Hijjawi N, Sulaiman I, Fayer R, Thompson RC, Olson M, Lal A, Xiao L (2002) *Cryptosporidium hominis* n. sp. (Apicomplexa: Cryptosporidiidae) from Homo sapiens. J Eukaryot Microbiol 49(6):433–440

3. Chalmers RM, Smith R, Elwin K, Clifton-Hadley FA, Giles M (2011) Epidemiology of anthroponotic and zoonotic human cryptosporidiosis in England and Wales, 2004-2006. Epidemiol Infect 139(5):700–712. https://doi.org/10.1017/S0950268810001688

4. Blackburn BG, Mazurek JM, Hlavsa M, Park J, Tillapaw M, Parrish M, Salehi E, Franks W, Koch E, Smith F, Xiao L, Arrowood M, Hill V, da Silva A, Johnston S, Jones JL (2006) Cryptosporidiosis associated with ozonated apple cider. Emerg Infect Dis 12(4):684–686. https://doi.org/10.3201/eid1204.050796

5. Fill MA, Lloyd J, Chakraverty T, Sweat D, Manners J, Garman K, Hlavsa MC, Roellig DM, Dunn JR, Schaffner W, Jones TF (2017) Cryptosporidiosis outbreak associated with a single hotel. J Environ Health 79(9):16–22

6. Hlavsa MC, Roellig DM, Seabolt MH, Kahler AM, Murphy JL, McKitt TK, Geeter EF, Dawsey R, Davidson SL, Kim TN, Tucker TH, Iverson SA, Garrett B, Fowle N, Collins J, Epperson G, Zusy S, Weiss JR, Komatsu K, Rodriguez E, Patterson JG, Sunenshine R, Taylor B, Cibulskas K, Denny L, Omura K, Tsorin B, Fullerton KE, Xiao L (2017) Using molecular characterization to support investigations of aquatic facility-associated outbreaks of cryptosporidiosis - Alabama, Arizona, and Ohio, 2016. MMWR Morb Mortal Wkly Rep 66(19):493–497. https://doi.org/10.15585/mmwr.mm6619a2

7. Feng Y, Tiao N, Li N, Hlavsa M, Xiao L (2014) Multilocus sequence typing of an emerging *Cryptosporidium hominis* subtype in the United States. J Clin Microbiol 52(2):524–530. https://doi.org/10.1128/JCM.02973-13

8. Xiao L, Hlavsa MC, Yoder J, Ewers C, Dearen T, Yang W, Nett R, Harris S, Brend SM, Harris M, Onischuk L, Valderrama AL, Cosgrove S, Xavier K, Hall N, Romero S, Young S, Johnston SP, Arrowood M, Roy S, Beach MJ (2009) Subtype analysis of *Cryptosporidium* specimens from sporadic cases in Colorado, Idaho, New Mexico, and Iowa in 2007: widespread occurrence of one *Cryptosporidium hominis* subtype and case history of an infection with the *Cryptosporidium* horse genotype. J Clin Microbiol 47(9):3017–3020. https://doi.org/10.1128/JCM.00226-09

9. Xiao L, Alderisio K, Limor J, Royer M, Lal AA (2000) Identification of species and sources of *Cryptosporidium* oocysts in storm waters with a small-subunit rRNA-based diagnostic and genotyping tool. Appl Environ Microbiol 66(12):5492–5498

10. Xiao L, Fayer R, Ryan U, Upton SJ (2004) *Cryptosporidium* taxonomy: recent advances and implications for public health. Clin Microbiol Rev 17(1):72–97

11. Alves M, Xiao L, Sulaiman I, Lal AA, Matos O, Antunes F (2003) Subgenotype analysis of *Cryptosporidium* isolates from humans, cattle, and zoo ruminants in Portugal. J Clin Microbiol 41(6):2744–2747

12. Sulaiman IM, Hira PR, Zhou L, Al-Ali FM, Al-Shelahi FA, Shweiki HM, Iqbal J, Khalid N, Xiao L (2005) Unique endemicity of cryptosporidiosis in children in Kuwait. J Clin Microbiol 43(6):2805–2809. https://doi.org/10.1128/JCM.43.6.2805-2809.2005

# Use of Markers to Determine *Cryptosporidium* Genotypes for Epidemiology Tracking and Detection

## Anson V. Koehler and Jan Šlapeta

## Abstract

Polymerase chain reaction (PCR) enables amplification of specific DNA fragments for the detection and tracking of *Cryptosporidium* spp. Newly obtained DNA are compared to an ever-growing database of *Cryptosporidium* sequences with uncertain or outdated metadata in primary public repositories (i.e., EMBL/DDBJ/GenBank). Here, we describe standard operating procedures to obtain DNA sequences from *Cryptosporidium* spp. marker genes. Small-subunit ribosomal RNA gene, large-subunit ribosomal RNA gene, and glycoprotein 60 are amplified using conventional PCR. Amplified and sequenced genes are compared to a reference library of up-to-date curated gene sequences to identify *Cryptosporidium* species and variants.

**Key words** PCR, DNA, Genotyping, LSU, SSU, gp60, *Cryptosporidium*

## 1 Introduction

*Cryptosporidium* spp. (Apicomplexa) is the causative agent of cryptosporidiosis which is responsible for diarrhea in humans around the world [1]. Over 30 species and 44 genotypes of *Cryptosporidium* have been found in a wide array of vertebrates [2–4]. Due to the small size of the oocysts and indistinguishable morphological features, methods such as PCR combined with Sanger sequencing are necessary for *Cryptosporidium* identification and characterization [5]. For species-level differentiation of *Cryptosporidium*, the small-subunit rRNA gene (18S) marker has served well for 20 years [6]. Recently, primers targeting the V8 portion of the large-subunit ribosomal rRNA gene (28S) were designed and the amplified region was able to distinguish species of *Cryptosporidium* [7]. For *Cryptosporidium* outbreaks, the glycoprotein 60 (gp60) gene including the serine repeat region is often used to further classify subtypes of the major *Cryptosporidium* species and genotypes of public health significance including species *C. parvum/pestis*, *C. hominis*, and *C. cuniculus* [8]. Due to the large variation found in

the gp60 gene, not all species and genotypes of *Cryptosporidium* are currently able to be subtyped using these markers, therefore additional primers (not demonstrated with this protocol) have been developed to target other *Cryptosporidium* including *C. fayeri* [9], *C. meleagridis* [10], *C. ubiquitum* [11], *C. viatorum* [12], *C.* sp. chipmunk genotype [13], and *C.* sp. skunk genotype [14].

Here, we present a standard operating procedure to obtain DNA sequences from *Cryptosporidium* spp. marker genes which can then be compared to a reference library of curated sequences in an effort to identify *Cryptosporidium* species and variants.

## 2    Materials

1. Sterile double-distilled water (PCR water).

2. Amplification primers stock solutions (100 pmol/μL or 100 μM each).

3. Amplification primers working solutions (10 pmol/μL or 10 μM each).

4. MyTaq™ Red Mix (Bioline, Meridian Life Science, Inc., Memphis, TN) or MangoTaq™ (Bioline).

5. dNTPs.

6. Agarose.

7. TBE buffer.

8. DNA molecular marker (1 kb ladder).

9. PCR purification kit.

10. NanoDrop ND-1000 spectrophotometer.

11. 200-μL thin-walled PCR tube.

12. Set of automatic pipettes (0.5–2, 2–20, 20–200 μL) with respective sterile filtered pipette tips.

13. Thermal cycler.

14. Agarose gel tank and power supply.

15. Nucleic acid stain (e.g., gel red, SYBR safe, ethidium bromide).

## 3    Methods

The methods require biological material confirmed or suspected to contain *Cryptosporidium* oocysts. Initially total genomic DNA is isolated using preferred DNA isolation protocol (*see* **Note 1**). Polymerase chain reaction (PCR) amplification of the genes— small-subunit rRNA gene (18S), large-subunit ribosomal rRNA gene (28S), and *Cryptosporidium* glycoprotein 60 (gp60)—is

followed by sequencing of the PCR products to determine the exact DNA. The sequences of the genes are conserved within, but differ across the different *Cryptosporidium* variants enabling tracking and detection.

All procedures were carried out on ice unless otherwise specified.

**3.1 PCR Amplification of *Cryptosporidium* Small-Subunit Ribosomal rRNA Gene**

1. Amplify a portion of the small-subunit rRNA gene (18S). Primers for amplification are listed in Table 1. These primers are sufficiently conservative to amplify most of the *Cryptosporidium* species [6, 15]. A two-step nested PCR protocol is used to amplify 18S with primers F2/RX followed by F3N/R3N.

**Table 1**

**Summary of primers used for the amplification of portions of the SSU, LSU, and gp60 gene regions of *Cryptosporidium***

| Gene region | PCR | Direction | Name | Primer 5′–3′ | Fragment size (bp) | Source |
|---|---|---|---|---|---|---|
| SSU | Primary | Forward | F2 | TTC TAG AGC TAA TAC ATG CG | ~1350 | [6] |
| | | Reverse | RX | CCC ATT TCC TTC GAA ACA GGA | ~1350 | [6] |
| | Secondary | Forward | F3N | GGA AGG GTT GTA TTT ATT AGA TAA AG | ~850 | [15] |
| | | Reverse | R3N | AAG GAG TAA GGA ACA ACC TCC A | ~850 | [15] |
| LSU | Primary | Forward | LSU2040F | CGA ATA GCG TTA TCT TTG CTA TTT | ~1000 | [7] |
| | | Reverse | LSU3020R | GTC TTC CGC GAA GAT CAG | ~1000 | [7] |
| | Secondary | Forward | LSU2065F | TTA CCA TGG AAT YAG TTC AGC | ~400 | [7] |
| | | Reverse | LSU2557R | AAC ACC ATT TTC TGG CCA TC | ~400 | [7] |
| gp60 | Primary | Forward | AL3531 | ATA GTC TCC GCT GTA TTC | ~1000 | [8] |
| | | Reverse | AL3535 | GGA AGG AAC GAT GTA TCT | ~1000 | [8] |
| | Secondary | Forward | AL3532 | TCC GCT GTA TTC TCA GCC | ~850 | [8] |
| | | Reverse | AL3534 | GCA GAG GAA CCA GCA TC | ~850 | [8] |

2. Prepare the following first-round PCR mix on ice in a 200-μL thin-walled PCR tube. Mix 2-μL genomic DNA (10–100 ng) with 15 μL of MyTaq™ Red Mix (Bioline) and 0.5 μL of forward and reverse primer F2/RX (10 pmol/μL or 10 μM each) and add up to 30 μL sterile double-distilled water (*see* **Note 2**).

3. Amplify using the following PCR protocol in a thermal cycler (*see* **Note 3**). Initial step of 2 min 95 °C (initial denaturation) followed by 35 cycles of amplification steps (15 s 98 °C denaturation, 15 s 55 °C annealing, 15 s 72 °C extension) and final extension step of 5 min 72 °C. Hold at 10 °C until ready to proceed with next step. Amplification with MyTaq™ Red Mix (Bioline) requires extension time of 10 s for amplicons under 1 kb.

4. Prepare the following second-round PCR mix on ice in a 200-μL thin-walled PCR tube. Use 0.5–2 μL first-round PCR product as template (*see* **Note 4**). Use 15 μL of MyTaq™ Red Mix (Bioline) and 0.5 μL of forward and reverse primer F3N/R3N (10 pmol/μL or 10 μM each) and add up to 30 μL sterile double-distilled water (*see* **Note 5**).

5. Amplify using the following PCR protocol in a thermal cycler. Initial step of 2 min 95 °C (initial denaturation) followed by 35 cycles of amplification steps (15 s 98 °C denaturation, 15 s 55 °C annealing, 10 s 72 °C extension) and final extension step of 5 min 72 °C. Hold at 10 °C until ready to proceed with next step. Amplification with MyTaq™ Red Mix (Bioline) requires extension time of 10 s for amplicons under 1 kb.

6. Verify positive PCR products by running a small aliquot (5 μL) of each reaction on a 1% agarose gel in 1× TBE buffer as per standard protocols (*see* **Note 6**).

7. Purify the PCR products using a commercial PCR purification kit following the manufacturer's recommendations and send the PCR products for sequencing using both primers used in the PCR, as per sequencing facility requirements (*see* **Note 7**).

8. Assemble the new sequence using software enabled for visual inspection of DNA sequencing chromatographs. Visually confirm the quality and unambiguity of the new sequence. Create a consensus, by removing the primer sequence (*see* **Note 8**).

9. Compare the assembled consensus DNA sequence to the published sequences. Go to the NCBI website for BLAST (http://www.ncbi.nlm.nih.gov/blast/) and use nucleotide-against-nucleotide (blastn) tab (*see* **Note 9**).

*3.2 PCR Amplification of Cryptosporidium Large-Subunit Ribosomal rRNA Gene*

1. Amplify a portion of the large-subunit rRNA gene (28S). Primers for amplification are listed in Table 1. These primers are sufficiently conservative to amplify most of the *Cryptosporidium* species [7]. A two-step nested PCR protocol is used to amplify 28S with primers LSU2040F/LSU3020R followed by LSU2065F/LSU2557R.

2. For a 50-μL reaction, prepare the following first-round PCR mix on ice in a 200-μL thin-walled PCR tube. Mix 2-μL genomic DNA (10–100 ng) with 1 μL of 25 mM dNTPs (6.25 mM each) (Promega), 2 μL of 25 mM $MgCl_2$, 10 μL of 5× PCR Mango buffer, 0.25 μL MangoTaq™ polymerase (Bioline), and 0.25 μL of forward and reverse primer LSU2040F/LSU3020R (100 pmol/μL or 100 μM each) and add 34.25 μL sterile double-distilled water (*see* **Note 10**).

3. Amplify using the following PCR protocol in a thermal cycler. Initial step of 5 min 94 °C (initial denaturation) followed by 35 cycles of amplification steps (30 s 94 °C denaturation, 30 s 58 °C annealing, 50 s 72 °C extension) and final extension step of 5 min 72 °C. Hold at 10 °C until ready to proceed with next step. Amplification with MangoTaq™ (Bioline) requires extension time of 15–30 s for amplicons of 1 kb in size.

4. Prepare the following second-round PCR mix on ice in a 200-μL thin-walled PCR tube. Use 1-μL first-round PCR product as template with 1 μL of 25 mM dNTPs (6.25 mM each), 2 μL of 25 mM $MgCl_2$, 10 μL of 5× PCR Mango buffer, 0.25 μL MangoTaq™ polymerase (Bioline), and 0.25 μL of forward and reverse primer LSU2040F/LSU3020R (100 pmol/μL or 100 μM each) and add 35.25 μL sterile double-distilled water (*see* **Note 11**).

5. Amplify using the same PCR protocol as before except reduce the extension time to 30 s.

6. Verify positive PCR products by running a small aliquot (5 μL) of each reaction on a 1% agarose gel in 1× TBE buffer as per standard protocols (*see* **Note 12**).

7. Purify the PCR products using a commercial PCR purification kit following the manufacturer's recommendations and send the PCR products for sequencing using both primers used in the PCR, as per sequencing facility requirements.

8. Assemble the new sequence using software enabled for visual inspection of DNA sequencing chromatographs. Visually confirm the quality and unambiguity of the new sequence (*see* **Note 13**). Create a consensus, by removing the primer sequence.

9. Compare the assembled consensus DNA sequence to the published sequences. Go to the NCBI website for BLAST (http://www.ncbi.nlm.nih.gov/blast/) and use nucleotide-against-nucleotide (blastn) tab.

*3.3 PCR Amplification of Cryptosporidium Glycoprotein 60*

*Cryptosporidium subtyping (gp60, synonymous with gp15 or gp15/45/60)*

1. Amplify a portion of the glycoprotein 60 gene (gp60). Primers for amplification are listed in Table 1. These primers are able to

amplify *C. hominis*, *C. parvum/pestis*, and *C. cuniculus* [8]. A two-step nested PCR protocol is used to amplify gp60 with primers AL3531/AL3535 followed by AL3532/AL3534.

2. Prepare the following first-round PCR mix on ice in a 200-μL thin-walled PCR tube. Mix 2-μL genomic DNA (10–100 ng) with 15 μL of MyTaq™ Red Mix (Bioline) and 0.5 μL of forward and reverse primer AL3531/AL3535 (10 pmol/μL or 10 μM each) and add up to 30 μL sterile double-distilled water (*see* **Note 2**).

3. Amplify using the following PCR protocol in a thermal cycler. Initial step of 2 min 95 °C (initial denaturation) followed by 35 cycles of amplification steps (15 s 98 °C denaturation, 15 s 50 °C annealing, 15 s 72 °C extension) and final extension step of 5 min 72 °C. Hold at 10 °C until ready to proceed with next step. Amplification with MyTaq™ Red Mix (Bioline) requires extension time of 10 s for amplicons under 1 kb.

4. Prepare the following second-round PCR mix on ice in a 200-μL thin-walled PCR tube. Use 0.5–2 μL first-round PCR product as template (*see* **Note 4**). Use 15 μL of MyTaq™ Red Mix (Bioline) and 0.5 μL of forward and reverse primer AL3532/AL3534 (10 pmol/μL or 10 μM each) and add up to 30 μL sterile double-distilled water (*see* **Note 5**).

5. Amplify using the following PCR protocol in a thermal cycler. Initial step of 2 min 95 °C (initial denaturation) followed by 35 cycles of amplification steps (15 s 98 °C denaturation, 15 s 50 °C annealing, 10 s 72 °C extension) and final extension step of 5 min 72 °C. Hold at 10 °C until ready to proceed with next step. Amplification with MyTaq™ Red Mix (Bioline) requires extension time of 10 s for amplicons under 1 kb.

6. Verify positive PCR products by running a small aliquot (5 μL) of each reaction on a 1% agarose gel in 1× TBE buffer as per standard protocols (*see* **Note 14**).

7. Purify the PCR products using a commercial PCR purification kit following the manufacturer's recommendations and send the PCR products for sequencing using both primers used in the PCR, as per sequencing facility requirements (*see* **Note 7**).

8. Assemble the new sequence using software enabled for visual inspection of DNA sequencing chromatographs. Visually confirm the quality and unambiguity of the new sequence. Create a consensus, by removing the primer sequence (*see* **Note 8**).

9. Compare the assembled consensus DNA sequence to the published sequences. Go to the NCBI website for BLAST (http://www.ncbi.nlm.nih.gov/blast/) and use nucleotide-against-nucleotide (blastn) tab (*see* **Note 9**).

*3.4 Detection of Cryptosporidium Genotype Using Reference Library*

1. Download reference sequence data for 18S, 26S, and gp60 that include reference sequences aligned for sequence comparison (sequences are provided in FASTA format, as of July 7, 2018 at LabArchives http://dx.doi.org/10.25833/2gm4-fc39). Open or download, then install and open MEGA7 (https://www.megasoftware.net/) [16] (*see* **Note 15**).

2. Open reference alignment, append the assembled consensus DNA sequence, and align in MEGA7. Visually inspect sequence alignment and manually edit as required (*see* **Note 16**). Once finished, save and open for phylogenetic analysis.

3. Construct a pairwise table. In the option table, select Kimura 2-parameter model and pairwise deletion option for gaps/missing data treatment. The resulting table is inspected for pairwise distance between the new sequence and the reference dataset.

4. Construct a phylogenetic tree. Select minimum evolution method. In the option table, select bootstrap method and 1000 bootstrap replicates, select Kimura 2-parameter model and pairwise deletion option for gaps/missing data treatment. The resulting phylogenetic tree is inspected for position of the new sequence compared to the reference dataset (*see* **Note 17**).

# 4 Notes

1. DNA is isolated with high-quality fecal or soil total DNA isolation kit, that is, PowerFecal® DNA Isolation Kit (Qiagen, Inc., Germantown, MD), FastDNA™ SPIN Kit for Feces (MP Biomedicals LLC, Santa Ana, CA) or ZR Fecal DNA MiniPrep™ (Zymo Research, Irvine, CA). Extraction of *Cryptosporidium* DNA from environmentally resistant oocysts requires homogenization with high-speed homogenizer such as Fast Prep-24 (MP Biomedicals). The number of *Cryptosporidium* oocysts in biological or environmental samples can be low. Any DNA isolation workflow should be validated in house by your laboratory for *Cryptosporidium* limit of detection and reproducibility of such workflow. DNA isolation workflow must include steps to control for cross-contamination by including a blank DNA isolation in each DNA isolation batch to be tested for potential contaminants.

2. PCRs are typically prepared with positive control (DNA that include *Cryptosporidium* DNA), nontarget negative control (DNA that does not include *Cryptosporidium* DNA), negative control (DNA template is substituted by sterile double-distilled water), and blank DNA extraction control. Therefore, even a single tested sample will include a minimum of 5 PCRs that are

prepared as the mastermix. The mastermix is prepared by multiplying the volumes of all the components of PCR by the number of planned reactions plus typically 20% (to account for pipetting inaccuracies). In our example, the mastermix will include 90 µL of MyTaq™ Red Mix (Bioline), 3 µL of F2 primer, 3 µL of RX primer, and 72 µL of sterile double-distilled water. The mastermix is mixed and 28 µL are dispensed into a 200-µL thin-walled PCR tube. Do not forget to label each tube with unique identifier. We routinely use MyTaq™ Red Mix (Bioline), however, many alternatives exist and can be sourced from a variety of molecular biology vendors including setup with individually added DNA polymerase, dNTPs, and $MgCl_2$. Our preference is to use premixes that include DNA polymerase, dNTPs, $MgCl_2$, and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR, because it limits number of pipetting steps and therefore minimizes opportunity for cross-contamination.

3. Thermocycler conditions will vary depending on the brand of thermocycler and the brand of *Taq* DNA polymerase used. Always consult the manufacturer's recommendations and adjust your protocol accordingly.

4. The volume of first-round PCR product as DNA template required for amplification will vary according to the initial amount of parasite DNA. Note that excess amount of DNA template from the primary PCR may result in the presence of nonspecific amplification. An initial trial with 1 µL is recommended, to be later adjusted as needed based on the presence of specific and/or nonspecific amplicons. Alternatively, PCR with 0.5, 1, and 2 µL is prepared simultaneously for direct comparison. Nested PCRs are prone to cross-contamination, and extreme care must be exercised to avoid cross-contamination. Primary PCRs must be spun down and opened in an environment that can be completely decontaminated with the use of 10% bleach and/or UV light for 10 min. Template is added last and only when all other material is packed away.

5. Similar to the first round of PCR, mastermix is prepared according to the abovementioned suggestions adjusting the volumes according to the template volume used. With any nested PCR extreme care must be exercised to avoid cross-contamination. Primary PCRs must be spun down and opened in environment that can be completely decontaminated. Template is added last and only when all other material is packed away.

6. Products such as Gel Red® (Biotium, Inc., Fremont, CA) and SYBR Safe® (Invitrogen Corp., Carlsbad, CA) can be substituted

for traditional nucleic acid stains such as ethidium bromide. You are expecting a single PCR amplicon for each sample. Multiple bands indicate nonoptimal PCR amplification requiring troubleshooting. If a PCR amplicon is present with additional minor bands of different sizes, you may consider cutting the PCR amplicon from the gel. The abovementioned protocol will yield ~780 nt sequence (excluding primers). An alternative protocol using two-step nested PCR with 18SiCF2/18SiCR2 [17] followed by 18SiCF1/18SiCR1 using identical mixing and PCR conditions as above protocol will yield ~530 nt sequence (excluding primers).

7. Some sequencing facilities will perform PCR purification; therefore you can submit PCR directly, assuming you have a clear single PCR amplicon on the gel.

8. Multiple bioinformatics software vendors offer solutions for intuitive sequence assembly, visual inspection, and DNA consensus sequence editing, that is, CLC Main Workbench, Sequencher, Geneious, or SeqMan.

9. The result should reveal top hits of GenBank entries within the genus *Cryptosporidium* and the respective sequenced marker gene. Note that the exact taxonomic identity of the entries is ambiguous, because of inconsistent status of annotation in GenBank.

10. The volume of PCR can be adjusted based on laboratory experience, volumes of 25–50 μL are commonly used. As for amplification of 18S, DNA needs to be prepared and controls need to be in place to minimize cross-contamination and false positives. In the case of 28S, we have extensive experience with MangoTaq (Bioline), however, use of alternative DNA polymerases as well as premixes that combine buffer, dNTPs, and DNA polymerase are also possible. Workflows must be optimized for performance and reproducibility by each laboratory.

11. Nested PCRs are prone to cross-contamination, and extreme care must be exercised to avoid cross-contamination (*see* **Note 4**). While 1 μL of template (first-round PCR) has been extensively tested as optimal from initial first-round 50 μL PCR, the volume should be adjusted to suit local needs and processes.

12. You are expecting a single PCR amplicon for each sample. Multiple bands indicate nonoptimal PCR amplification requiring troubleshooting, consider using the troubleshooting guides provided with your DNA polymerase. The above mentioned protocol will yield ~400 nt sequence (excluding primers).

13. The presence of multiple peaks could indicate a mixed infection.

14. You are expecting a single PCR amplicon for each sample. Multiple bands indicate nonoptimal PCR amplification requiring troubleshooting. If PCR amplicon is present with additional minor bands of different sizes, you may consider cutting the PCR amplicon from the gel. The above mentioned protocol will yield ~800–850 nt sequence (excluding primers).

15. The aim is to compare the newly obtained DNA sequence to the curated sequences from public repositories such as GenBank that represent named *Cryptosporidium* and unnamed genotypes. We will use MEGA7 for comparison. For this exercise, we commonly use MEGA7, but other alternative bioinformatics suites are available.

16. The usual expectation is that you compare sequences that have had the primer region trimmed, and if necessary, you trim them again to the shortest sequence you are comparing it to. During this stage it is important to make sure that the sequences are correctly orientated. Therefore, sequences must be checked to see if any need to be reverse complemented. The multiple sequence alignment programs will not alert the user therefore it is the responsibility of the operator to confirm correct sequence orientation. For amino acid coding gene (gp60), prior to the alignment stage it is important to confirm that the coding sequence is free from any stop codons, as these primers amplify an internal region of the gp60 gene. Any stop codons should warrant reevaluation of the raw chromatographs.

17. Reference sequences are provided for quick and reliable access to entries that have been selected from all GenBank entries as representative sequences with reliable taxonomic annotation. For the above example, we used the Kimura 2-parameter model, pairwise deletion option for gaps/missing data treatment, and minimum evolution method for distance table to infer phylogenetic tree. While such an approach will yield a good result for most routine applications, it is recommended that dedicated phylogenetic manuals are further consulted for further validation of the results.

## References

1. Checkley W, White AC Jr, Jaganath D, Arrowood MJ, Chalmers RM, Chen XM, Fayer R, Griffiths JK, Guerrant RL, Hedstrom L, Huston CD, Kotloff KL, Kang G, Mead JR, Miller M, Petri WA Jr, Priest JW, Roos DS, Striepen B, Thompson RC, Ward HD, Van Voorhis WA, Xiao L, Zhu G, Houpt ER (2015) A review of the global burden, novel diagnostics, therapeutics, and vaccine targets for *Cryptosporidium*. Lancet Infect Dis 15(1):85–94. https://doi.org/10.1016/S1473-3099(14)70772-8

2. Zahedi A, Paparini A, Jian F, Robertson I, Ryan U (2016) Public health significance of zoonotic *Cryptosporidium* species in wildlife: critical insights into better drinking water management. Int J Parasitol Parasit Wildl 5(1):88–109. https://doi.org/10.1016/j.ijppaw.2015.12.001

3. Ryan U, Zahedi A, Paparini A (2016) *Cryptosporidium* in humans and animals-a one

health approach to prophylaxis. Parasite Immunol 38(9):535–547. https://doi.org/10.1111/pim.12350

4. Šlapeta J (2013) Cryptosporidiosis and *Cryptosporidium* species in animals and humans: a thirty colour rainbow? Int J Parasitol 43(12–13):957–970. https://doi.org/10.1016/j.ijpara.2013.07.005

5. Šlapeta J (2018) DNA barcoding of *Cryptosporidium*. Parasitology 145(5):574–584. https://doi.org/10.1017/S0031182017001809

6. Xiao L, Morgan UM, Limor J, Escalante A, Arrowood M, Shulaw W, Thompson RC, Fayer R, Lal AA (1999) Genetic diversity within *Cryptosporidium parvum* and related *Cryptosporidium* species. Appl Environ Microbiol 65(8):3386–3391

7. Koehler AV, Korhonen PK, Hall RS, Young ND, Wang T, Haydon SR, Gasser RB (2017) Use of a bioinformatic-assisted primer design strategy to establish a new nested PCR-based method for *Cryptosporidium*. Parasit Vectors 10(1):509. https://doi.org/10.1186/s13071-017-2462-4

8. Alves M, Xiao L, Sulaiman I, Lal AA, Matos O, Antunes F (2003) Subgenotype analysis of *Cryptosporidium* isolates from humans, cattle, and zoo ruminants in Portugal. J Clin Microbiol 41(6):2744–2747

9. Power ML, Cheung-Kwok-Sang C, Slade M, Williamson S (2009) *Cryptosporidium fayeri*: diversity within the GP60 locus of isolates from different marsupial hosts. Exp Parasitol 121(3):219–223. https://doi.org/10.1016/j.exppara.2008.10.016

10. Stensvold CR, Beser J, Axen C, Lebbad M (2014) High applicability of a novel method for gp60-based subtyping of *Cryptosporidium meleagridis*. J Clin Microbiol 52(7):2311–2319. https://doi.org/10.1128/JCM.00598-14

11. Li N, Xiao L, Alderisio K, Elwin K, Cebelinski E, Chalmers R, Santin M, Fayer R, Kvac M, Ryan U, Sak B, Stanko M, Guo Y, Wang L, Zhang L, Cai J, Roellig D, Feng Y (2014) Subtyping *Cryptosporidium ubiquitum*, a zoonotic pathogen emerging in humans. Emerg Infect Dis 20(2):217–224. https://doi.org/10.3201/eid2002.121797

12. Stensvold CR, Elwin K, Winiecka-Krusnell J, Chalmers RM, Xiao L, Lebbad M (2015) Development and application of a gp60-based typing assay for *Cryptosporidium viatorum*. J Clin Microbiol 53(6):1891–1897. https://doi.org/10.1128/JCM.00313-15

13. Guo Y, Cebelinski E, Matusevich C, Alderisio KA, Lebbad M, McEvoy J, Roellig DM, Yang C, Feng Y, Xiao L (2015) Subtyping novel zoonotic pathogen *Cryptosporidium* chipmunk genotype I. J Clin Microbiol 53(5):1648–1654. https://doi.org/10.1128/JCM.03436-14

14. Yan W, Alderisio K, Roellig DM, Elwin K, Chalmers RM, Yang F, Wang Y, Feng Y, Xiao L (2017) Subtype analysis of zoonotic pathogen *Cryptosporidium* skunk genotype. Infect Genet Evol 55:20–25. https://doi.org/10.1016/j.meegid.2017.08.023

15. Xiao L, Bern C, Limor J, Sulaiman I, Roberts J, Checkley W, Cabrera L, Gilman RH, Lal AA (2001) Identification of 5 types of *Cryptosporidium* parasites in children in Lima, Peru. J Infect Dis 183(3):492–497. https://doi.org/10.1086/318090

16. Kumar S, Stecher G, Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33(7):1870–1874. https://doi.org/10.1093/molbev/msw054

17. Ryan U, Xiao L, Read C, Zhou L, Lal AA, Pavlasek I (2003) Identification of novel *Cryptosporidium* genotypes from the Czech Republic. Appl Environ Microbiol 69(7):4302–4307

# Chapter 9

# Preparation of *Cryptosporidium* DNA for Whole Genome Sequencing

## Guy Robinson and Rachel M. Chalmers

## Abstract

The application of whole genome sequencing (WGS) to *Cryptosporidium* has been challenged by the relatively small numbers of oocysts present in feces and lack of suitable culture systems to generate sufficient DNA that is required. Here, we provide a method for the preparation of sufficient, high-quality *Cryptosporidium* DNA for WGS from feces, incorporating parasite purification stages and an optional whole genome amplification step.

**Key words** *Cryptosporidium*, DNA extraction, Whole genome sequencing, WGS, Whole genome amplification

## 1   Introduction

Whole genome sequencing (WGS) has revolutionized our understanding of pathogens and is now being used routinely, for example, in diagnosis, antibiotic resistance monitoring, and genotyping many bacteria, as predicted by Didelot and colleagues in 2013 [1]. For *Cryptosporidium* and other hard to culture organisms, these technologies remain firmly in the domain of research because of the demands of efficient DNA extraction and adequate genome coverage, paucity of reference genomes, and lack of quality assured and standardized bioinformatic pipelines. Nevertheless, applications are being made to improve classification, genotyping and molecular epidemiology, seek new drug and vaccine targets, and investigate host–pathogen interactions and virulence factors [2, 3]. New *Cryptosporidium* genomes are made available on the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov) and can be analyzed using the CryptoDB resource (www.cryptoDB.org). Some of these developments have been enabled by the use either of rigorous oocyst purification procedures [4, 5] or whole genome amplification [6];

*Cryptosporidium*-positive stools are often low in volume and may contain relatively small numbers of oocysts to generate sufficient DNA required for WGS. These protozoa are also outnumbered by the presence of non-*Cryptosporidium* DNA in feces (e.g., bacterial and human DNA).

Here, we provide a method for high-quality DNA preparation for WGS from feces (Fig. 1). The method incorporates semipurification of oocysts by salt flotation (which may also be used prior to regular DNA extraction and PCR, *see* Chapter 2), further purification and concentration by immunomagnetic separation (IMS)

**Saturated salt flotation**
- Semi purifies oocysts from faeces by removing the majority of debris, non-target cells and DNA present (e.g. from bacteria, host cells, food and beverages)

**Immunomagnetic separation**
- Further purifies the sample and concentrates the *Cryptosporidium* oocysts

**Oocyst surface sterilization by chlorine**
- The chlorine-resistant nature of oocysts allows treatment to destroy any remaining non-target DNA carried through the IMS (e.g. cells caught up in the oocyst/bead complexes)

**DNA extraction**
- Extracts highly purified *Cryptosporidium* DNA from concentrated oocysts

**Whole genome amplification**
- (*Optional step)* Allows almost any sample to be processed regardless of the amount of starting material, number of oocysts present or high amount of DNA required for library preparation

**Library preparation & whole genome sequencing**
- DNA can be stored at 4°C in the short term or at -20°C if library preparation is not planned immediately

**Fig. 1** Workflow for *Cryptosporidium*-positive fecal sample preparation for whole genome sequencing

| a. Stool smear | b. Oocyst suspension after salt flotation | c. Oocyst suspension after IMS and bleach |
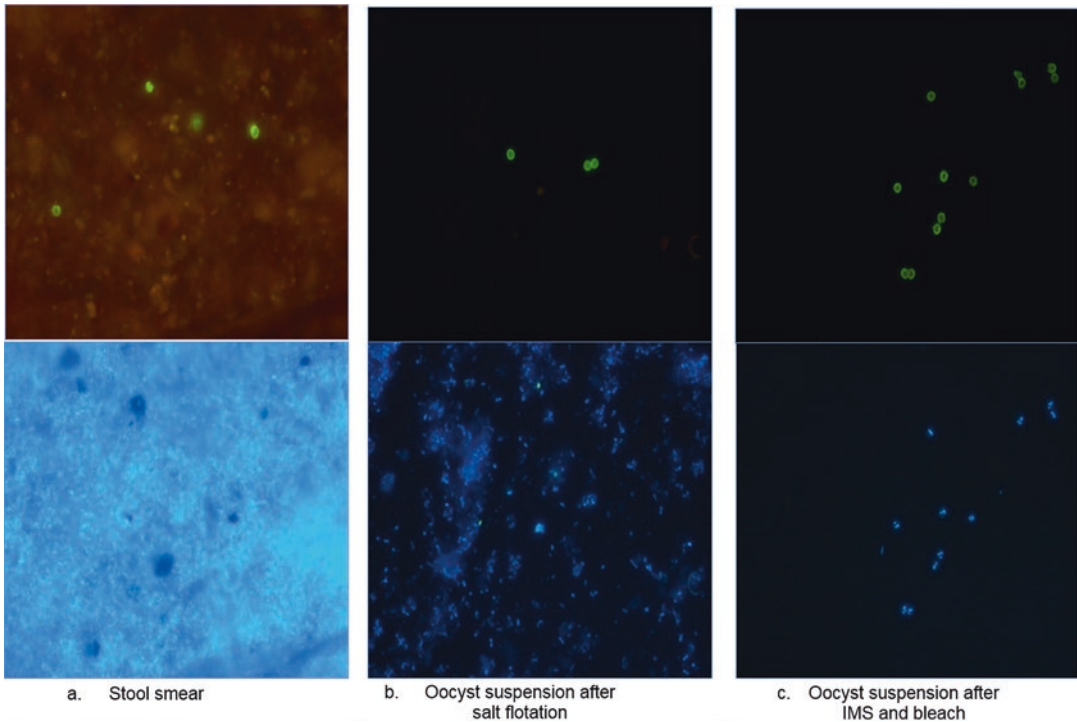
**Fig. 2** Stool sample before salt flotation (**a**), after salt flotation (**b**), and after IMS and bleaching (**c**), stained with immunofluorescent antibody (top image) and DAPI (bottom image) to demonstrate the purification of oocysts and reduction in nontarget DNA by each step. DAPI is a DNA-intercalating dye and the bright blue in image **a** is from the bacterial load in the stool sample, much reduced in image **b**, and eliminated in image **c**, where there is a greater concentration of fluorescing oocysts

(Fig. 2), surface sterilization using bleach, and finally spin-column DNA extraction [4]. An option to use whole genome amplification (WGA) is also described.

## 2    Materials

### 2.1   Preparation of DNA From Next-Generation Sequencing (NGS)

1. Saturated salt solution (cold preparation method): NaCl solution, 1.2 SG. Approximately 1.5 kg of sodium chloride is required to saturate 4 L of oocyst-free distilled water by adding the sodium chloride in small quantities, taking care to maintain continuous, brisk stirring (e.g., with a magnetic stirrer). Continue to add sodium chloride until 1.2 SG is achieved as measured by hydrometer. Store in the dark at ambient temperature.

2. Immunomagnetic separation (IMS) kit, adapted from [7], usually containing 10× reagent A (detergent solution), 10× reagent B (protein-blocking solution), and paramagnetic beads coated with anti-*Cryptosporidium* antibodies.

3. 1× Reagent A: Dilute the supplied 10× reagent A 1:9 with oocyst-free distilled water on the day of processing. Prepare enough for 1 mL per sample, plus an additional wastage 1 mL.

4. Glass Leighton tubes appropriate for the commercial IMS kit (*see* **Note 1**).

5. Hydrochloric acid solution: 0.1 M HCl. Add 90 μL of concentrated hydrochloric acid (1.18 SG) to 90 mL of oocyst-free distilled water. Mix well and make up to 100 mL with oocyst-free distilled water. The solution may be stored in the range 20 ± 5 °C for up to 1 month. Commercial supplies are available.

6. Sodium hydroxide solution: 1 M NaOH. Dissolve 40 g of sodium hydroxide in 900 mL of oocyst-free distilled water. Cool the solution and make up to 1000 mL with oocyst-free distilled water. The solution may be stored in the range 20 ± 5 °C for up to 1 month. Commercial supplies are available.

*2.2 Oocyst Surface Sterilization*

1. Sodium hypochlorite: NaOCl solution (1.2% active chlorine). Starting with a 14% NaOCl stock (other concentrations are available and adjustments must be made accordingly), add 860 μL to 9.14 mL nuclease-free water in a universal container. Prepare this solution on the day of processing.

*2.3 DNA Extraction*

1. Liquid nitrogen (where liquid nitrogen is not available, a dry ice/methanol slurry may be used as an alternative).

2. Column-based commercial DNA extraction kits usually contain some sort of lysis buffer, proteinase K, wash buffers, and a membrane-based spin column. Several different kits are available and reported in the published literature for the extraction of *Cryptosporidium* DNA. The example given below which is used in our laboratory is the QIAamp DNA mini kit (Qiagen, Inc., Germantown, MD).

3. Nuclease-free water.

*2.4 Optional Step: Whole Genome Amplification (WGA) (See* **Note 2***)*

1. Commercial WGA kit. The use of WGA to boost *Cryptosporidium* DNA for WGS has been reported in the published literature [6]. Several different kits are available.

## 3 Methods

Wear appropriate personal protective equipment (PPE) including protective clothing, disposable gloves, and eye protection during all sample processing steps.

*3.1 Preparation of DNA for WGS, Modified From [4]*

1. Salt flotation procedure: Before use, ensure that the salt solution is mixed by inversion and allowed to settle for 5 min.

2. Transfer 10 mL of saturated salt solution in a 15-mL centrifuge tube (one per sample).

3. Add approximately 1–2 g of feces (*see* **Note 3**) with an applicator stick, or pipette 1 to 2 mL liquid feces, to the saturated salt solution, cap the tube, and mix thoroughly.

4. Gently overlay the salt/feces slurry with 3 mL of oocyst-free distilled water.

5. Place the centrifuge tube in a benchtop centrifuge with swing out buckets, add a balance tube, if necessary, and centrifuge at $1100 \times g$ for 5 min (*see* **Note 4**).

6. Gently swirl the top 3 mL of fluid (the overlain fraction) and transfer to a clean 15 mL tube.

7. Wash the oocysts by topping up with oocyst-free distilled water, centrifuging at $1100 \times g$ for 5 min (*see* **Note 4**), and decanting (tipping off) the supernatant.

8. Resuspend the pellet in 1 mL of oocyst-free distilled water.

*3.2 Immuno-magnetic Separation*

1. Take the IMS reagents from the refrigerator and allow them to reach ambient temperature (*see* **Note 5**).

2. Add 1 mL of 10× reagent A and 1 mL of 10× reagent B to each clean, labeled Leighton tube.

3. Transfer the entire 1 mL oocyst suspension to the Leighton tube and rinse out the sample tube with 1 mL of oocyst-free distilled water before adding this to the Leighton tube.

4. Add 8 mL of oocyst-free distilled water to the Leighton tube.

5. Mix the antibody-coated paramagnetic beads thoroughly and add 100 μL to each suspension in the Leighton tubes.

6. Incubate the samples by placing the Leighton tubes into rotating mixer at ambient temperature for at least 60 min, rotate typically at approximately 20 rpm.

7. Remove from the rotor and place each Leighton tube into a magnetic particle concentrator with the flat side against the magnet.

8. Rock gently according to the manufacturer's instructions (typically either through a 90° motion or attached to a rotating mixer) (*see* **Note 6**).

9. One by one, with the remaining Leighton tubes still moving on the rocker, remove the magnet block containing first tube from the rocker and pour the noncaptured particular matter carefully into a 15-mL centrifuge tube and retain in case further processing is required (*see* **Note 7**). Using a serological

pipette, aspirate and dispose any remaining supernatant from the Leighton tube without disturbing the beads (and captured oocysts) against the magnet.

10. Remove the Leighton tube from the magnet, add 0.8 mL of 1× reagent A, and rinse the beads (and captured oocysts) from the side of the tube by repeatedly pipetting the solution over them.

11. Transfer the beads (and captured oocysts) to a clean, labeled 1.5-mL flip-top microcentrifuge tube.

12. Add 0.2 mL of 1× reagent A to the Leighton tube (*see* **Note 8**), rinse out any remaining beads, and add this to the microcentrifuge tube.

13. Repeat the transfer of beads to 1.5 mL tubes with any remaining samples.

14. Gently resuspend the samples in the microcentrifuge tube by inversion several times and place the microcentrifuge tubes into a second particle concentrator (appropriate for the 1.5 mL tubes).

15. Rock according to the manufacturer's instructions (typically either through a 180° motion or attached to a rotating mixer).

16. One by one, with the remaining tubes still moving on the rocker, remove the first sample, still attached to the magnet, taking care not to disturb the pellet.

17. Immediately remove the supernatant from the inside of the cap and the tube (taking care not to disturb the pellet) using a clean fine-tipped disposable transfer pipette (Pastette), discard, and repeat with each of the remaining samples.

18. Remove all of the 1.5 mL tubes from the magnets and add 50 μL of 0.1 M HCl to each tube.

19. Vortex the tubes thoroughly for 10 s to separate the oocysts from the beads and stand the tubes at room temperature for 5 min (*see* **Note 9**).

20. Vortex each tube again for 10 s immediately afterward placing the tubes into the dissociation block (*see* **Note 10**).

21. Lay the dissociation block flat on the workbench so that the tubes are horizontal with the magnets below them and leave for at least 30 s.

22. During this time, add 5 μL of 1.0 M NaOH to clean labeled 1.5 mL screw-capped microcentrifuge tubes (one for each test sample).

23. Return the dissociation block to an upright position and transfer the supernatant from each tube to the labeled NaOH-containing 1.5 mL screw-capped tube, without disturbing the

beads at the back wall of the tube. Gently mix the sample using the same pipette tip.

24. Perform a second acid treatment on each sample by repeating the above steps and transfer this into the same tube.

25. Add 90 μL of oocyst-free distilled water to each of the tubes and mix well.

26. Proceed immediately to treat the suspension with NaOCl as described below. If this is not possible straightaway, store the suspensions at 2–8 °C for no longer than 20 h.

*3.3 Oocyst Surface Sterilization*

1. Add 200 μL of 1.2% NaOCl solution to each 200 μL oocyst suspension.

2. Immediately vortex briefly and centrifuge at $1100 \times g$ for 5 min with soft acceleration–deceleration to minimize damage to oocysts (*see* **Note 4**).

3. Gently aspirate the supernatant into a suitable plastic waste container (*see* **Note 11**).

4. Wash the oocyst pellet by adding 200 μL of nuclease-free water to the 1.5 mL tube, vortex briefly to mix, centrifuge at $1100 \times g$ for 5 min with soft acceleration–deceleration profile to minimize damage to oocysts, and remove supernatant as previously to the waste container.

5. Repeat the nuclease-free water wash step twice more and finally resuspend the oocysts in 200 μL of nuclease-free water.

6. Proceed to DNA extraction immediately. If DNA extraction is not possible straightaway, perform the freeze–thaw process, but following the final freeze transfer the tubes to a chilled rack and store at −20 °C until ready for DNA extraction to be completed.

*3.4 DNA Extraction*

There are several commercial kits available to perform DNA extraction, but this example method using Qiagen's QIAamp DNA mini kit has been successfully used during the preparation of DNA for NGS [4, 5].

1. Disrupt the oocysts by eight freeze–thaw cycles of 1 min in liquid nitrogen and 1 min at 100 ± 5 °C, gently flicking the tubes between each cycle to resuspend any settled oocysts/debris.

2. Microcentrifuge briefly to spin any condensation back into the tube.

3. Add 200 μL of AL buffer and 20 μL of proteinase K (*see* **Note 12**) to each tube (*see* **Note 13**) before mixing by vortex for 15 s and incubating for 30 min at 54–58 °C.

4. After incubation at 54–58 °C for 30 min, remove the tubes from the dry block, cool in a rack for 5 min, and briefly vortex, followed by brief centrifugation then place back in the tube rack.

5. Add 200 μL ethanol to each tube and vortex for 15 s before briefly centrifuging to remove any drops of sample from around the lid.

6. Carefully apply the mixture to the top of the appropriate QIAamp spin column sitting in a 2-mL collection tube, without wetting the rim and centrifuge at $6000 \times g$ for 1 min.

7. Discard the collection tube containing the filtrate and place the spin column in a second collection tube.

8. Add 500 μL of buffer AW1 to each tube (*see* **Note 14**) and centrifuge using at $6000 \times g$ for 1 min.

9. Discard the collection tube containing the filtrate into a waste disposal container (not containing bleach waste) and place the spin column in a third collection tube.

10. Add 500 μL of buffer AW2 to each tube (*see* **Note 14**) and centrifuge at $20,000 \times g$ for 4 min.

11. Discard the collection tube containing the filtrate into a waste disposal container and place the spin column into a clean, labeled 1.5 mL tube (*see* **Note 15**).

12. Add 50 μL of nuclease-free water to the membrane in each tube and incubate at room temperature for 5 min before centrifuging at $6000 \times g$ for 1 min.

13. Discard the spin columns and store the extracted DNA at −18 to −22 °C until use, either for library preparation if sufficient DNA is present or by WGA to increase the amount of available DNA.

*3.5 Optional Step: Whole Genome Amplification*

1. Follow the manufacturer's instructions of the commercially available kit.

2. Store WGA DNA at 4 °C in the short term; for longer term storage, keep at 18–22 °C.

# 4    Notes

1. Different manufacturers use different sized Leighton tubes, so check the product insert before purchasing.

2. Several factors can influence whether WGA is required prior to WGS including the number of oocysts present in a sample, amount of sample available, and the amount of start DNA required for NGS library preparation (library kit requirements

can vary between 1 ng to over 100 ng of starting DNA). We have used multiple displacement amplification (MDA) for WGA to enable WGS from any sample, but have not extensively validated it. However, several published studies use this technology prior to WGS [6].

3. All stools should be well mixed prior to transferring to the salt solution.

4. Centrifugation at speeds higher than $1100 \times g$ for longer periods of time (>5 min) is not advised as some parasites may deform or rupture and collapse.

5. Make sure that any crystals present in the reagents have dissolved.

6. It is important that the tubes are continuously moved at this stage to prevent additional unwanted particulate matter becoming stuck to the beads. If the motion stops for more than 10 s, samples will need removing from the magnet and resuspending, before being fully recaptured.

7. If only a few beads remain in the Leighton tube against the magnet, the majority still need to be recovered from the supernatant. Return the supernatant back to the Leighton tube and incubate for a further incubation as before. If this still returns few beads, then return the supernatant back to the tube and incubate again, but reduce the speed of rocking.

8. Take care not to disturb any fecal debris on the front side of the tube when rinsing.

9. Vortexing breaks the bonds between the oocysts and the beads. The acid stops the oocysts from rebinding with the beads.

10. Make sure all of the sample is at the bottom of the tube.

11. Do not discard waste into container with DNA extraction waste, as some buffers contain guanidine thiocyanate that can react with the bleach to create toxic gases.

12. Aliquot sufficient reagents at the start to reduce the risk of contaminating stock reagents.

13. A new tip must be used for each sample tube and each tube lid should be removed individually at the point of adding reagents.

14. Providing the tip does not touch any of the tubes, and there is no bridging, one tip can be used for all samples as the DNA is immobilized on the column and is no longer a splash back risk.

15. This tube should remain closed at all times after labeling and during the extraction procedure to prevent cross-contamination, and should only be opened to admit the spin column as described.

## Acknowledgments

## References

1. Didelot X, Bowden R, Wilson DJ (2012) Transforming clinical microbiology with bacterial genome sequencing. Nat Rev Genet 13:601–612

2. Khan A, Shaik JS, Grigg ME (2018) Genomics and molecular epidemiology of *Cryptosporidium* species. Acta Trop 184:1–14

3. Ryan U, Hijjawi N (2015) New developments in *Cryptosporidium* research. Int J Parasitol 45:367–373

4. Hadfield SJ, Pachebat JA, Swain MT (2015) Generation of whole genome sequences of new *Cryptosporidium hominis* and *Cryptosporidium parvum* isolates directly from stool samples. BMC Genomics 16:650

5. Gilchrist CA, Cotton JA, Burkey C, Arju T, Gilmartin A, Lin Y, Ahmed E, Steiner K, Alam M, Ahmed S, Robinson G, Zaman SU, Kabir M, Sanders M, Chalmers RM, Ahmed T, Ma JZ, Haque R, Faruque ASG, Berriman M, Petri WA (2018) Genetic diversity of *Cryptosporidium hominis* in a Bangladeshi community as revealed by whole genome sequencing. J Infect Dis 218(2):259–264

6. Guo Y, Li N, Lysén C, Frace M, Tang K, Sammons S, Roellig DM, Feng Y, Xiao L (2015) Isolation and enrichment of *Cryptosporidium* DNA and verification of DNA purity for whole-genome sequencing. J Clin Microbiol 53:641–647

7. Anon (2010) The microbiology of drinking water (2010) - part 14 - methods for the isolation, identification and enumeration of *Cryptosporidium* oocysts and *Giardia* cysts. Environment Agency, Bristol. http://standing-committeeofanalysts.co.uk/methods/Microbiology/drinkw.html

# Chapter 10

# Accessing *Cryptosporidium* Omic and Isolate Data via CryptoDB.org

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## Abstract

*Cryptosporidium* has historically been a difficult organism to work with, and molecular genomic data for this important pathogen have typically lagged behind other prominent protist pathogens. CryptoDB (http://cryptodb.org/) was launched in 2004 following the appearance of draft genome sequences for both *C. parvum* and *C. hominis*. CryptoDB merged with the EuPathDB Bioinformatics Resource Center family of databases (https://eupathdb.org) and has been maintained and updated regularly since its establishment. These resources are freely available, are web-based, and permit users to analyze their own sequence data in the context of reference genome sequences in our user workspaces. Advances in technology have greatly facilitated *Cryptosporidium* research in the last several years greatly enhancing and extending the data and types of data available for this genus. Currently, 13 genome sequences are available for 9 species of *Cryptosporidium* as well as the distantly related *Gregarina niphandrodes* and two free-living alveolate outgroups of the Apicomplexa, *Chromera velia* and *Vitrella brassicaformis*. Recent years have seen several new genome sequences for both existing and new *Cryptosporidium* species as well as transcriptomics, proteomics, SNP, and isolate population surveys. This chapter introduces the extensive data mining and visualization capabilities of the EuPathDB software platform and introduces the data types and tools that are currently available for *Cryptosporidium*. Key features are demonstrated with *Cryptosporidium*-relevant examples and explanations.

**Key words** Apicomplexa, Bioinformatics, Parasite, Pathogen, Genomics, Transcriptomics, Orthology, Proteomics, SNP, Sequence analysis

## 1 Introduction

A member of the Eukaryotic Pathogen Bioinformatics Resource Center (https://eupathdb.org/) [1, 2], CryptoDB (http://cryptodb.org/) serves as the functional genomics database for *Cryptosporidium* and related species. CryptoDB is a free, online resource for accessing and exploring genome sequence and annotation,

functional genomics data, isolate sequences, and orthology profiles across organisms. Data mining via CryptoDB requires no prior bioinformatics or computational experience since all integrated data are pre-analyzed according to standard workflows and accessed via an easy-to-use interactive web interface. CryptoDB brings a genome-wide perspective to the exploration of *Cryptosporidium* and related species by integrating the pre-analyzed data with advanced search capabilities, convenient data visualization, analysis tools, and a comprehensive record system describing genomic features and pathways.

Data mining in CryptoDB proceeds along four general paths:

1. Direct examination of record pages which compile all database information about a feature (genes, SNPs, pathways, EST, isolates, etc.) into tables and graphs.

2. An advanced search strategy system which facilitates genome-wide data mining within and across data sets, data types, and organisms (e.g., retrieve all *Cryptosporidium* orthologs that contain signal peptides or are regulated at 48 h).

3. Data visualization tools, such as a genome browser, which facilitate visualization of sequence-based data to explore gene models, single-nucleotide polymorphisms, or synteny of genomic regions across any or all integrated genome sequences.

4. Data analysis tools for functional enrichment or primary analysis via a private EuPathDB Galaxy [3, 4] instance (e.g., analysis of your RNA-seq data or variant calling).

This chapter starts with a brief overview of the database content and then describes the routine data mining methodologies outlined above. For maximum benefit, this chapter is best utilized at a computer with CryptoDB.org open in a browser. Screenshots of all relevant actions are provided as a guide for completing examples and in case offline reading is preferred.

## 2    Using CryptoDB

### 2.1    Database Content and Download

CryptoDB integrates a wide range of data types including genome sequence and annotation, whole-genome sequencing of clinical or environmental isolates, GenBank Popset [5] sequences from surveys, RNA sequence, microarray, proteomics, genome-wide RT-PCR, ESTs, and metabolic pathways. Versions of the database are released about every 2 months and incorporate new data, update existing data, and launch new features. Central to the database is the genome sequence and annotation. CryptoDB enhances these data with a series of in-house bioinformatic analyses for domain prediction, functional label assignments, and genome feature identification. In addition, functional genomics data are mapped to the genome sequence for interrogation and visualization of sequence-based data in a genomic context.

**Fig. 1** Homepage layout. (**a**) Header section. Gray menu bar (arrow 2) for quick access to all CryptoDB searches and tools. When scrolling CryptoDB pages, this menu bar remains available at the top of every page. Gene ID search (arrow 3) navigates to the gene record page of the ID entered. Gene Text Search (arrow 4) returns a list of genes whose records contain the desired text. About menu (arrow 5) with links for Help, Login, Registration, and to Contact Us via email with questions and suggestions. (**b**) Side panel with expandable sections containing a Data Summary (arrow 1) table listing data types available for each genome in CryptoDB, news which documents changes per release, tweets which inform on items of interest to the Crypto research community, links to other community resources, and educational materials. (**c**) Searches and tools section used to access all searches that return genes (arrow 6), other record types (Popset Isolates, ESTs, etc.) (arrow 7) or tools (arrow 8) for BLAST, analysis or annotation, among others. (d) Footer section with links to all EuPathDB home pages
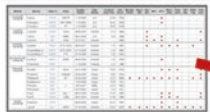
The data summary table provides an overview of available data as of the submission of this chapter. Open the *Data Summary* section in the home page left panel (Fig. 1, arrow 1) and click on the table to retrieve a table that provides a quick view of the data available for each organism (Fig. 2a) including the data provider and version.

*2.1.1 Genome Sequences and Annotation*

1. CryptoDB contains 13 annotated genome sequences including sequences for 9 *Cryptosporidium* species (including several strains), the closely related *Gregarina niphandrodes* [6] and the proto-apicomplexans *Chromera velia* and *Vitrella brassicaformis* [7]. None of the genome sequences is fully complete, telomere-to-telomere without gaps. Contigs have been assigned to scaffolds, super-scaffolds, and chromosomes by the data providers as possible. Currently, *C. hominis* UdeA01, *C. parvum* Iowa II, and *C. tyzzeri* UGA55 are represented as 8 chromosomes each, with gaps and at times some left over, unplaced

**Fig. 2** Data summary table and downloads. (a) Use the Data Summary Table, accessed from the Data Summary tab on the left side of the home page, for a quick overview of the data types available for each organism. (**b**) Genome scale data files are accessed from Downloads, Data Files section in the gray menu bar. Previous releases are archived, and organism folders contain data that cannot be unambiguously assigned to species, such as ESTs

contigs. It is important to bear in mind the relative completeness of each of the sequences when searching the database. The observation that a particular gene is not found in a species does not necessarily indicate that it is not present, as the genome sequences do contain gaps, in some instances hundreds of gaps. The same caution applies to gene or sequence copy number. Paralogous gene families are abundant in the Apicomplexa and parasitic protists in general. Genome assembly algorithms often overassemble multiple copies of highly similar genes into a single gene or are unable to assemble the additional family members if the similarity is lower. In such cases, additional gene family members are often found in the unassembled contigs for the species. This happened with the *Toxoplasma gondii* rhoptry proteins [8].

As there has been very little functional genomic data to assist with genome annotation until very recently, *Cryptosporidium* annotations are also underinformed. The lack of strand-specific RNA-seq or cDNA experimental data has led to under annotation of intron sequences and misannotation of transcripts with overlapping untranslated regions (UTRs). As a user of this and other genomic resources, please bear in mind these rather universal caveats regarding the completeness the genome and annotation.

2. Downloading genome-scale data: The downloads section is accessed from the gray menu bar located near the top of the browser page (see Fig. 1, arrow 2 for the bar and Fig. 2b). This section contains sequence data from the current release as well as archived files from previous releases. Contents are arranged by organism. Folders without a species designation contain data, such as expressed sequence tags, that cannot be assigned unambiguously to a strain. Species folders for unannotated genome sequences contain the genome sequence and predicted open reading frames (ORFs) in fasta format. Genome sequences that are annotated have additional fasta files that contain the predicted protein sequences, transcripts, coding sequence and ORF amino acid translations, as well as GAF- and GFF-formatted annotation files and text files of codon usage, and protein domain associations.

3. Genome analyses/predictions performed at CryptoDB: Sequence and annotation files are integrated into CryptoDB from repositories such as GenBank [9, 10] or, in rare cases, obtained directly from researchers. CryptoDB runs a series of analyses that supplement the existing information and annotation with data such as protein domain prediction, synteny information, and protein structure and function prediction (Table 1).

**Table 1**
**Genome analyses and access in CryptoDB**

| Analysis | Data | Data category | Appearance |
|---|---|---|---|
| WU-BLAST | Sequence similarities in NRDB (genomic or protein sequence vs. NRDB) | Protein features and properties | Records |
| HMM-Pfam | Domains | Protein features and properties | Search, records |
| Protein PI | Isoelectric points for annotated proteins | Protein features and properties | Search, records |
| Splign | Exon–intron boundaries, splice-junctions, potential frameshifts, and alternative models when there is more than one possibility | Gene models | Search, records, |
| TMHMM | Predicted transmembrane domain topology | Protein features and properties | Search, records |
| SignalP | Predicted signal peptides and their likely cleavage sites | Protein features and properties | Search, records |
| Repeat masker | Interspersed repeats and low complexity DNA sequences | Sequence composition, complexity, and repeats | GBrowse |
| Tandem repeat finder | Two or more adjacent, approximate copies of a pattern of nucleotides | Sequence composition, complexity, and repeats | GBrowse |
| Low complexity regions | Sequences containing regions of low complexity are masked with the character "x" | Sequence composition, complexity, and repeats | GBrowse |
| Pspired | Secondary structure prediction | Structure analysis, protein features, and properties | Search, records |
| tRNAScan | Predicted tRNA genes | Gene models | Search |
| InterProScan | Domains and functional site assignments from known proteins | Protein features and properties | Search, records |
| CAP4 EST Assembly | EST assemblies | Sequence analysis | Records, GBrowse |
| BLAST EST alignments | | Sequence analysis | Records, GBrowse |
| OrthoMCL | Groups based on clustered ortholog pairs from 150 proteomes across the tree of life | Orthology and synteny | Search, records, GBrowse |
| ORF generation | Translations in all six reading frames | Sequence analysis | GBrowse |

The OrthoMCL [11] analysis creates ortholog profiles across all genome sequences in CryptoDB and beyond. An initial analysis of 150 genome sequences gathered from across the tree of life included several *Cryptosporidium* genome sequences and consisted of an all-vs-all BLASTP of quality screened protein sequences followed by clustering of reciprocal best BLAST hits. The analysis produces groups of similar proteins that include proteins that span the tree of life. The ortholog groups created in this analysis facilitate comparative genomic analysis.

*2.1.2 Isolates*

1. PopSet Isolates: CryptoDB integrates isolate sequences from the NCBI PopSet database [5]. These sequences originate from phylogenetic, population, environmental, or mutational studies and represent one facet of the known variation for a specific gene, often well-characterized genes commonly used for genotyping like SSU *18S rRNA*, *gp60*, and *cowp*.

2. Whole-genome sequencing: Decreasing sequencing costs and improved technologies enable whole-genome sequencing (WGS) on a larger scale, and CryptoDB has integrated several WGS projects as isolates of *C. parvum* and *C. hominis* [12–15]. The isolate data are aligned to the reference genome and analyzed for single-nucleotide polymorphisms (SNPs) and copy number variation (CNV).

*2.1.3 Functional and Structural Predictions*

1. Gene Ontology assignments: GO terms and their associated identifiers are part of the Gene Ontology controlled vocabulary that describe the biological process, molecular function, and cellular component of a gene product. GO terms in CryptoDB are either manually curated or electronically inferred from orthologs.

2. EC numbers: Enzyme commission numbers are assigned to genes and classify the enzymatic activity of the gene product. CryptoDB receives EC numbers with genome annotation and supplements this with assignments retrieved from other sources [16, 17].

3. InterPro domains: Protein domains and functional insights associated with them are assigned to predicted proteins using InterProScan [18]. The presence of annotated protein domains facilitates sequence- and feature-based searching of identifiable features in known proteins possible. Several protein motif signatures are available in CryptoDB, including Pfam [19], InterPro [20], and Smart [21].

*2.1.4 Functional Data*

CryptoDB maps functional genomics data (Express Sequence Tag (EST), RNA sequence data, genome-wide RT-PCR, proteomics) [22, 23] to the genome sequence. These data sets document transcription and translational events under different experimental conditions.

1. EST: ESTs represent portions of the sequences expressed by an organism and result from single-pass sequencing of a clone from a cDNA library. ESTs in CryptoDB are downloaded NCBI's database of ESTs [24] and aligned to genomes.

2. RNA Seq: RNA sequence data represent the full complement of RNA in the sample at the time it was collected, e.g., the intracellular transcriptome. RNA sequence reads are downloaded from archival repositories like the SRA [25] and analyzed through an in-house pipeline that aligns the reads, calculates expression values, and determines differential expression [26–28].

3. Microarray: Spotted glass slide as well as oligonucleotide array data are obtained from the research community and analyzed to determine expression values with an in-house workflow [29].

4. RT-PCR: A large-scale RT-PCR study [30] determined expression values for 3281 *C. parvum* genes.

5. Proteomics: CryptoDB integrates peptide sequence, abundance, and translational modification data from published studies as well [31–34].

*2.1.5 Metabolic Pathways and Compounds*

Metabolic pathways are downloaded from KEGG [35–37] and MetaCyc [38–40] as networks of enzymes (EC numbers) and compounds (Compound IDs from PubChem Compounds [41]). Genes that are annotated with EC numbers contained in the metabolic pathway are mapped to the pathways, creating a network of gene, pathway, and compound records. Using the strategy system described below, any list of genes can be transformed into the pathways or compounds associated with these genes.

### 2.2 Home Page and Basic Functions

*2.2.1 Layout of Home Page*

The CryptoDB home page (Fig. 1) provides quick access to the four data mining resources—record pages, genome-wide searches and strategies, analysis tools, and visualization tools. The page is organized into four sections. The header (Fig. 1a) provides easy access to gene ID (Fig. 1a, arrow 3) and text searches (Fig. 1a, arrow 4) that return genes for further exploration. The header also contains links (Fig. 1a, arrow 5) for registration, login, and a "Contact Us" form for emailing EuPathDB's active support hotline with questions or suggestions. The gray menu bar (Fig. 1a, arrow 2) provides access to all CryptoDB functionality and remains visible on all CryptoDB pages. Registered users can bookmark record pages in *My Favorites*, save searches and strategies in *My Strategies*, add comments on record pages (Fig. 7), and use the EuPathDB Galaxy site [42] for private analysis of large-scale data accessed through *Analyze My Experiment*. Hover your computer cursor over the *New Search* tab to reveal a dropdown menu for

accessing all searches. The *My Basket* tool provides a mechanism to save records for later viewing or strategy integration. The side panel (Fig. 1b, red headers) contains links to useful information including a summary of data in CryptoDB (Fig. 2a), community resources, educational materials including exercises and video tutorials, news documenting updates implemented with each release, and the EuPathDB twitter feed. The center panels (Fig. 1c, blue headers) contain expandable categories for accessing all searches that return genes (Fig. 1c, arrow 6), searches that return other records (Fig. 1c, arrow 7), and links to useful tools (Fig. 1c, arrow 8) such as BLAST for retrieving records based on sequence similarity, the Companion annotation tool [43] for easy, first-pass, reference-based genome annotation, and the Genome Browser for visualization of sequence-based data aligned to a reference genome. The footer (Fig. 1d) is also available from any page and provides links to the home pages of all EuPathDB sites.

*2.2.2 Retrieval of Records Based on Gene ID Searches*

Genome sequence annotation specifies a unique identifier for each gene in the genome sequence, and CryptoDB offers several ways to retrieve genes based on their ID. The Gene ID search in the header (Fig. 3a, expansion) navigates directly to the record page of the corresponding gene ID (locus tag). The header appears on every page, making it convenient to reach gene records from any page on the site. A search capable of retrieving multiple records from a list of input gene IDs is available in the *Search for Genes* panel under Annotation, curation, and identifiers (Fig. 3b, arrow 1). Popset Isolate, Genomic sequence, SNP, EST, ORF, Metabolic Pathway, and Compound records can also be retrieved with similar ID searches located in the home page Search for Other Data Types panel (Fig. 3b, e.g., arrows 2, 3).

1. *Visit the gene record page for the gene encoding GP-60.* To visit the record page for the isolate typing gene Glycoprotein _ GP40, also known as sporozoite antigen *gp40/15* or *gp60*, enter the gene ID (cgd6_1080) into the *Gene ID* search in the header and then click the search icon to the right of the box. (*See* Subheading 2.3 for discussion of the gene record page).

2. *Retrieve multiple genes from the database:* Navigate to the *Identify Genes based on Gene ID(s)* search (Fig. 3c) using the home page *Search for Genes* panel (Fig. 3b, arrow 1), enter the following gene IDs (cgd7_230, cgd6_1080, cgd5_4560, GY17_00002758, CTYZ_00003987) in the text box or upload a text file containing the IDs (sample in Electronic Supplemental File 1), and click *Get Answer*. The search results, 5 genes corresponding to the IDs entered in the *Gene ID input set* parameter, appear in the *My Strategies* section (Fig. 3d), which begins with a graphic representation of the search and a summary of results in the top strategy panel (Fig. 3d, blue
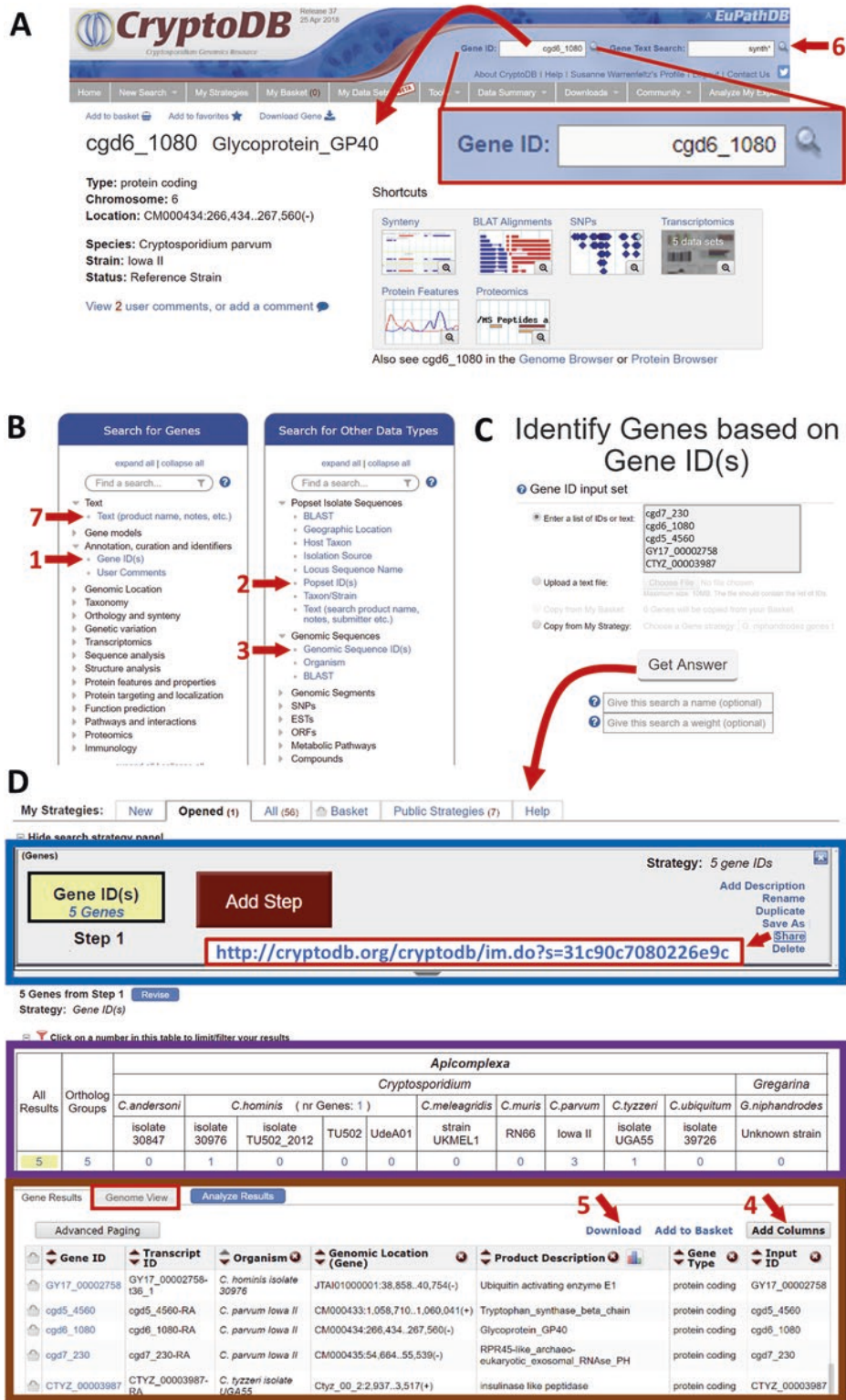
**Fig. 3** Find records based on IDs. (**a**) The header Gene ID search navigates directly to the gene page for the ID entered. (**b**) Multiple records may be retrieved using the dedicated ID searches for each record type, for example,

box). Several options for strategy actions appear at the right of the panel and include an option to share the strategy via a dedicated URL. The URL for this strategy is http://cryptodb. org/cryptodb/im.do?s=31c90c7080226e9c. The organism table (Fig. 3d, purple box) shows the distribution of results across the organisms queried. Clicking a number in the organism table filters the result to display only hits from the selected organism. Yellow highlighting in the strategy panel and organism table indicates the active result that appears in the *Gene Results* table (Fig. 3d, brown box). The *Gene Results* section tabulates and displays data for the active result (highlighted with yellow). All CryptoDB data associated with genes, such as GO terms or gene expression values, are available for display in the *Gene Results* tab via the *Add Columns* tool (Fig. 3d, arrow 4). The download tool (Fig. 3d, arrow 5) retrieves either custom (one row per ID) or preconfigured tab-delimited TXT files of chosen data, sequences in FASTA format or annotations in GFF3 format for the active result. The *Genome View* tab (Fig. 3d, red box) displays genomic sequences (contigs or chromosomes) "painted" with the genes from the active result.

*2.2.3 Record Retrieval Based on Text*

CryptoDB text searches are available for gene, Popset isolate, and compound record retrieval. These searches query the textual content of each record to assemble a set of genes that contain a user-defined text term or phrase. For example, genome annotation, as well as other data in the gene record (e.g., UniProt [17, 44] data, protein domain predictions), contains text that can be used to retrieve genes with similar characteristics.

CryptoDB offers two searches that return genes based on the text in their records: the *Gene Text Search* is available in the header (Fig. 3a, arrow 6) and the dedicated search *Identify Genes based on Text (product name, notes, etc.)* is available in the *Search for Genes, Text (product name, notes, etc.)* panel (Fig. 3b, arrow 7). A text term or phrase (within quotations) entered in the header *Gene Text Search* initiates a preconfigured search that queries a broad range of data fields in all gene records of all annotated genome sequences in CryptoDB. The dedicated gene text search (Fig. 3b, arrow 7) offers control over the organisms and fields that will be searched. For example, the dedicated gene text search can be configured to search only the annotated gene product name of genes in *C. parvum* Iowa II. Both searches support a wild card (wild card = ∗ and means any character) used for broadening the search to include partial text. For example, a text search of the term *fructo∗* will

**Fig. 3** (continued) genes (arrow 1), Popset IDs (arrow 2), and ESTs (arrow 3). (**c**) Gene ID search page configured to return 5 genes. (**d**) My Strategies page depicting results of the ID search shown in (**c**) and consisting of the strategy panel summary and actions (blue box), the organism filter (purple box) showing the distribution of IDs, and the result table (brown box) with returned IDs and links for downloading and viewing results

retrieve genes whose records contain words that start with the pre-fix "fructo." Surrounding a multiword phrase in quotation marks configures the search to find the exact phrase. For example, search-ing for *"protein kinase"* will return genes with the exact term "pro-tein kinase"; whereas searching for *protein kinase* (without quotations) will return genes with the term "protein," genes with the term "kinase," and some may have the phrase "protein kinase."

1. Use the Gene Text Search to retrieve genes that are likely kinases and download the GeneIDs and download the IDs, product description, and coding sequence length for all results. Navigate to the dedicated gene text search from the home page (Fig. 3b, arrow 6) and enter the term kinase in the box for the Text term parameter (Fig. 4a). Notice the default set-tings for the Organism parameter (all organisms selected) and Fields (exclude similar proteins (BLAST hits v. NRDB/PDB)). Click Get Answer to initiate a search of all annotated genome sequences for genes whose records contain the term kinase.

2. Examine the search results. The result (over 3500 genes) appears in the My Strategies section and genes from every organism are returned by the search as seen in the Organism table. Results may vary in subsequent database releases due to annotation changes and new database content. The URL for this strategy is http://cryptodb.org/cryptodb/im. do?s=4be050ac9f4150fe.

3. Download the product description and coding sequence length for your result. Briefly described above, the download tool is available from any result table. Click Download (Fig. 4b, arrow 1) to open the tool (Fig. 4c). Choose Tab Delimited Excel— Choose columns to make a custom table to retrieve the custom table tool. Since IDs are downloaded by default, choose the Product Description and CDS length as columns, change the Download Type to Text File, and click Get Genes (Fig. 4b, arrow 2). An example of the text file downloaded from CryptoDB 39 is in Electronic Supplemental File 2.

*2.2.4 Record Retrieval Based on Sequence Similarity, BLAST Search*

Laboratory and bioinformatic research often generate sequence data for genes and isolates. The CryptoDB BLAST search (Fig. 5) employs the NCBI-BLAST algorithm deployed locally on the CryptoDB collection of organisms, to retrieve records based on similarity to a user-defined input query sequence. Input sequences can be compared to CryptoDB genomic sequences, transcripts, proteins, Popset Isolate sequences obtained from GenBank, ESTs, and ORFs. Furthermore, an option exists for comparing an input

**Fig. 4** (continued) (*Fields* = exclude Similar proteins (BLAST hits vs. NRDB/PDB)). (**b**) Search results in the My strategies page showing the link to the Download Tool (arrow 1). (**c**) Download tool configured to retrieve a text file containing IDs, product descriptions, and coding sequence lengths for 3519 genes returned by the text search in (**a**)

**Fig. 4** Dedicated text search page and download tool. (**a**) Search page shown configured to return genes from all annotated genome sequences (*Organism* parameter = 13 selected out of 13) that contain the word "kinase" (*Text term (use * as wildcard)* = kinase) from the majority of text fields in the gene record Fig. 4

**Fig. 5** Retrieve records based on BLAST similarity. (**a**) BLAST search page configured to return Popset records (arrow 1) from a comparison of the input sequence against the *Cryptosporidiidae SSU_18S rRNA Reference Isolates* (arrow 2) database in CryptoDB. (**b**) Blast search result page in the My Strategies

18S sequence to the community curated *Cryptosporidiidae* SSU_18S rRNA Reference Isolate set to determine the putative identity of the input sequence relative to reference sequences that are considered standards by the *Cryptosporidium* community.

1. *Find the genotype of an unclassified Cryptosporidium isolate.* Consider that the following sequence originated from a laboratory study of the 18S small subunit ribosomal RNA gene from an isolate retrieved during a *Cryptosporidium* outbreak at a swimming pool. The sequence (below and Electronic Supplemental File 3) was identical from all isolates. Use the CryptoDB BLAST search to find a similar reference sequence.

   AAGCTCGTAGTTGGATTTCTGTTAATAA
   TTTATATAAAATATTTTGATGAATATTTATATAATA
   TTAACATAATTCATATTACTATATATTTTAG
   TATATGAAATTTTACTTTGAGAAAATTAGAG
   TGCTTAAAGCAGGCATATGC
   CTTGAATACTCCAGCATGGAATAATA
   TTAAAGATTTTTATCTTTCTTATTGGTTCT
   AAGATAAGAATAATGATTAATAGGGACA
   GTTGGGGGCATTTGTATTTAACAGTCAGAGGTGAA
   ATTCTTAGATTTGTTAAAGACAAACTAATGCGAAAG
   CATTTGCCAAGGATGTTTTCATTAATCAAGAACG
   AAAGTTAGGGGATCGAAGACGATCAGATACCGT
   CGTAGTCTTAACCATAAACTATGCCAACT
   AGAGATTGGAGGTTGTTCCTTAC
   TCCTTCAGCACCTTA

2. Navigate to the BLAST search from the tools menu and set the *Target Data Type* to Popset (Fig. 5a, arrow 1) which will configure the query to return Popset Isolate records and to use the BLASTN algorithm. For *Target Organism,* choose *Cryptosporidiidae SSU_18S rRNA Reference Isolates* (Fig. 5a, arrow 2) to BLAST your input sequence against the isolate reference database. Paste the sequence into *Input Sequence* and click *Get Answer* to initiate the BLAST.

3. The results appear in *My Strategies* (Fig. 5b) with a graphic summary of the strategy at the top followed by three tabbed pages for the results: BLAST (Fig. 5b, arrow 3), Popset Isolate Sequences (Fig. 5c), and Popset Isolate Sequences Geographic Location (Fig. 5c, inset). The BLAST result (BLAST tab) reveals two hits with 100% identity, to *C. parvum* over the length of the sequence provided. One hit was to a *C. parvum*

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**Fig. 5** (continued)  section tabulates the 50 best Popset sequences based on Score and E value. (**c**) Other tabbed pages in the BLAST search results. *Popset Isolate Sequences* tab lists the record IDs with associated CryptoDB data and offers a sequence alignment tool, while the *Popset Isolate Sequences Geographical Location* displays the collection site location

bovine strain (AF093490) and the other to *C. parvum* Iowa II (AF164102), indicating that the outbreak is likely caused by *C. parvum.*

**2.3    Record Pages**    CryptoDB's extensive record system is a rich data mining resource. Records compile all database information into tables and graphs for genes and nongene entities including genomic sequences, Popset Isolate Sequences, Genomic Sequences, Genomic Segments, SNPs, ESTs, ORFs, Metabolic Pathways, and Compounds. All record pages open with a summary section at the top (Fig. 6a) and employ a floating, collapsible *Contents* panel (Fig. 6b) on the left for navigating the data section (Fig. 6c) on the right. This section of the chapter will explore the structure of the record page and highlight important data content using record pages for the gene that encodes GP60 (cgd6_1080), the isolate record page for *Cryptosporidium parvum isolate 830 surface glycoprotein 900 gene*, and the metabolic pathway for *Glycolysis/Gluconeogenesis (KEGG).*

*2.3.1    Gene Record Page Organization and Content*

1. *Use the Gene ID search in the header to open the record page* for the gene encoding GP60 *(cgd6_1080)* (Fig. 6a, inset). The gene page opens with summary information at the top (Fig. 6a) including gene ID, gene product name, genomic location, and species/strain information. In this case, the official gene name, Glycoprotein_GP40 differs from the gene and protein name commonly used by the research community. See **Step 2** below for more information about gene aliases and other names. Links to bookmark (*Add to favorites*) or save the gene for later use (*Add to basket*) as well as to download sequences or gene data appear at the top of the page (Fig. 6a, arrow 1). The *Shortcuts* (Fig. 6a arrow 2) provide quick visualizations of the gene page data via the magnifying glass icon and navigate to the corresponding image in the data when the image or the label above is clicked. The collapsible *Contents* section (Fig. 6b) directs the data section (Fig. 6c) of the page to the desired content. Uncheck a box to hide that data from the page. Data (Fig. 6, arrow 3) are represented in images, graphs, and downloadable tables (Fig. 6, arrow 4). The *Data sets* link next to the section title reference the data sets from which the data were taken (Fig. 6, arrow 4). *View in genome browser* links offer easy access to the genome browser in the genomic region containing the gene (Fig. 6d). All mapped sequence-based data are available for interactive display via the *Select Tracks* page (Fig. 6d, arrow 5).

2. *Annotation, curation, and identifiers section. Use the Contents navigation tool to view this section* (Fig. 7a), which offers alternative gene product descriptions, aliases, notes from the annotator, and a *User Comments* table. As new versions of genome
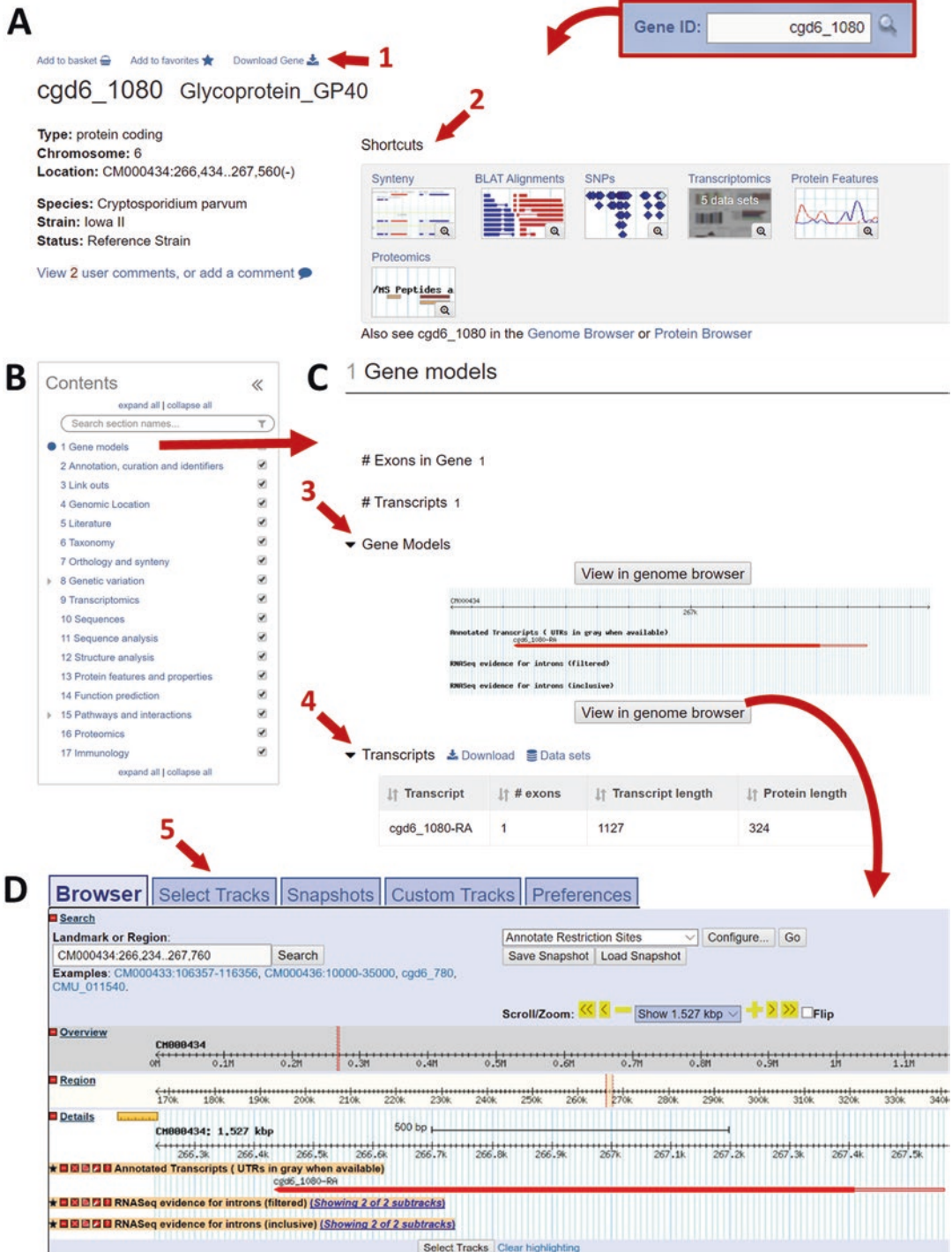
**Fig. 6** Gene record page for cgd6_1080, Glycoprotein_GP40. (**a**) Summary section containing basic location and identity information. Quick links to send the gene to Basket and Favorites or download (arrow 1) are available. Shortcuts (arrow 2) direct the data section to display the associated data. (**b**) Searchable Contents panel for navigating the data on the page showing the data section directed to the Gene Models section. (**c**) Data section focused on Gene Models which are represented in images (arrow 3) or downloadable tables (arrow 4). (**d**) Browser tab of the genome browser accessed from the *View in genome browser* button in the gene page data section. The *Select Tracks* tab (arrow 5) is for choosing tracks to display in the Browser tab

**Fig. 7** Gene page section for annotation, curation, and identifiers. (**a**) Contents panel focused on the Annotation, curation, and identifiers section of the gene page. Previous names are listed in a table (arrow 1) as are comments entered by users that serve to enhance the official genome annotation. (**b**) Comment form accessed from the "Add a comment" link (long downward arrow) which is used to add information to the gene page and can contain files and images

annotation are accepted by the community and integrated into CryptoDB, gene IDs and product descriptions can change. CryptoDB maps the aliases within table *Names, Previous Identifiers, and Aliases* (Fig. 7a, arrow 1) to the current ID so that an ID search performed with an alias (CPW_00001274) will return the record page of the current ID, cgd6_1080.

CryptoDB encourages the community to enhance the gene page with information added via the *User Comments* platform. Comments can offer valuable additions to the official annotation such as alternative gene models, unpublished data, and associated publications or references. The comment form (Fig. 7b) used for entering comments is retrieved from the *Add a comment* link (Fig. 7a, down arrow). Users must be registered and logged in to enter user comments. Once added, the comment immediately becomes part of the record page and the CryptoDB searchable text. For example, the comments entered here (Fig. 7a, arrow 2) provide the alternative gene names "gp40/15" and "GP60." Although "GP60" does not appear in the gene product name, it does appear in the user comment which is queried during the *Gene Text Search*, so searching for the text term "GP60" will return the gene: cgd6_1080, Glycoprotein_GP40.

3. *Orthology and synteny section. Explore orthology and synteny for cgd6_1080, Glycoprotein_GP40 and compare the results to the cgd2_60, ABC_transporter.* Navigate to the Ontology and Synteny section with the *Contents* navigation tool and explore orthology for cgd6_1080, Glycoprotein_GP40 (Fig. 8a). The ID of the ortholog group to which this gene belongs appears as a link to the OrthoMCL group record (Fig. 8a). Use this link to go to OrthoMCL and explore the group features (scroll down in OrthoMCL.org to the *List of Sequences* table). Only two sequences are assigned to this group, supporting the species-specific and single-copy nature of cgd6_1080, Glycoprotein_GP40. Out of the 150 proteomes contained in OrthoMCLDB, which included *C. hominis* and *C. parvum* representing the *Cryptosporidiiae*, only one sequence each from *C. hominis* and *C. parvum* is represented in the group.

*Visit the Orthologs and Paralogs within CryptoDB table which lists all orthologs from organisms within EuPathDB* (Fig. 8b). This table expands the local list of orthologs compared to the Ortholog group at OrthoMCL. As CryptoDB integrates newly available annotated genome sequences, orthology is determined by assigning their proteomes to existing Ortholog groups without updating the original 150 proteome analysis that forms the underlying data set for OrthoMCLDB. Orthologs of cgd6_1080 do not appear in *Gregarina*, *Chromera*, or *Vitrella*. Only one gene from each *Cryptosporidium* species is orthologous and all are syntenic, in the same genomic location,

**Fig. 8** Orthology and synteny. (**a**) Navigation route from the gene page Contents tool to the *Orthology and synteny* section of the gene page and finally to the gene's OrthoMCL group page. (**b**) Orthologs and Paralogs within EuPathDB table for cgd6_1080

reflecting the conserved nature of this gene. Also notice the product name does not always reflect the activity of the gene, reflecting the maturity of the annotation. Use the *Gene Text* search in the header to search for *gp40*. The search returns less than 10 genes, one for each organism, again confirming the conserved single-copy nature of the gene.

*Contrast the orthology information for cgd6_1080, Glycoprotein_GP40 with cgd2_60, ABC_transporter*. Navigate to the gene record page for ABC_transporter by entering the ID (cgd2_60) in the *Gene ID* search in the header. Use the

*Contents* section to view the *Orthology and synteny* data section and notice the *Orthologs and Paralogs within EuPathDB* table. The table contains multiple entries for each *Cryptosporidium* species suggesting that gene duplication events may have occurred.

*Return to the cgd6_1080, Glycoprotein_GP40 gene page by clicking the browser back button or using the Gene ID search in the header.* The *Retrieve multiple sequence alignment or multi-FASTA* tool part of the *Orthology and Synteny* section can be used to produce a ClustalW multiple sequence alignment in the region of the gene. Choose all organisms (Fig. 9a, arrow 1) and click Run *alignment*. The alignment appears in a new tab and is an alignment between 9 sequences, the gene itself, and 8 additional genomic sequences found to have similarity. It is interesting to note that the *C. andersoni* sequence in the alignment is missing from the Ortholog table. A closer look at the *Synteny* graph (Fig. 9b), which shows the chromosomal organization of genes across organisms with orthology indicated by shadows connecting the genes, shows that no gene is annotated for the region in *C. andersoni* or *C. muris* (Fig. 9b, red box). Since the MSA returns an alignment for *C. andersoni*, this suggests that a gene should be called for the region and this information would make a good comment for the genomic sequence record page. Also notice that *Gregarina*, *Chromera*, and *Vitrella* do not have syntenic sequences or orthologs to GP60, indicative of the distant evolutionary relationship between the organisms.

4. *Genetic Variation section: Compare variation in the gp60 gene among isolates from China, Egypt, and Uganda.* From the *Synteny* graph, scroll down to the *Genetic Variation* section which contains the *Isolate Alignments in this Gene Region* tool and a summary of *DNA Polymorphism* determined from an analysis of whole-genome sequencing of isolates. The isolate alignment tool (Fig. 10a) produces an alignment of the whole-genome sequence data within the region of the gene. The tool opens with all isolates selected for the alignment and includes the option to reduce the number of isolates included in the alignment based on isolate characteristics such as geographic location of isolate collection or gp60 genotype. The left panel of the tool shows the isolate characteristics, while the right panel shows the data distribution for the characteristic chosen on the left. Navigate the filter tool (Fig. 10a, arrow 1) to choose only isolates from China, Egypt, and Uganda and retrieve the alignment with the *View Results* button. The results appear in a new tab with variation highlighted in pink. Figure 10b shows an area of the alignment with a SNP in the Ugandan isolate (TU114) (Fig. 10a, arrow 2) and another area where all but one Egyptian isolate (35090) diverge from

**Fig. 9** Gene alignments and synteny on the gene page. (**a**) Use the *Retrieve multiple sequence alignment or multi-FASTA* tool in the "Orthology and synteny" section for quick alignments in chosen organisms (arrow 1). (**b**) Synteny graph on the gene page is a static representation. Click the "View in genome browser" button to navigate to GBrowse with the possibility to change settings for the synteny graph

**Fig. 10** Isolate alignments and SNPs on the gene page. (**a**) *Isolate Alignments in this Gene Region* tool for retrieving alignments of isolate whole-genome sequence data with options for choosing isolates based on isolate characteristics (metadata). As shown, the tool is configured to align isolates from China, Egypt, and

the reference *C. parvum* sequence (CM000434) (Fig. 10a, arrow 3).

*Close the Isolate Alignment tool by clicking the triangle next to the title* (Fig. 10a, arrow 4) and notice the *DNA polymorphism* section which includes a summary of the coding potential, the incidence of all SNPs across all isolates, and an image of SNPs mapped to the gene model. In your browser, hover your mouse over the SNPs in the image to reveal a panel of detailed information including a link to the SNP record page (Fig. 10c).

5. *Transcriptomics Section: Scroll down from the DNA polymorphism section to view the Transcriptomics table* (Fig. 11), *which includes all transcript expression data sets with data that map to this gene*. Data types include genome-wide RT-PCR, microarray, and RNA-seq data. Table rows expand to reveal the results of CryptoDB transcriptomic analysis presented in graphs and tables. Click the triangle in the header next to the data set "Expression profiling of life cycle stages post-infection" to view the data graphs and tables (Fig. 11, arrow 1). For this experiment, transcript abundance was determined using Real Time-PCR for >3200 genes over a 72-h infection of HCT8 cells [30]. The data graph reveals that the gene is maximally expressed at 12 h postinfection.

*Compare the oocyst and intracellular stage expression for this gene*. A comparison of oocyst and intracellular stage transcriptomes is also available, and the graphs and tables associated with this RNA-sequence analysis can be viewed by expanding "Transcriptome of oocyst and intracellular stages" (Fig. 11, arrow 2). The data indicate that the gene is highly expressed in the intracellular stages with very little expression in oocysts. Open *Coverage* (Fig. 11, arrow 3) to reveal a snapshot of the RNA sequence coverage plots and these can be further interrogated using the *View in genome browser* link.

6. *Proteomics section: Use the Content section to navigate to the proteomics data*. Proteomics data are tabulated in the *Mass Spec.-based Expression Evidence* table as well as associated data such as sequence counts and observed spectra. The *Mass Spec.-based Expression Evidence Graphic* depicts individual peptides mapped to the protein sequence. In your browser, using your mouse to hover over the rectangle peptide glyphs retrieves details concerning the mapped peptide sequence and its spectral count and the experiment in which it was detected (Fig. 12).

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**Fig. 10** (continued)  Uganda. Isolates are chosen based on sample metadata, in this case the country of isolate collection (arrow 1) with China, Egypt, and Uganda as values. (**b**) A portion of the resulting alignment indicating variation highlighted in pink. (**c**) SNPs image from the *DNA Polymorphism* section of the gene page. SNPs are colored by coding potential and reveal detailed information upon hover

**A**



**Fig. 11** Transcriptomics section of the gene page. Transcriptomics table with expandable rows (arrows 1 and 2) that reveal detailed information concerning the experimental data. Data are represented in graphs and tables as well as coverage plots for RNA sequence data (arrow 3)

# 16 Proteomics

▶ Mass Spec.-based Expression Evidence ▤ Data sets

▼ Mass Spec.-based Expression Evidence Graphic ▤ Data sets
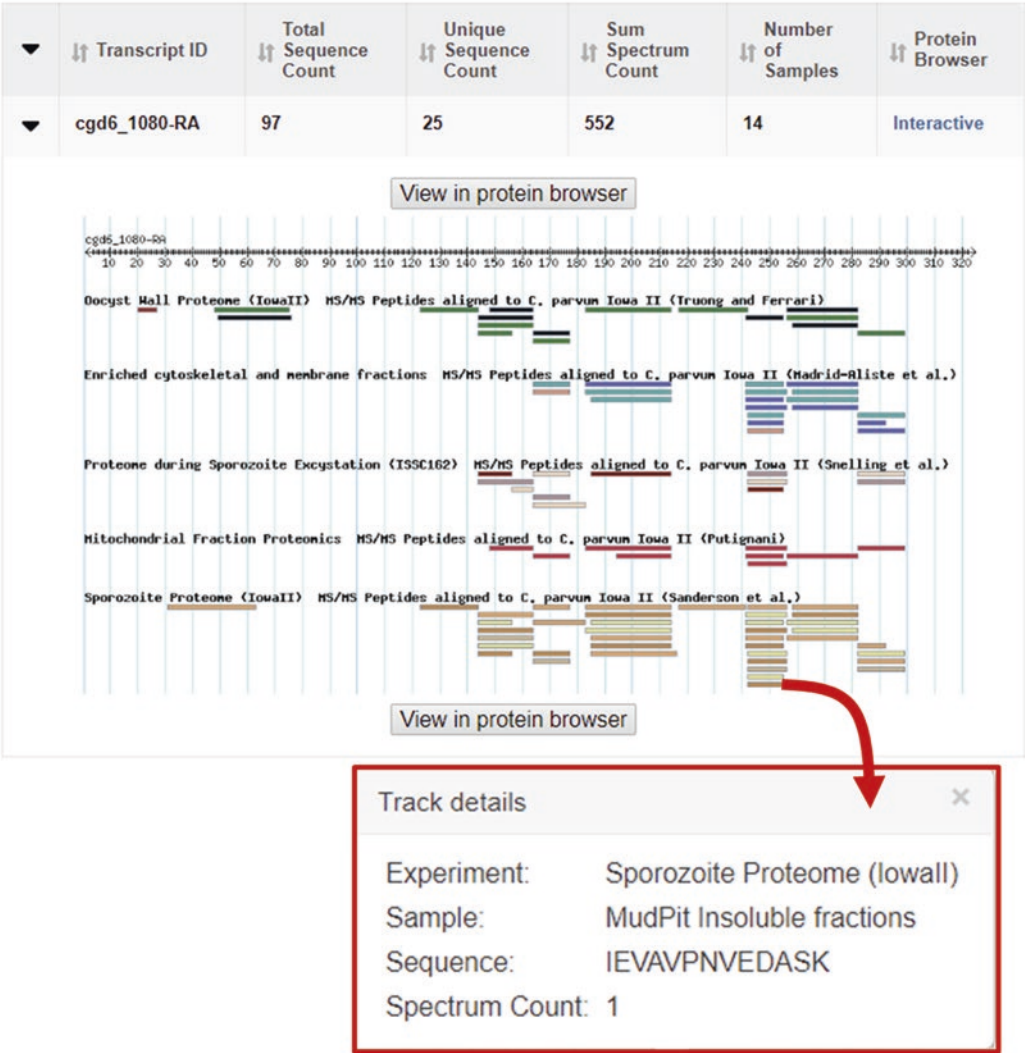


**Fig. 12** Proteomics section of the gene page. *Mass Spec.-based Expression Evidence Graphic* expandable table displaying graphic of peptides mapped to the protein sequence for five different proteomics experiments in CryptoDB. Mapping details are revealed when hovering over the peptides glyphs

*2.3.2 Isolate Record Organization and Content*

1. Retrieve the record for isolate AF527844, *Cryptosporidium parvum* isolate 830 surface glycoprotein 900 gene, partial cds by entering the ID into the ID search that returns Popset isolate records on the CryptoDB home page (Fig. 13a). Since the

search was run on only one ID, the result navigates to the record page instead of My Strategies. The layout of the isolate record page is similar to the gene page with a summary section, a floating Contents section (which can be closed using the double arrowhead on the right top), and a data section. The summary section contains pertinent information such as isolation source, location, organism, and product description information (Fig. 13b). Links to bookmark, save, and download the record appear above the ID and product description. The sequence section provides the nucleic acid sequence associated with the isolate, while the Blast Similarity Alignments and Overlapping Genes table shows the results of a BLAST of the isolate sequence against all genome sequences in CryptoDB (Fig. 13c). Overlapping genomic sequences and genes are indicated for each BLAST hit.

*2.3.3 Metabolic Pathway Record Organization and Content*

The Cryptosporidiiae are known to be missing several metabolic pathways [45, 46]. While basic pathways such as glycolysis are present, surprisingly, both purine and pyrimidine biosynthesis pathways are missing in Cryptosporidiiae, although salvage pathways are present [47]. Amino acid biosynthetic pathways are also missing for lysine, tyrosine, alanine, serine, selenocysteine, threonine, and thiamine. [48] Another pathway, the tricarboxylic acid (TCA) cycle is present in *C. muris* but absent from *C. parvum* and *C. hominis* [48]. These qualities can be investigated in the CryptoDB representation of metabolic pathways.

1. Retrieve the Glycolysis/Gluconeogenesis (KEGG) and explore the basic functions of the page. Navigate to the Pathway Name/ID search under Metabolic Pathways in the Search for Other Data Types category on the home page (Fig. 14a). Set the Pathway Source to KEGG and begin typing "glycolysis" in the Pathway Name or ID parameter and then choose the pathway from the list. Click Get Answer to retrieve the pathway.

   The organization of the page is similar to other record pages with a Summary at the top, a collapsible Contents section, and the data presented in images and searchable tables. An interactive Cytoscape [49] image (Fig. 14b) shows the pathway as a series of enzymatic reactions with square enzyme nodes and circular compounds. The image can be repositioned by dragging or with the navigation tool (Fig. 14b, arrow 1). Resizing the image is accomplished with the zoom tool (Fig. 14b, arrow 2) or by scrolling a mouse in or out while hovering over any portion of the image. At low zoom levels, pathway nodes appear as glyphs. At mid zoom levels, the main compound nodes appear as molecular formulas. At high zoom levels, enzymes and side nodes appear as formulas, terms, or Enzyme Commission numbers. Clicking on a node retrieves details

**Fig. 13** Popset isolate records. (**a**) Popset Isolate ID search is accessed from the *Other Data Types panel, Popset Isolate Sequences, Popset ID(s)* search. Shown configured to return one record—AF527844. (**b**) Summary section of the Popset Isolate record. (**c**) *Blast Similarity Alignments and Overlapping Genes* table showing the results of a CryptoDB BLAST analysis of the sequence against all genome sequences integrated into CryptoDB. Links to matching genomic sequences and overlapping genes are provided

**Fig. 14** Metabolic pathway record page. (**a**) Metabolic Pathway Name/ID search is accessed from the *Other Data Types* panel, *Metabolic Pathways, Pathway Name/ID*. The search is shown configured to query for KEGG Pathways and use the type-ahead function of the *Pathway Name or ID* parameter.

about the node, regardless of zoom level (Fig. 14b, arrow 3 and expansion). Enzyme nodes highlighted in orange represent enzymes for which at least one gene in CryptoDB is annotated with the EC number of the enzyme. Pathway reactions are tabulated in Metabolic Pathway Reactions (Fig. 14, arrow 4) below the pathway image, and Pathways with Shared Reactions offer links to related pathways.

2. Annotate the Glycolysis/Gluconeogenesis (KEGG) pathway using the Paint Enzymes tool. Click on the yellow part of the glyph representing Glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) (Fig. 14b, arrow 3) to retrieve the node details. Open the Paint Enzymes tool in the Cytoscape Drawing menu bar and choose By Genera (Fig. 15, arrow 1). Use the Genera Selector tool to choose Cryptosporidium, Gregarina, Chromera, and Vitrella. Click Paint to retrieve a graph depicting which organisms have genes with this EC number. The graph (Fig. 15, arrow 2) indicates that all organisms have at least one gene with this enzymatic activity. To retrieve a list of the genes, click the Show genes which match this EC Number link (Fig. 15, arrow 3) to initiate a search for genes ("Identify Genes based on EC Number") that are annotated with the EC number. The search returns 25 genes. Scroll to the organism table in the search result and notice that the search returned one gene for each Cryptosporidium organism in CryptoDB, while multiple genes annotated with the EC number are found in Gregarina, Chromera, and Vitrella. These are likely paralogs/orthologs since all genes belong to the same ortholog group.

Pathways may also be annotated with expression data from Microarray and RNA sequence data to give a quick overview of pathway expression. Using a similar workflow as above, explore the expression of enzymes within the glycolysis pathway (Fig. 15, inset). Paint the pathway with the RNA sequence data set called "*C. parvum* Iowa II Transcriptome of oocyst and intracellular stages (Widmer et al.)" in which the authors determined the transcriptomes of oocysts and *C. parvum*-infected cultures.

3. Explore the TCA cycle for Cryptosporidium species. Navigate to the MetaCyc pathway record for TCA cycle I (prokaryotic) as above and annotate the pathway by genera with Cryptosporidium, Gregarina, Chromera, and Vitrella. Click on the nodes to inspect the genera graphs and notice that at least

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**Fig. 14** (continued) **(b)** Glycolysis/Gluconeogenesis pathway record page depicting the Cytoscape representation. Navigation (arrow 1) and zoom (arrow 2) tools are available and will reveal enzyme EC numbers and compound structures at sufficient zoom. Node details appear once an enzyme (square) or compound (circle) has been clicked. *Metabolic Pathway Reactions* (arrow 4) tabulate all reactions in the pathway
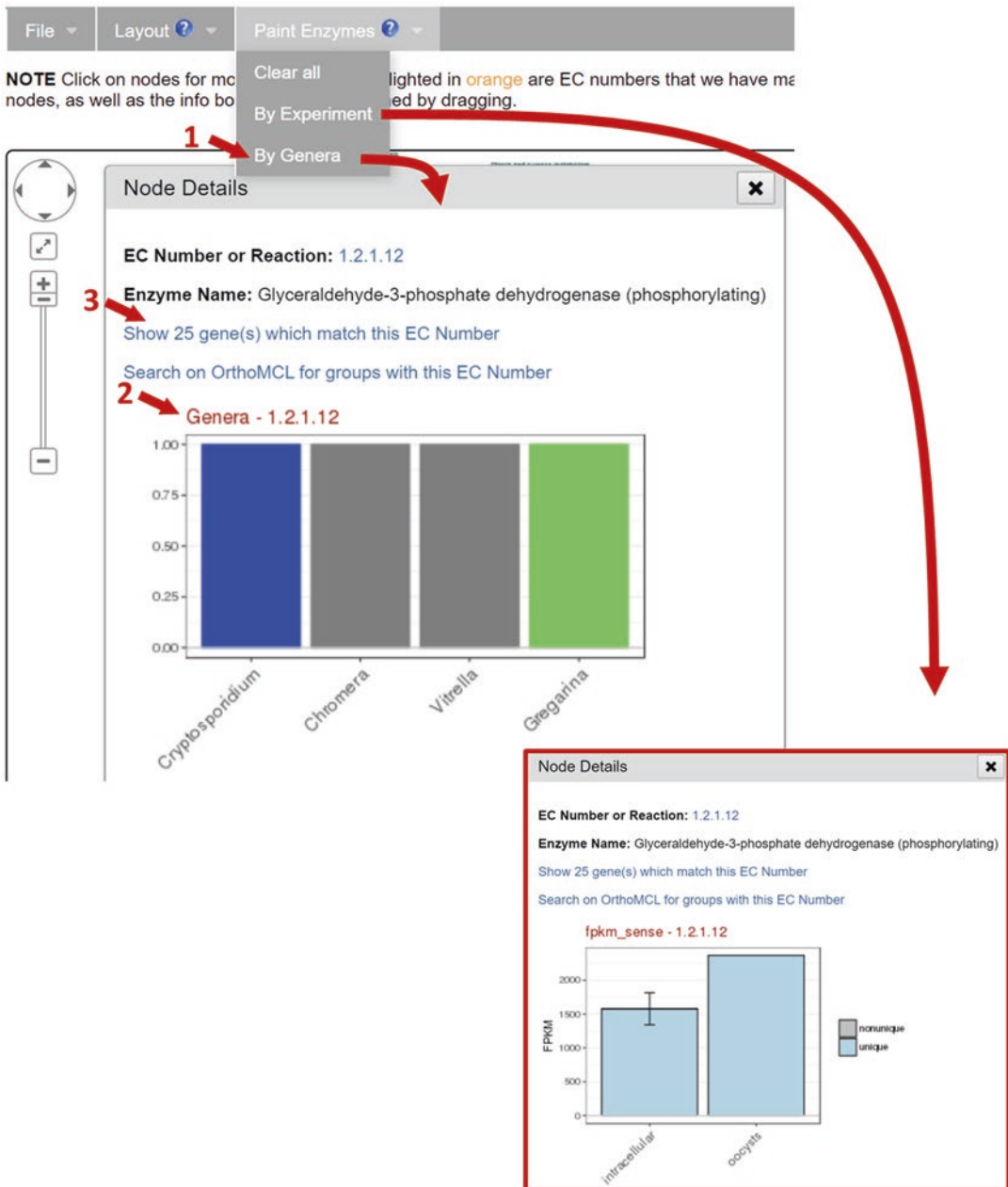
**Fig. 15** Annotate metabolic pathways. (**a**) Metabolic pathways can be annotated with orthology and functional genomics data using the *Paint by Genera* (arrow 1) and *Paint by Experiment* tools, respectively. Once annotated, *Node Details* will include data graphs (arrow 2 and inset). The *Show gene(s) which match this EC Number* (arrow 3) link initiates a search for genes with that EC number

one gene encoding each enzyme is present in Cryptosporidium. To learn the distribution of enzymes across the CryptoDB organisms, use the Show genes with matching EC numbers link within the node details panel or the link in the Gene Count column of the Metabolic Pathway Reactions table. The organism table in the search result page reveals that *C. muris* and *C. andersoni* have genes representing the enzyme EC 1.1.1.42, but it is missing from the other Cryptosporidium strains. Using the table links to investigate the remaining enzymes in the pathway, *C. muris* is the only Cryptosporidium species to have all the enzymes, supporting the finding that the TCA cycle exists in *C. muris* but is missing from *C. parvum* and *C. hominis* [48, 50].

### 2.4 Searches, Strategies, and Result Analyses

The CryptoDB strategy system is a unique and powerful tool for exploring relationships across data sets, data types, and organisms. The system offers over 100 preconfigured searches that query individual data sets ranging from genome-wide analyses for predicted signal peptides to GO annotation, transcriptomic analyses, or SNP analyses. Each search provides evidence for a specific biological property, returning a list of records that meet the search criteria and therefore share the biological property intrinsic to the data set. For a comparative genomics perspective, searches can be configured to query any or all genome sequences integrated into CryptoDB. The system can easily answer questions concerning stage-specific expression, expression timing, biological function, and more.

Strategies are created by adding, subtracting, intersecting, joining, or collocating the results of individual searches. An orthology transform tool can convert a gene result in one organism to their orthologs in another, making it possible to take advantage of a data-rich organism such as *C. parvum* to garner information about less studied organisms such as *Gregarina*. A colocation tool is used to explore relationships based on relative genomic location, for example, to find SNPs located within 500 bp of annotated genes. A nesting tool can be used to control the strategy logic while combining search results. The "*Analyze Results*" tools offer GO, metabolic pathway, and gene product word enrichment analyses for any gene search or strategy result. Isolate search results can be analyzed by MSA. The following examples illustrate how to create and analyze a gene strategy and an isolate strategy in CryptoDB.

### 2.4.1 Gene Strategy Example

This example assembles a list of *G. niphandrodes* genes that are likely kinases expressed in oocysts by creating a four-step strategy. The first step is a text search that retrieves genes from all organisms in CryptoDB whose records contain the word kinase (Fig. 16a, arrow 1). The second search (Fig. 16a, arrow 2) retrieves genes annotated with GO terms associated with kinase activity which provides a second line of evidence for kinase activity. The first two
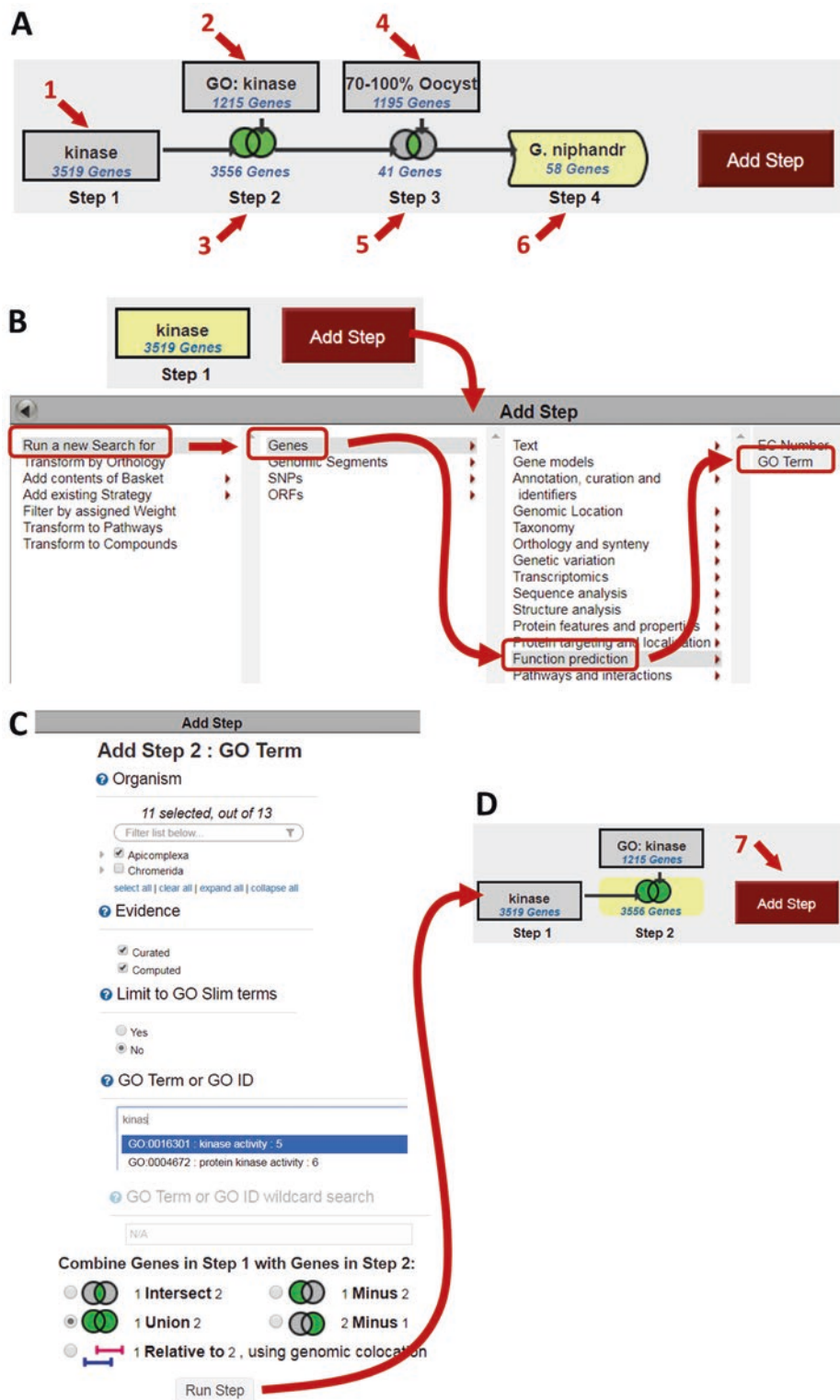
**Fig. 16** Example 1 strategy, Step 1 result and adding Step 2. (**a**) Strategy panel depicting the final four-step strategy with search (arrows 1, 2, 4) and step results (arrows 3, 5, 6). (**b**) Strategy result after the text search for kinase and the *Add Step* panel for extending the strategy. (**c**) GO Term search page accessed from the *Add Step* panel configured to combine the GO Term search with previous results using a union operator. (**d**) The two-step strategy results after running the GO Term search and combining it with the text search

searches are combined by adding (union) the two search results to create the Step 2 result (Fig. 16a, arrow 3), which is a more comprehensive set of kinases across all organisms. Next, we want to restrict the list of kinases to only those with evidence of expression during the oocyst stage. Since no transcriptomic data are publicly available for *G. niphandrodes*, we will query *C. parvum* data and then transform those results into *G. niphandrodes*, thus inferring expression is conserved between organisms (potentially an invalid assumption, but it serves for illustrative purposes). The third search in the strategy (Fig. 16a, arrow 4) queries the expression values determined in an RNA sequence analysis comparing the oocyst and intracellular stages of *C. parvum*. The oocyst expressed genes are intersected with the kinases to produce a Step 3 result (Fig. 16a, arrow 5), including kinases that are also expressed in the oocyst stage. An intersection returns genes in common between the two search results. Since Step 2 is a multi-organism set of kinases, while Step 3 contains only *C. parvum* oocyst genes; the Step 3 result (intersection) contains only *C. parvum* genes. The fourth step (Fig. 16a, arrow 6) transforms the *C. parvum* oocysts-expressed kinases into their *G. niphandrodes* orthologs creating a set of *G. niphandrodes* genes that are likely oocyst-expressed kinases. The full strategy can be found here: http://cryptodb.org/cryptodb/im.do?s=88749e65a931cae9

1. *Use the text search to find genes whose records contain the work kinase in their records.* As in Subheading 2.2.3, **Step 1** above, initiate a text search for the term "kinase" from the dedicated Gene text search (Fig. 4a). The search returns over 3500 genes across all organisms in CryptoDB. Future versions of CryptoDB may return different results as new genome sequences become available and are integrated, and the annotation of existing genome sequences is updated.

2. *Expand the set of kinases by adding a search for genes that are annotated with the GO term GO:0016301: kinase activity.* GO Terms are part of a controlled vocabulary describing the biological function, molecular process, or location of gene products. Terms are assigned to genes during the annotation process. CryptoDB enhances GO annotations by running InterproScan on all genome sequences and integrating the analysis results. A search for genes annotated with the kinase activity GO term may identify genes with kinase activity that do not contain the word kinase in their records. To extend the strategy with this search, click the *Add Step* button (Fig. 16b) and navigate the *Add Step* panel through *Run a new Search for, Genes, Function Prediction, Go Term*. When the GO Term search page appears, begin typing the GO term in the *GO Term or GOID* parameter and then choose the correct item (*GO:0016301: kinase activity*) from the list.

**Table 2**
**Methods for combining search results**

| Operator | Symbol | Combined result will contain |
|----------|--------|------------------------------|
| 1 intersect 2 |  | IDs in common between 1 and 2 |
| 1 union 2 |  | A nonredundant set of IDs from list 1 and list 2 |
| 1 minus 2 |  | IDs unique to 1 |
| 2 minus 1 |  | IDs unique to 2 |
| 1 relative to 2 |  | IDs whose features are new each other (collocated) in the genome |

Since this search is the second step of a strategy, the method of combining the GO Term search results with the Text term search results must be indicated before running the search (Table 2). Choose *1 Union 2* to produce a Step 2 result that contains all genes from both searches. The GO term search returns over 1200 genes annotated with the GO term kinase activity, and the combined result is a set of over 3500 genes with gene products that likely have kinase activity (Fig. 16c).

3. *Reduce the Step 2 result to only those kinases that are highly expressed in oocysts.* To ensure that the kinases in Step 2 are expressed during the oocyst stage, intersect the Step 2 result (kinases) with a search for genes that are highly expressed in the oocyst. Click *Add Step* (Fig. 16c, arrow 7) and navigate the Add Step panel through *Run a new Search for, Genes, Transcriptomics, RNA Sequence* (similar to Fig. 16b). Notice that *G. niphandrodes* is not represented in the *Organism* column, indicating that CryptoDB does not contain RNA sequence data sets associated with *G. niphandrodes.* However, CryptoDB does offer a data set that contains expression values from the *C. parvum* oocyst and intracellular transcriptomes determined by RNA sequence analysis [27]. Searching these data will return genes expressed in oocyst, and the intersection of the oocyst genes with the Step 2 result will find genes in common between the two lists: kinases that are expressed during the oocyst stage.

Choose the *Percentile* search for the data set "Transcriptome of oocyst and intracellular stages (Widmer et al.)" (Fig. 17a).

**Fig. 17** Example 1 strategy, adding Step 3. (**a**) RNA sequence evidence table for choosing data set to query. (**b**) Percentile RNA sequence search page specific to the *Transcriptome of oocyst and intracellular stages* data set. The search is configured to search the Oocyst sample (arrow 1) for genes in the 70–100% percentile (arrow 2) of expression in the sample and intersect (arrow 3) the oocyst expressing genes with the Step 2 result. (**c**) The three-step strategy result

This search returns genes based on the percentile rank of expression values within a sample. CryptoDB analyzed the raw RNA sequence data to produce expression values and determined the percentile rank of each gene within samples. To configure the search to find genes that are highly expressed in oocysts, choose *Oocysts* for the *Samples* parameter (Fig. 17b, arrow 1) and set the *Minimum expression percentile* to 70 (Fig. 17b, arrow 2). Choose to intersect the results of this new search with Step 2 using *2 Intersect 3* (Fig. 17b, arrow 3). The Step 3 result will have genes that are present in the Step 2 result as well as the expression search and, therefore, have both biological properties, kinase activity, and high expression in oocysts. Click *Run Step* to initiate the expression search and combine the results with Step 2. The expression search returns over 1100 genes that are highly expressed in oocysts and over 40 of these are likely to have kinase activity (Fig. 17c).

4. *Use the Transform by Orthology tool to convert the C. parvum oocyst kinases to their G. niphandrodes orthologs.* This step of the strategy takes advantage of expression data available in *C. parvum* to make inferences about possible expression of *G. niphandrodes* genes. Click *Add Step* (Fig. 17c, arrow 4) and choose *Transform by Orthology* in the first column of the Add Step panel (Fig. 18a). Set the transform to *Gregarina niphandrodes* using the *Filter list below* tool. Begin typing the organism name and select the correct organism from the list (Fig. 18a, arrow 1). Click *Run Step* to initiate the transform. The transform returns over 50 *G. niphandrodes* genes. This final strategy result is a set of *G. niphandrodes* genes that likely proteases expressed in oocysts. The full strategy can be found here: http://cryptodb.org/cryptodb/im.do?s=88749e65a931cae9

5. *Explore your results.* A critical review of the results is advisable to ensure credibility. For example, review the *Product Description* column of the *Gene Result* to see if they seem like kinase (Fig. 18b, arrow 2). Use the links in the *Gene ID* column (Fig. 18b, arrow 3) to visit a few gene pages which may contain information about domains associated with kinase activity.

6. *Analyze your results*: Determine functional and metabolic pathway enrichment for the *G. niphandrodes* gene set. CryptoDB offers enrichment analyses that can be accessed from the *Analyze Results* tab (Fig. 18c, arrow 4) of any search result. Focus the strategy on the Step 4 result by clicking on the search box in the strategy panel. The active result is highlighted in yellow and the genes from that result populate the Gene Result table. Click the blue *Analyze Results* tab to create a *New Analysis* tab that will contain the analysis results and choose to run a *Gene Ontology Enrichment* (Fig. 19a). Click *Submit* to run the default analysis (Fig. 19b, arrow 1) which will apply a Fischer's exact test to
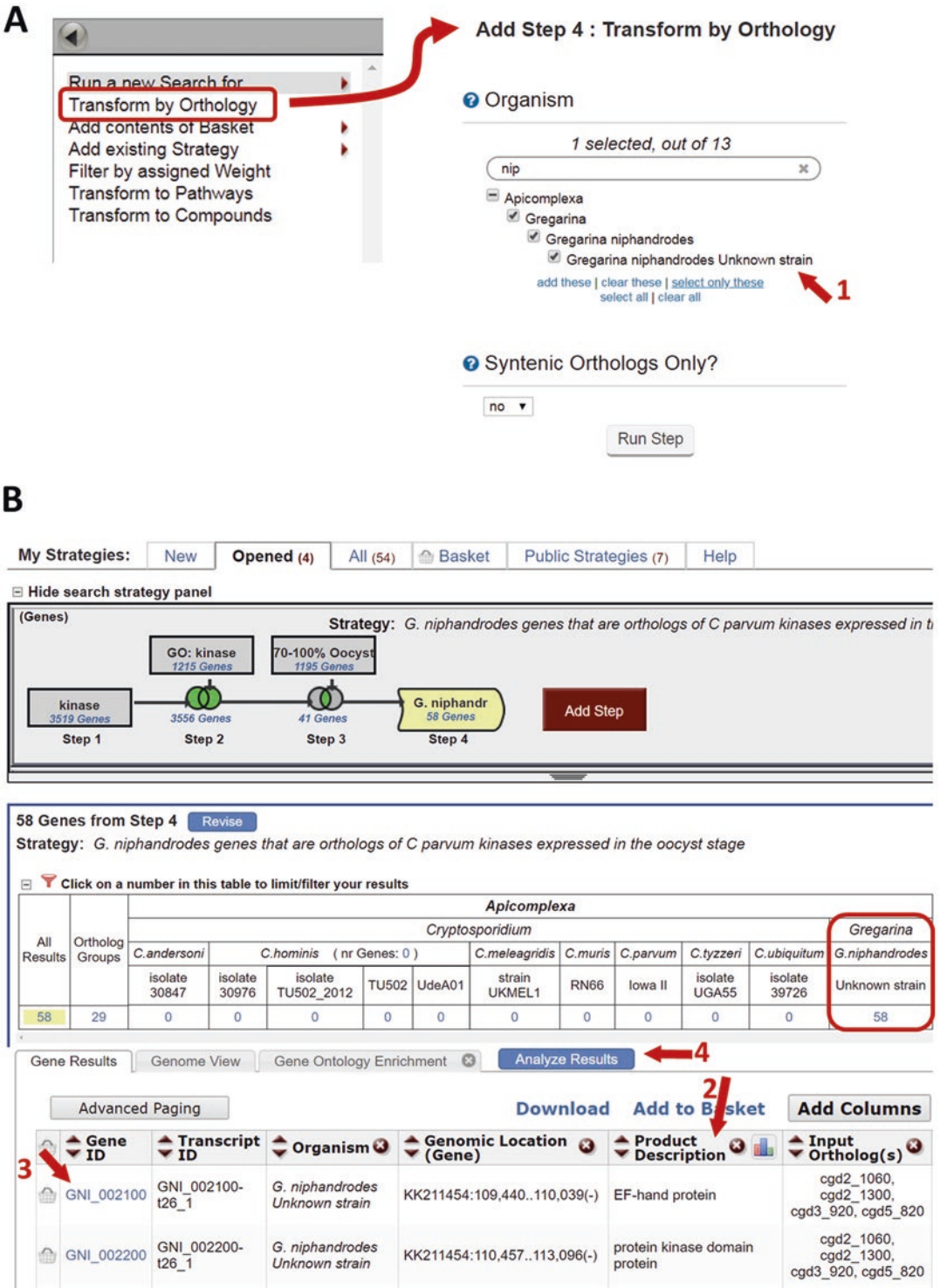
**Fig. 18** Example 1 strategy, transform by orthology. (**a**) *Transform by Orthology* tool accessed from the *Add Step Panel* and configured to convert the Step 3 result into their *G. niphandrodes* orthologs. (**b**) The final strategy result shown in *My Strategies*. The organism filter reflects the transform into *G. niphandrodes* (red border). Enrichment analyses are available in the *Analyze Results* tab (arrow 4)

**Fig. 19** Example 1 strategy, enrichment analyses of the strategy result. (**a**) The blue *Analyze Results* tab for accessing the enrichment analyses, which include *Gene Ontology Enrichment*, *Metabolic Pathway Enrichment*, and *Word Enrichment*. (**b**) *Gene Ontology Enrichment* tool configured to search the active strategy result for enriched biological process GO terms that are supported by computed and curated evidence, will not limit to GO Slim, and have an enrichment p-value of 0.05 or less. (**c**) GO enrichment analysis results shown tabulated with one row per enriched GO term and include a link to the Revigo visualization tool (arrow 2 and inset)

compare the biological process GO terms assigned to genes in the gene set to a background consisting of all genes in the genome. The analysis returns GO terms that are statistically enriched, appearing more often in the gene result than in all genes in the genome. The default GO enrichment of the *G. niphandrodes* oocyst expressed kinases returns over 120 enriched GO terms with p-values as low as $1 \times 10^{-28}$. The *Open in Revigo* button above the enrichment result table (Fig. 19c, arrow 2) opens the Revigo search page and populates the search with the GO enrichment results. Revigo is a clustering and visualization tool for enrichment analyses (Fig. 19c).

*2.4.2 Isolate Strategy Example*

Find isolates collected from the feces of humans in Europe that were genotyped with GP60. While Strategy Example 1 returned genes with shared biological properties, this strategy retrieves isolate sequences that share characteristics such as geographic location and isolation source. The sample characteristics (metadata) that CryptoDB downloads with the NCBI Popset isolate sequences are used to assemble a set of isolates that share your desired characteristics. This four-step strategy creates a set of isolates that were collected from the feces of humans in Europe and were typed using the gene encoding GP 40/15. The full strategy can be accessed here: http://cryptodb.org/cryptodb/im.do?s=2418e5fa79e56fde

1. *Find Popset Isolates Sequence records that were collected in Europe*. Navigate to *Identify Popset Isolate Sequences based on Geographic Location* from the home page *Search for Other Data Types* panel (Fig. 20a). Set the *Geographic Location* parameter to Europe and click *Get Answer*. The search returns over 1700 Popset sequences known to be collected in Europe.

2. *Filter the European isolates to include only those isolated from feces*. Click *Add Step* in the strategy panel (Fig. 20a, arrow 1) and navigate the panel through *Run a new Search for, Popset Isolate Sequences, Isolation Source* (Fig. 20b). From the *Add Step* search form, set the *Isolation Source* to feces (Fig. 20c). Choose to intersect the results of Step 1, the geographic location search, with the isolation source search so that the Popset Isolates in the Step 2 result have both properties, isolated in Europe from feces. Click Run Step to initiate the search. The Isolate Source search returns over 3400 isolate sequence records, and the Step 2 result contains over 600 isolates that were isolated in Europe from feces (Fig. 20c).
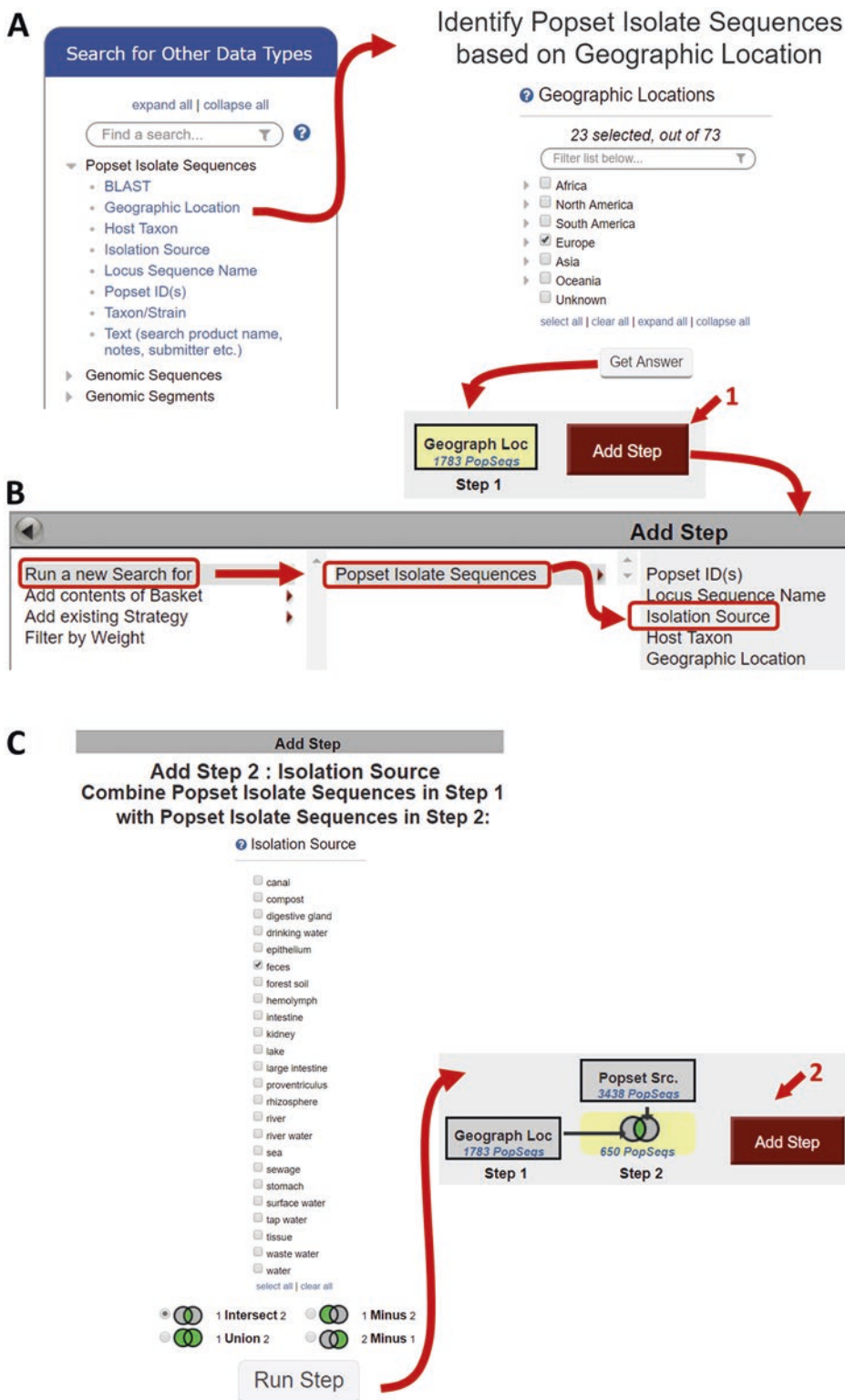
**Fig. 20** Example strategy 2, Steps 1 and 2. (**a**) Home panel showing the link for the search that returns Popset Isolate records based on *Genomic Location*. Search page shown configured to return isolates collected in Europe. Strategy panel depicting results of the search. (**b**) *Add Step* panel showing navigation to the *Popset Isolate by Isolation Source* search. (**c**) *Isolate Source* search form in the *Add Step* panel configured to search for Popset Isolates that were isolated from feces and intersect that result with the Step 1 result

3. *Filter the European isolates from feces to include only those geno-typed with sporozoite antigen gp40/15.* Click *Add Step* in the strategy panel (Fig. 20c, arrow 2) and navigate through *Run a new Search for, Popset Isolate Sequences, Locus Sequence Name.* Set the *Locus Sequence Name* parameter to sporozoite antigen GP40/15 (an alias for GP60) (Fig. 21a) and choose *2 Intersect 3* to combine the Locus Sequence search with the Step 2 result before clicking *Run Step.* The new search returns over 2600 isolates and the intersection with Step 2 returns over 180 iso-lates that were isolated from feces in Europe and genotyped with sporozoite antigen GP40/15.

4. *Filter the Step 3 result by host to include only sequences isolated from human.* Click *Add Step* after the Step 3 result (Fig. 21a, arrow 1) and navigate the *Add Step* panel through *Run a new Search for, Popset Isolate Sequences, Host Taxon.* To choose only *Homo sapiens* for the *Host* parameter, begin typing *Homo sapiens* in the search box and then click *Select Only These* when the tree is reduced to the correct choice. Choose *3 Intersect 4* the Host Taxon search with the Step 3 result and click *Run Step* to initiate the search. The search returns over 2200 Popset isolate sequences from human and the final strategy result returns 95 Popset isolate sequences that were geno-typed with sporozoite antigen GP40/15 and isolated from the feces of humans in Europe. The full strategy can be accessed here: http://cryptodb.org/cryptodb/im.do?s=2418e5fa79e56fde

5. *Explore and analyze your results by running a multiple sequence alignment for the Croatian and Czech Republic isolates.* Click on the *Popset Isolates Geographic Location* tab to see a map of the collection sites for isolates in the result (Fig. 21b, inset). Pin color represents the number of isolates from that location. Return to the *Popset Isolate Sequences* tab and sort the Geographic location column to place the isolates from Croatia and Czech Republic at the top (Fig. 21b, arrow 2). Click the box next to the Popset Sequence IDs for the two Croatian isolates and scroll to the bottom of the list to click *Run ClustalW on Checked Strains.* The alignment appears in a new window (Fig. 21c, left side). The sequences appear to be very similar with few differences noted in pink. Return to the *Popset Isolate Sequences* result and run the multiple sequence alignment

**Fig. 21** (continued) strategy result showing the addition of the *Locus Sequence Name* results. (**b**) Final strategy result shown in *My Strategies* including an expansion of the *Popset Isolate Sequences Geographical Location* tab which displays a map of isolates in geographic space (inset). Popset Isolate Sequences result with sortable columns (arrow 2) and a built in sequence alignment tool to easily return isolate ClustalW alignments of checked Popset Sequence IDs. (**c**) Example of alignment results between Croatian isolates (left) and Croatian plus Czech Republic isolates. Pink highlights indicate sequence divergence
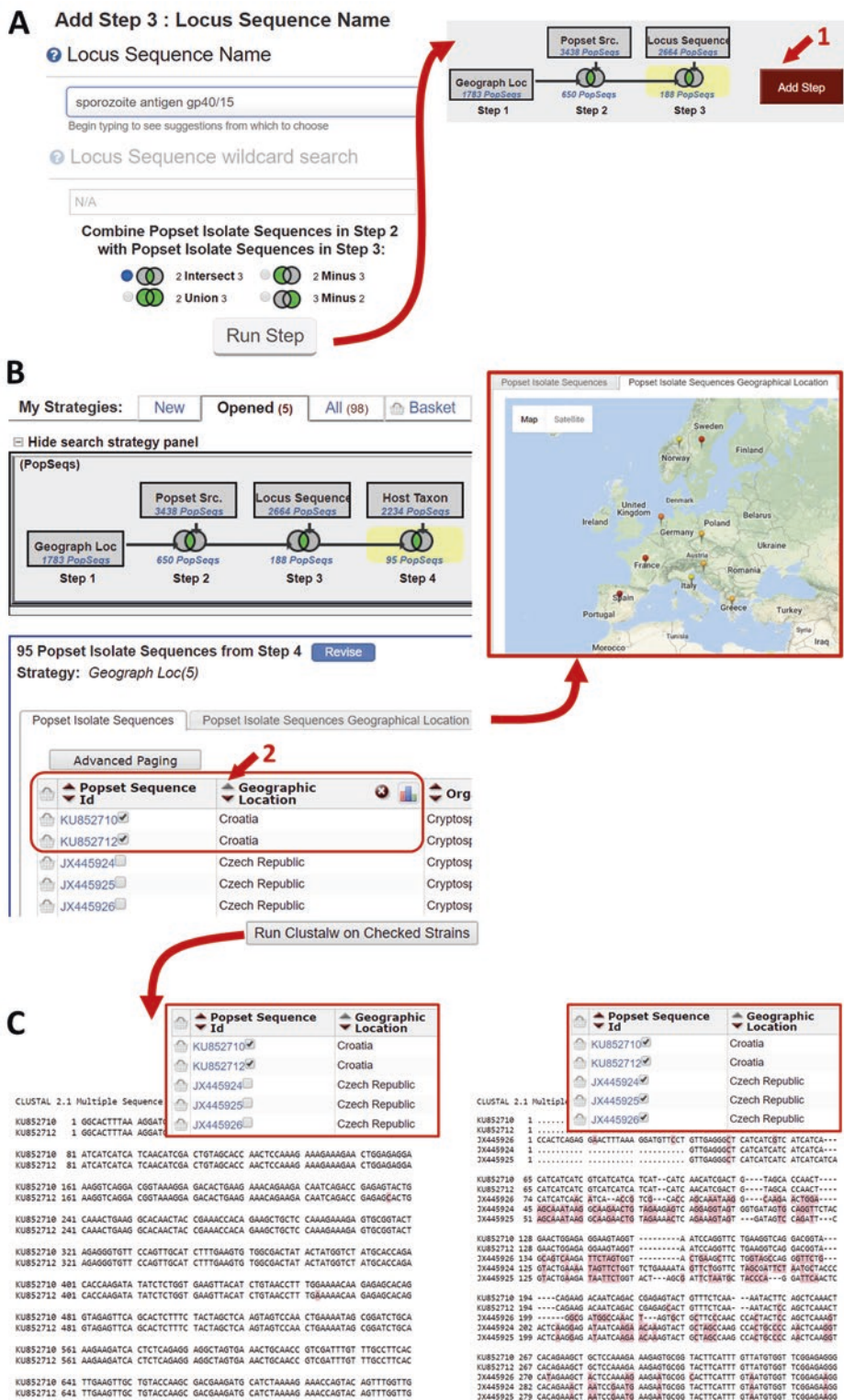
**Fig. 21** Example strategy 2, Step 3 and a sequence alignment of results. (**a**) *Locus Sequence Name* search that returns Popset isolates based on the locus that was used for genotyping. Shown configured to return isolates typed with sporozoite antigen gp40/15 and intersect those results with the Step 2 result. Three-step

on the three Czech Republic isolates in addition to the Croatian isolates. The results reveal (Fig. 21c, right side) differences between the groups of isolates. The Croatian isolates are similar to each other as are the Czech Republic isolates. However, many differences are noted between the Croatian and Czech Republic isolates.

*2.5   Data Analysis of Your Sequence Data*

Sequence alignment and enrichment analysis tools are an integral part of CryptoDB record pages and strategy results. Comparative genomics often relies on sequence alignments to reveal differences between strains or isolates. For easy access, tools for aligning gene sequence from annotated genome sequences (Fig. 9a) or isolate sequence in the region of the gene (Fig. 10a, b) are integrated into the gene record page. Popset Isolate sequence alignments can be performed from any isolate search or strategy result page (Fig. 21b, c).

While the strategy system facilitates reducing large volumes of data to meaningful gene sets, enrichment analyses support interpretation by identifying over-represented functional annotations such as GO Terms or metabolic pathways in a gene set. The CryptoDB enrichment analyses apply a Fischer's exact test to compare annotations assigned to the genes in a search result to the entire genome. Tools to determine gene ontology term, metabolic pathway, and gene product description term enrichment are readily accessible from a result page under the blue *Analyze Results* tab on any gene search or strategy result page (Fig. 18b, arrow 3 and Fig. 19).

CryptoDB also offers an environment for analyzing raw large-scale data such as for differential expression from RNA sequence or variant calling from whole-genome sequence of isolates. The following section describes how to use the EuPathDB Galaxy data analysis service developed in partnership with Globus Genomics to analyze your own high-throughput next-generation sequence data. Galaxy is an analysis tool which is available on all EuPathDB sites.

*2.5.1   CryptoDB/ EuPathDB Galaxy Data Analysis Service*

Galaxy is an open-source web-based platform for data intensive research that reduces the barrier of access to bioinformatic analysis since command-line computing is not required. A variety of bioinformatics tools are available and can be used one at a time or dropped into a graphic workspace and chained together to create workflows. Workflows and their results can be shared between users or published to the community. The CryptoDB Galaxy is preloaded with reference genome sequences and offers preassembled workflows for reference-based RNA sequence analysis as well as variant calling (SNP detection). Analysis results are easily ported to CryptoDB for private viewing in the genome browser side-by-side with data already integrated into CryptoDB.

The following example employs a shared EuPathDB workflow to call variants between a diarrheal isolate and the reference genome. A recent study of genetic diversity in Bangladesh performed whole-genome sequencing on 63 *Cryptosporidium hominis* isolates [13]. The published analysis calls single-nucleotide polymorphisms between the *C. hominis* isolates and *C. parvum* IowaII. We will use EuPathDB Galaxy to repeat a portion of this analysis. The data are available in the sequence read archive repositories (Project ID = PRJEB24168 at ENA and SRA) [51, 52] and can easily be imported to a Galaxy account. The workflow performs FastQC [53] to check the isolate's read quality and uses Sickle [54] for adapter trimming, Bowtie2 [55] for aligning the isolate sequences to the reference genome sequence, FreeBayes [56] for calling variants, SNPEff [57] for annotating and predicting the effects of genetic variants, and SNPsift [58] for manipulating the variant call files produced by the workflow.

1. *Visit and explore the CryptoDB Galaxy page by clicking Analyze My Experiment in the header menu bar.* Registration with both Globus Genomics and CryptoDB are required. First-time users will be prompted with a series of screens to create and log into their accounts. Once registered, Galaxy opens to a welcome page in the center panel (Fig. 22a, arrow 1) which contains links to tutorials as well as the EuPathDB preconfigured workflows and is controlled by the center top menu bar (Fig. 22a, red border). The center panel also serves as a workspace for configuring tools, starting and editing workflows, and file management. The tools panel (Fig. 22a, arrow 2) offers bioinformatics tools for a variety of purposes, including importing data into Galaxy, next-generation sequencing applications, data manipulation, and statistical analysis. Choosing a tool from the left panel (Fig. 22a, arrow 3) opens the tool in the center panel (Fig. 22b) where one can change default parameter values (Fig. 22b, left) and initiate the analysis. The History panel presents a running history of files imported or created during the analyses (Fig. 22a, arrow 4 and 22b, right). The Tools and History panels can be collapsed (Fig. 22a, arrow 5) creating a larger workspace in the center.

2. *Import isolate sequence data into Galaxy.* To import the isolate sequence data directly from EBI, open the *Get Data* via *Globus from the EBI server* tool from the tools panel (Fig. 22a, arrow 3) and enter SAMEA104459070 for the parameter *Enter your ENA Sample ID.* In this case, the sample ID is provided but retrieving Sample IDs is easily accomplished by searching the ENA or SRA repositories with a keyword (such as author name) the project ID (PRJEB24168, which is usually documented in the publication) to retrieve the data set record.
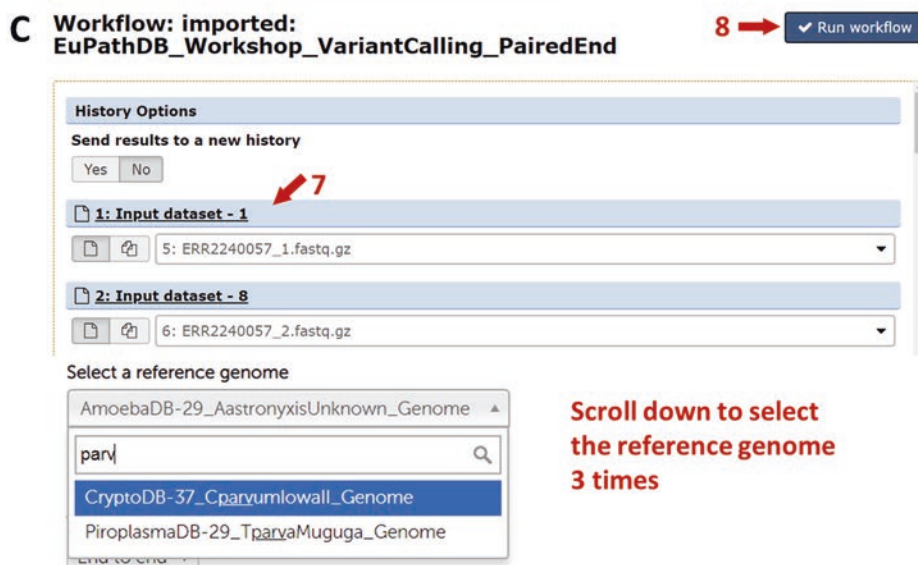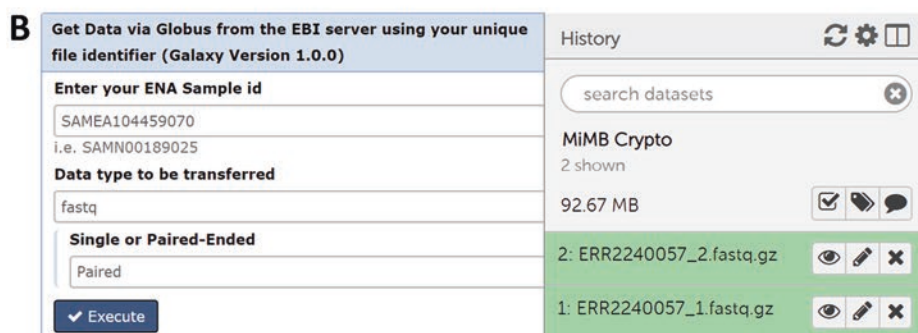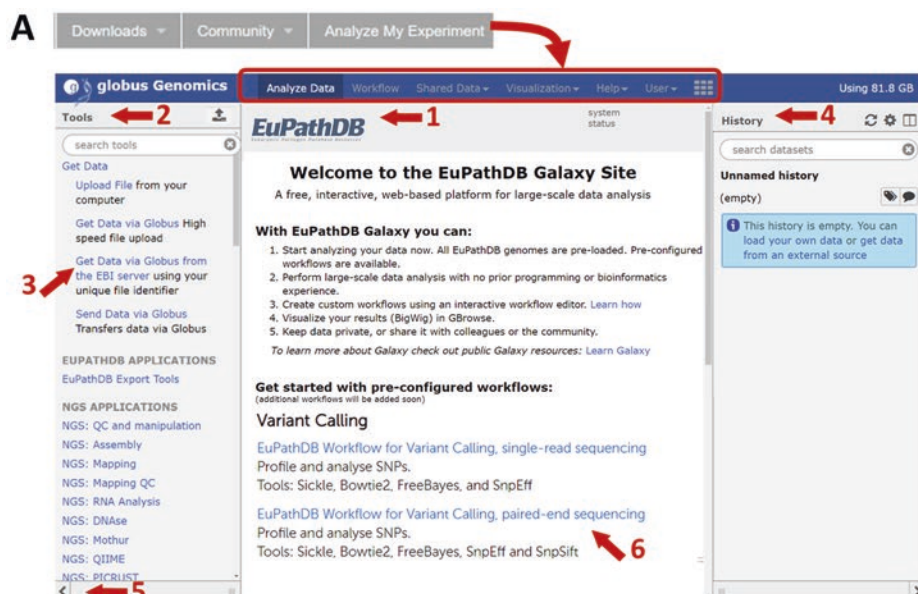
**Fig. 22** EuPathDB Galaxy, layout, data upload, and workflow. (**a**) EuPathDB Galaxy home page accessed through the *Analyze My Experiment* link in the gray menu bar. The center workspace (arrow 1) is flanked by the *Tools* (arrow 2) and *History* (arrow 4) panels. Shown are the Get Data via Globus from the EBI server

Enter parameter values for the *Data type to be transferred* as fastq and the *Single or Paired-Ended* as paired (Fig. 22b, left). Click execute to initiate the upload. Tiles representing files appear in the history panel and will turn from gray to yellow when the job starts and then to green when the job is finished. The files are named ERR2240057_1 and ERR2240057_2 which represent forward and reverse RNA sequence runs associated with the sample ID.

3. *Run the preconfigured variant calling workflow shared by EuPathDB.* Return to the welcome page by clicking *Analyze Data* in the center panel (Fig. 22a, red border) and choose the *EuPathDB Workflow for Variant Calling, paired-end sequencing* (Fig. 22a, arrow 6). Indicate the *Input data* sets (Fig. 22c ERR2240057_1 for forward reads; ERR2240057_2 for reverse reads). Three tools in the workflow (Bowtie2, FreeBayes and SNPEff) require the reference genome as input. Since all EuPathDB genomes are preloaded into EuPathDB Galaxy, defining the reference genome is as easy as choosing it from a pick list. Scroll down carefully to change the reference genome to *CryptoDB-37_Cparvum*IowaII_*Genome* in three places (Fig. 22c, arrow 7). Click *Run workflow* (Fig. 22c, arrow 8) to start the workflow. History files appear and will indicate successful completion of a step when green or failure when red.

4. *Explore the results.* The history panel contains a tile for each output file created by the workflow (Fig. 23a). Some files are hidden from the history panel but can be shown by clicking the *hidden* link (Fig. 23a, arrow 1). Clicking the name of the file reveals file attributes within the history tile, whereas clicking the eye icon populates the center panel with file contents. Files can also be downloaded for use in local programs such as excel (Fig. 23a, red border).

5. *View variants in CryptoDB GBrowse.* The FreeBayes output vcf file (variant call file) can easily be converted to a graphic file (bigwig) that can be exported to CryptoDB for viewing. Click the edit icon next and open the Convert Format tab that appears in the center panel (Fig. 23b). Choose *Convert BED, GFF, or VCF to BigWig* and click *Convert.* A new history tile representing the new file will appear. Once the file conversion is complete, use the *EuPathDB Export Tool, Bigwig Files to EuPathDB*, to transfer the file to the *My Data Sets* section of CryptoDB (Fig. 23c). This tool requires an entry for all five

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**Fig. 22** (continued)  tool (arrow 3) for uploading data from EBI to your EuPathDB Galaxy account and the link for the preconfigured workflow for variant calling (arrow 6). (**b**) *Get Data* via *Globus from the EBI server* tool (left) and a completed upload in the history panel (right). (**c**) Variant calling workflow showing parameters for choosing input data sets (arrow 7) and the dropdown menu for selecting a reference genome. The *Run Workflow* button (arrow 8) for initiating the workflow

**Fig. 23** EuPathDB Galaxy, results and file conversion. (**a**) History panel actions for retrieving file information (click the Filename) or for displaying the file contents in the center panel (click the eye icon) and for file download (red border). (**b**) Tool for converting text files with genomic location to graphic bigwig files which can be displayed in the genome browser. Access the conversion tool through the Edit icon (pencil) in the history panel. (**c**) *EuPathDB Export Tools* section showing the *Bigwig Files to EuPathDB* tool

**Fig. 24** Bigwig files to EuPathDB tool. Tool is shown configured to create a data set called *C hominis* isolate which will contain one Bigwig file that is associated with the *C. parvum* reference genome. All five parameters need entries, or the tool will return a request for parameter values

parameters before executing. Open the export tool and make an entry for all parameters, choose the file to transfer, and make an entry for all parameters, *My Data Set name*, *Bigwig files*, *Reference genome*, *My Data Set summary*, and *My Data Set description* (Fig. 24). A history tile representing the export will appear. Once the export is finished and your history tile is green, visit CryptoDB *My Data Sets* (Fig. 25a) page to install the file into GBrowse. The *My Data Sets* page tabulates all private data sets that are shared with or uploaded by you. Open the record for the new data set by clicking the data set name in the *My Data Sets* table (Fig. 25a, arrow 1). The *GBrowse Tracks* table contains a *Send to GBrowse* button that installs the file in GBrowse (Fig. 24b) and, upon file installation, becomes a link (*View in GBrowse*) to directly view the file in GBrowse (Fig. 24c). GBrowse is itself a complex analysis tool. Consulting
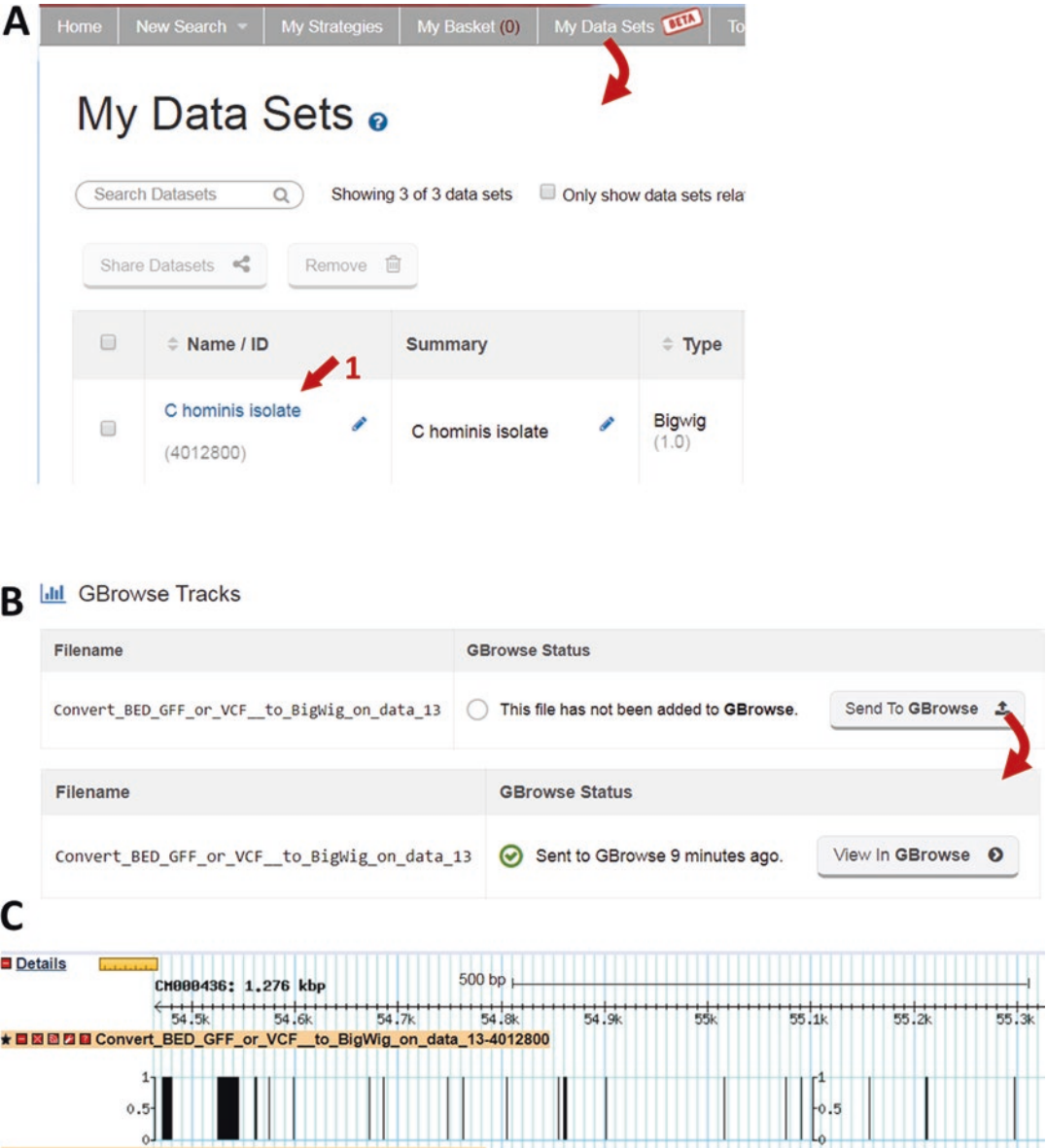
**Fig. 25** My data sets section. (**a**) *My Data Sets* page, accessed through the gray menu bar, tabulates your personal data sets. (**b**) GBrowse Tracks table from the *My Data Sets* page with options to send the file to GBrowse for viewing and to *View in GBrowse* after the file has been sent. (**c**) Variant file (vcf converted to bigwig) displayed in GBrowse

a GBrowse user guide [59] is recommended for assistance with configuring visualizations, especially navigation and adjusting scales when viewing quantitative results.

*2.6 Summary*     This exciting time for *Cryptosporidium* omics-based research supports biological discovery and a changing view of *Cryptosporidium* as a pathogen. CryptoDB and its associated analysis tools reduce

the threshold of access to valuable omics data and provide a powerful data mining and analysis platform for *Cryptosporidium* researchers and related research communities. CryptoDB is updated roughly every 2 months with new data and new features. Changes made to the database will affect data mining outcomes; thus, strategies should be saved for easy execution when new data are available. As laboratory breakthroughs render *Cryptosporidium* more amenable to advanced molecular and phenotypic analyses such as genetic crosses, high-throughput phenotypic screens, and CRISPR-Cas, additional tools developed for other organisms represented in the EuPathDB family of databases will be provisioned for CryptoDB.

## References

1. NIAID-Funded Bioinformatics Resource Centers (2018). https://www.niaid.nih.gov/research/bioinformatics-resource-centers

2. Eukaryotic Pathogen Database Resources (EuPathDB) (2018). https://eupathdb.org/eupathdb/

3. Afgan E, Baker D, Batut B, van den Beek M, Bouvier D, Cech M, Chilton J, Clements D, Coraor N, Gruning BA, Guerler A, Hillman-Jackson J, Hiltemann S, Jalili V, Rasche H, Soranzo N, Goecks J, Taylor J, Nekrutenko A, Blankenberg D (2018) The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. Nucleic Acids Res 46(W1):W537–W544. https://doi.org/10.1093/nar/gky379

4. Giardine B, Riemer C, Hardison RC, Burhans R, Elnitski L, Shah P, Zhang Y, Blankenberg D, Albert I, Taylor J, Miller W, Kent WJ, Nekrutenko A (2005) Galaxy: a platform for interactive large-scale genome analysis. Genome Res 15(10):1451–1455. https://doi.org/10.1101/gr.4086505

5. Popset (2018). https://www.ncbi.nlm.nih.gov/popset

6. Gregarina niphandrodes genome sequencing project (2018). https://www.ncbi.nlm.nih.gov/bioproject/PRJNA59799/

7. Templeton TJ, Pain A (2016) Diversity of extracellular proteins during the transition from the 'proto-apicomplexan' alveolates to the apicomplexan obligate parasites. Parasitology 143(1):1–17. https://doi.org/10.1017/S0031182015001213

8. Reese ML, Zeiner GM, Saeij JP, Boothroyd JC, Boyle JP (2011) Polymorphic family of injected pseudokinases is paramount in *Toxoplasma* virulence. Proc Natl Acad Sci U S A 108(23):9625–9630. https://doi.org/10.1073/pnas.1015980108

9. GenBank (2018). https://www.ncbi.nlm.nih.gov/genbank/

10. Benson DA, Cavanaugh M, Clark K, Karsch-Mizrachi I, Lipman DJ, Ostell J, Sayers EW (2013) GenBank. Nucleic Acids Res 41(Database issue):D36–D42. https://doi.org/10.1093/nar/gks1195

11. Li L, Stoeckert CJ Jr, Roos DS (2003) OrthoMCL: identification of ortholog groups for eukaryotic genomes. Genome Res 13(9):2178–2189

12. Feng Y, Li N, Roellig DM, Kelley A, Liu G, Amer S, Tang K, Zhang L, Xiao L (2017) Comparative genomic analysis of the IId subtype family of *Cryptosporidium parvum*. Int J Parasitol 47(5):281–290. https://doi.org/10.1016/j.ijpara.2016.12.002

13. Gilchrist CA, Cotton JA, Burkey C, Arju T, Gilmartin A, Lin Y, Ahmed E, Steiner K, Alam M, Ahmed S, Robinson G, Zaman SU, Kabir M, Sanders M, Chalmers RM, Ahmed T, Ma JZ, Haque R, Faruque ASG, Berriman M, Petri WA (2018) Genetic diversity of *Cryptosporidium hominis* in a Bangladeshi community as revealed by whole-genome sequencing. J Infect Dis 218(2):259–264. https://doi.org/10.1093/infdis/jiy121

14. Hadfield SJ, Pachebat JA, Swain MT, Robinson G, Cameron SJ, Alexander J, Hegarty MJ, Elwin K, Chalmers RM (2015) Generation of whole genome sequences of new *Cryptosporidium hominis* and *Cryptosporidium parvum* isolates directly from stool samples. BMC Genomics 16:650. https://doi.org/10.1186/s12864-015-1805-9

15. Widmer G, Lee Y, Hunt P, Martinelli A, Tolkoff M, Bodi K (2012) Comparative

genome analysis of two *Cryptosporidium parvum* isolates with different host range. Infect Genet Evol 12(6):1213–1221. https://doi.org/10.1016/j.meegid.2012.03.027

16. Bairoch A (2000) The ENZYME database in 2000. Nucleic Acids Res 28(1):304–305

17. UniProt C (2012) Reorganizing the protein space at the Universal Protein Resource (UniProt). Nucleic Acids Res 40(Database issue):D71–D75. https://doi.org/10.1093/nar/gkr981

18. Jones P, Binns D, Chang HY, Fraser M, Li W, McAnulla C, McWilliam H, Maslen J, Mitchell A, Nuka G, Pesseat S, Quinn AF, Sangrador-Vegas A, Scheremetjew M, Yong SY, Lopez R, Hunter S (2014) InterProScan 5: genome-scale protein function classification. Bioinformatics 30(9):1236–1240. https://doi.org/10.1093/bioinformatics/btu031

19. Bateman A, Coin L, Durbin R, Finn RD, Hollich V, Griffiths-Jones S, Khanna A, Marshall M, Moxon S, Sonnhammer ELL, Studholme DJ, Yeats C, Eddy SR (2004) The Pfam protein families database. Nucleic Acids Res 32:D138–D141

20. Finn RD, Attwood TK, Babbitt PC, Bateman A, Bork P, Bridge AJ, Chang HY, Dosztanyi Z, El-Gebali S, Fraser M, Gough J, Haft D, Holliday GL, Huang H, Huang X, Letunic I, Lopez R, Lu S, Marchler-Bauer A, Mi H, Mistry J, Natale DA, Necci M, Nuka G, Orengo CA, Park Y, Pesseat S, Piovesan D, Potter SC, Rawlings ND, Redaschi N, Richardson L, Rivoire C, Sangrador-Vegas A, Sigrist C, Sillitoe I, Smithers B, Squizzato S, Sutton G, Thanki N, Thomas PD, Tosatto SC, Wu CH, Xenarios I, Yeh LS, Young SY, Mitchell AL (2017) InterPro in 2017-beyond protein family and domain annotations. Nucleic Acids Res 45(D1):D190–D199. https://doi.org/10.1093/nar/gkw1107

21. Letunic I, Bork P (2018) 20 years of the SMART protein domain annotation resource. Nucleic Acids Res 46(D1):D493–D496. https://doi.org/10.1093/nar/gkx922

22. Marzano V, Mancinelli L, Bracaglia G, Del Chierico F, Vernocchi P, Di Girolamo F, Garrone S, Tchidjou Kuekou H, D'Argenio P, Dallapiccola B, Urbani A, Putignani L (2017) "Omic" investigations of protozoa and worms for a deeper understanding of the human gut "parasitome". PLoS Negl Trop Dis 11(11):e0005916. https://doi.org/10.1371/journal.pntd.0005916

23. Kissinger JC, Hermetz KE, Woods KM, Upton SJ (2018) Enrichment of *Cryptosporidium parvum* from in vitro culture as measured by total RNA and subsequent sequence analysis. Mol Biochem Parasitol 220:5–9. https://doi.org/10.1016/j.molbiopara.2017.12.004

24. dbEST (2018). https://www.ncbi.nlm.nih.gov/dbEST/

25. Sequence Read Archive (2018). https://www.ncbi.nlm.nih.gov/sra/

26. Isaza JP, Galvan AL, Polanco V, Huang B, Matveyev AV, Serrano MG, Manque P, Buck GA, Alzate JF (2015) Revisiting the reference genomes of human pathogenic *Cryptosporidium* species: reannotation of *C. parvum* Iowa and a new *C. hominis* reference. Sci Rep 5:16324. https://doi.org/10.1038/srep16324

27. Mirhashemi ME, Noubary F, Chapman-Bonofiglio S, Tzipori S, Huggins GS, Widmer G (2018) Transcriptome analysis of pig intestinal cell monolayers infected with *Cryptosporidium parvum* asexual stages. Parasit Vectors 11(1):176. https://doi.org/10.1186/s13071-018-2754-3

28. Woo YH, Ansari H, Otto TD, Klinger CM, Kolisko M, Michalek J, Saxena A, Shanmugam D, Tayyrov A, Veluchamy A, Ali S, Bernal A, del Campo J, Cihlar J, Flegontov P, Gornik SG, Hajduskova E, Horak A, Janouskovec J, Katris NJ, Mast FD, Miranda-Saavedra D, Mourier T, Naeem R, Nair M, Panigrahi AK, Rawlings ND, Padron-Regalado E, Ramaprasad A, Samad N, Tomcala A, Wilkes J, Neafsey DE, Doerig C, Bowler C, Keeling PJ, Roos DS, Dacks JB, Templeton TJ, Waller RF, Lukes J, Obornik M, Pain A (2015) Chromerid genomes reveal the evolutionary path from photosynthetic algae to obligate intracellular parasites. Elife 4:e06974. https://doi.org/10.7554/eLife.06974

29. Zhang H, Guo F, Zhou H, Zhu G (2012) Transcriptome analysis reveals unique metabolic features in the *Cryptosporidium parvum* oocysts associated with environmental survival and stresses. BMC Genomics 13:647. https://doi.org/10.1186/1471-2164-13-647

30. Mauzy MJ, Enomoto S, Lancto CA, Abrahamsen MS, Rutherford MS (2012) The *Cryptosporidium parvum* transcriptome during in vitro development. PLoS One 7(3):e31715. https://doi.org/10.1371/journal.pone.0031715

31. Rowe JS, Shah SS, Motlhagodi S, Bafana M, Tawanana E, Truong HT, Wood SM, Zetola NM, Steenhoff AP (2010) An epidemiologic review of enteropathogens in Gaborone, Botswana: shifting patterns of resistance in an HIV endemic region. PLoS One 5(6):e10924

32. Madrid-Aliste CJ, Dybas JM, Hogue Angeletti R, Weiss LM, Kim K, Simon I, Fiser A (2009)

EPIC-DB: a proteomics database for studying Apicomplexan organisms. BMC Genomics 10(1):38

33. Snelling WJ, Lin Q, Moore JE, Millar BC, Tosini F, Pozio E, Dooley JSG, Lowery CJ (2007) Proteomics analysis and protein expression during sporozoite excystation of *Cryptosporidium parvum* (Coccidia, Apicomplexa). Mol Cell Proteomics 6(2):346–355. https://doi.org/10.1074/mcp.M600372-MCP200

34. Sanderson SJ, Xia D, Prieto H, Yates J, Heiges M, Kissinger JC, Bromley E, Lal K, Sinden RE, Tomley F, Wastling JM (2008) Determining the protein repertoire of *Cryptosporidium parvum* sporozoites. Proteomics 8(7):1398–1414. https://doi.org/10.1002/pmic.200700804

35. Kanehisa M (2002) The KEGG database. Novartis Found Symp 247:91–101. discussion 101–103, 119–128, 244–152

36. Kanehisa M, Goto S, Hattori M, Aoki-Kinoshita KF, Itoh M, Kawashima S, Katayama T, Araki M, Hirakawa M (2006) From genomics to chemical genomics: new developments in KEGG. Nucleic Acids Res 34(Database issue):D354–D357

37. Kanehisa M, Sato Y, Kawashima M, Furumichi M, Tanabe M (2016) KEGG as a reference resource for gene and protein annotation. Nucleic Acids Res 44(D1):D457–D462. https://doi.org/10.1093/nar/gkv1070

38. Caspi R, Billington R, Ferrer L, Foerster H, Fulcher CA, Keseler IM, Kothari A, Krummenacker M, Latendresse M, Mueller LA, Ong Q, Paley S, Subhraveti P, Weaver DS, Karp PD (2016) The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. Nucleic Acids Res 44(D1):D471–D480. https://doi.org/10.1093/nar/gkv1164

39. Karp PD, Riley M, Paley SM, Pellegrini-Toole A (2002) The MetaCyc Database. Nucleic Acids Res 30(1):59–61

40. Krieger CJ, Zhang P, Mueller LA, Wang A, Paley S, Arnaud M, Pick J, Rhee SY, Karp PD (2004) MetaCyc: a multiorganism database of metabolic pathways and enzymes. Nucleic Acids Res 32(Database issue):D438–D442

41. Kim S, Thiessen PA, Bolton EE, Chen J, Fu G, Gindulyte A, Han LY, He JE, He SQ, Shoemaker BA, Wang JY, Yu B, Zhang J, Bryant SH (2016) PubChem substance and compound databases. Nucleic Acids Res 44(D1):D1202–D1213. https://doi.org/10.1093/nar/gkv951

42. Welcome to the EuPathDB Galaxy Site (2018). https://eupathdb.globusgenomics.org/

43. Steinbiss S, Silva-Franco F, Brunk B, Foth B, Hertz-Fowler C, Berriman M, Otto TD (2016) Companion: a web server for annotation and analysis of parasite genomes. Nucleic Acids Res 44(W1):W29–W34. https://doi.org/10.1093/nar/gkw292

44. UniProt C (2015) UniProt: a hub for protein information. Nucleic Acids Res 43(Database issue):D204–D212. https://doi.org/10.1093/nar/gku989

45. Abrahamsen MS, Templeton TJ, Enomoto S, Abrahante JE, Zhu G, Lancto CA, Deng M, Liu C, Widmer G, Tzipori S, Buck GA, Xu P, Bankier AT, Dear PH, Konfortov BA, Spriggs HF, Iyer L, Anantharaman V, Aravind L, Kapur V (2004) Complete genome sequence of the apicomplexan, *Cryptosporidium parvum*. Science 304(5669):441–445. https://doi.org/10.1126/science.1094786

46. Xu P, Widmer G, Wang Y, Ozaki LS, Alves JM, Serrano MG, Puiu D, Manque P, Akiyoshi D, Mackey AJ, Pearson WR, Dear PH, Bankier AT, Peterson DL, Abrahamsen MS, Kapur V, Tzipori S, Buck GA (2004) The genome of *Cryptosporidium hominis*. Nature 431(7012):1107–1112. https://doi.org/10.1038/nature02977

47. Striepen B, Pruijssers AJP, Huang JL, Li C, Gubbels MJ, Umejiego NN, Hedstrom L, Kissinger JC (2004) Gene transfer in the evolution of parasite nucleotide biosynthesis. Proc Natl Acad Sci U S A 101(9):3154–3159

48. Shanmugasundram A, Gonzalez-Galarza FF, Wastling JM, Vasieva O, Jones AR (2013) Library of apicomplexan metabolic pathways: a manually curated database for metabolic pathways of apicomplexan parasites. Nucleic Acids Res 41(Database issue):D706–D713. https://doi.org/10.1093/nar/gks1139

49. Franz M, Lopes CT, Huck G, Dong Y, Sumer O, Bader GD (2016) Cytoscape.js: a graph theory library for visualisation and analysis. Bioinformatics 32(2):309–311. https://doi.org/10.1093/bioinformatics/btv557

50. Mogi T, Kita K (2010) Diversity in mitochondrial metabolic pathways in parasitic protists *Plasmodium* and *Cryptosporidium*. Parasitol Int 59(3):305–312

51. Takashima Y, Xuan X, Kimata I, Iseki M, Kodama Y, Nagane N, Nagasawa H, Matsumoto Y, Mikami T, Otsuka H (2003) Recombinant bovine Herpesvirus-1 expressing p23 protein of *Cryptosporidium parvum* induces neutralizing antibodies in rabbits. J Parasitol 89(2):276–282

52. European Molecular Biology Laboratory (2018) European Nucleotide Archive. https://www.ebi.ac.uk/ena

53. Bioinformatics B (2018) FastQC. https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

54. Core UDB (2018) Software: Sickle. http://bioinformatics.ucdavis.edu/research-computing/software/

55. University JH (2018) Bowtie 2: Fast and sensitive read alignment. http://bowtie-bio.sourceforge.net/bowtie2/index.shtml

56. Erik Garrison GM (2012) Haplotype-based variant detection from short-read sequencing. https://arxiv.org/abs/1207.3907

57. Cingolani P (2018) SnpEff Documentation. http://snpeff.sourceforge.net/SnpEff_manual.html

58. Cingolani P (2018) SnpSift: filter and manipulate annotated files. http://snpeff.sourceforge.net/SnpSift.html#filter

59. Stein LD (2013) Using GBrowse 2.0 to visualize and share next-generation sequence data. Brief Bioinform 14(2):162–171. https://doi.org/10.1093/bib/bbt001

# Chapter 11

# A Novel Method to Silence Genes in *Cryptosporidium*

**Alejandro Castellanos-Gonzalez**

## Abstract

The parasites of the genus *Cryptosporidium* are important causes of diarrheal diseases, specifically cryptosporidiosis, worldwide. A major bottleneck for developing drugs and vaccines against cryptosporidiosis is the lack of methods to study gene function in this parasite. Silencing of genes by RNA interference (RNAi) is a powerful method to investigate gene function that has been widely used in the identification of targets for several pathogens. Unfortunately, as *Cryptosporidium* does not possess the enzymes of the RNAi pathway, its genes cannot be silenced by standard siRNA technology. To circumvent that problem, we have developed a novel strategy to knock down *Cryptosporidium* genes by reconstituting the effector arm of the siRNA pathway. We have induced silencing of several genes in *Cryptosporidium* by transfecting parasites with hybrid complexes formed between recombinant human Argonaute (hAgo2) and *Cryptosporidium* single-stranded RNA (ssRNA). This novel methodology provides an effective strategy to study the role of selected genes in host–parasite interactions, and also can be used to identify potential targets for chemotherapy. The standardized methodology based on this strategy is described in this chapter.

**Key words** *Cryptosporidium*, Gene silencing, RNAi

## 1 Introduction

Gene silencing by RNA interference (RNAi) was described for the first time in 1998 [1]. RNAi technology is based on the use of small double-stranded RNA (siRNA) complementary to the mRNA target; once the siRNA is introduced into the cell it is processed by the RNA-induced silencing complex (RISC). During this process, the sense strand of the siRNA is removed and the antisense strand is used as guide to bind to the mRNA target. This association suppresses gene expression by inducing degradation of the targets [2], and this process of degradation is conducted for the "slicer protein" Argonaute (Ago) [3]. Silencing of genes by RNAi interference is a powerful method to study gene function in both prokaryotic and eukaryotic organisms [2]. Therefore, this method has been widely used to study the biology of multiple parasites and pathogens [4]. Some of these studies have even led to rapid identification of targets needed for drug and vaccine
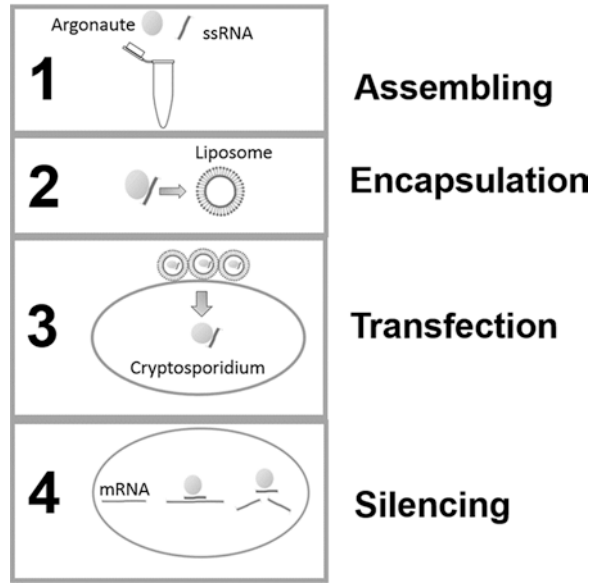
**Fig. 1** Silencing genes in *Cryptosporidium*. (**a**) Assembling: hAgo2 protein is loaded with *Cryptosporidium* ssRNA complementary to mRNA target. (**b**) Encapsulation: hAgo2–ssRNA complex is encapsulated within liposomes (protein transfection reagent). (**c**) Transfection: *Cryptosporidium* oocysts are transfected with complexes. (**d**) Silencing: hAgo2–ssRNA binds to mRNA target, translation is blocked, and mRNA target is sliced by hAgo2, then expression of target is reduced

development [5]. However, the main limitation of this method lies in the fact that some organisms do not have the RISC enzymes and, thus, siRNA is not feasible [6–8]. Genomic data demonstrated that *Cryptosporidium* does not have RISC machinery. Therefore, the RISC-dependent silencing method cannot be used to study gene function in this organism [7, 8]. To circumvent this problem, we recently developed a novel alternative method for silencing genes in this parasite [9].

Previous studies demonstrated that human Argonaute (hAgo2) associated with ssRNA induced specific degradation of mRNA targets in vitro [3]. We hypothesized that the recombinant human Ago (hAgo2) could be loaded with *Cryptosporidium* ssRNA to form a hybrid complex, then we pondered that if we could introduce this complex into live parasites then it should bind specifically mRNA target and then produce specific silencing due to the slicer activity of enzyme Ago2 (Fig. 1). Since bioactive proteins can be transported efficiently into cells using protein transfection reagents (PTR), first we demonstrated the feasibility to introduce proteins into the parasites using PTR [9]. After these experiments, we coupled *Cryptosporidium* ssRNA and hAgo2 and showed that transfection of complexes into parasites did not affect parasite viability

nor sporozoites excystation. The next step in the development of the method was to show the feasibility of silencing specific genes in *Cryptosporidium* by using preassembled ssRNA–hAgo2 complexes [9]. For these experiments, two sporozoite genes were selected as targets: *Cryptosporidium parvum* surface protein of 15 kDa (Cp15) and calcium-dependent kinase (CDPK1). Both of these molecules play key roles during the invasion process. Also, we targeted a glycoprotein gene gp900, which is not expressed during the invasive stage. We transfected oocysts with ssRNA–hAgo2 complexes targeting Cp15, CDPK1, and gp900, and after 4 h of transfection, we evaluated the effect of silencing by analyzing the expression of mRNA target by reverse transcriptase PCR (RT-PCR). All three ssRNA–hAgo2 complexes reduced expression of the target genes (~70–90%). No reduction was observed when we treated parasites with scrambled ssRNA or PRT alone. In these experiments, we conducted western blot analysis which confirmed that silencing correlates with reduction of the protein [9]. Once we demonstrated gene silencing in parasites, then next question was to show the usefulness of the method to evaluate the role of targeted genes in a biological process. Therefore, we developed an invasion assay model to evaluate the effect of silencing during the infection of human epithelial cells (HCT-8) cultured in the laboratory. The hypothesis was that silencing of essential genes would block entrance of the parasite to the cell which may lead into a reduction in the number of parasites in HCT-8 evaluated by RT-PCR. Previous reports showed that inhibition (with antibodies and drugs) of *Cryptosporidium* Cp15 and CDPK1 leaded to a reduction of parasite invasion in host cells [10, 11], we hypothesized that silencing of these genes would reduce parasite invasion. By contrast, no reduction was expected with silencing of gp900, since this protein is involved in oocyst formation. As predicted, silencing of Cp15 and CDPK1 led to a 70% and 60% reduction, respectively, in the number of parasites found inside host cells compared with infection with nontransfected parasites. By contrast, silencing of gp900 did not significantly reduce parasite numbers. Overall, these data demonstrate that our siRNA methods can be used to evaluate the phenotype of targeted genes during infection [9].

The major gap addressed by our novel methodology is the lack of methods to identify novel drug targets, which are desperately needed for the treatment of cryptosporidiosis. The goal of this chapter is to describe in detail the methodology to silence genes in *Cryptosporidium* [9]. The novel aspects of our method include the use of preassembled hybrid complexes of human Ago2 and *Cryptosporidium* ssRNA to suppress gene expression and the transfection of *Cryptosporidium*. The major advantage of our method over existing technology (i.e., CRISPR-Cas systems) is the simplicity and speed of analysis of the silencing. The use of preloaded Ago–ssRNA complexes "ready-to-silence" completely bypasses the

transcription–translation process associated with gene expression, reducing the time until the cells can be assayed from days to less than 2 h. This may allow scale-up of the method for high-throughput analysis. Therefore, we anticipate the method described here will be useful to identify novel targets and novel functions for genes with previously unknown functions in *Cryptosporidium*.

## 2   Materials

1. Recombinant protein human Argonaute 2 (hAgo2) from human (Sino Biologicals, North Wales, PA).

2. RNAse-free water.

3. Assembling buffer: 30 mM HEPES, pH 7.4, 150 mM KOAc, 2 mM Mg(OAc)$_2$, 5 mM dithiothreitol (DTT).

4. Protein transfection reagent (PTR): Pierce™ Protein Transfection Reagent (Thermo Fisher Scientific, Inc., Waltham, MA).

5. HEPES-buffered saline (HBS) for PTR: 10 mM HEPES, 150 mM NaCl, pH 7.0.

6. Acidic water: H$_2$O at pH 2.5–3.

7. Excystation media: RPMI 1640 medium, 1× antibiotic/anti-mycotic mix, and 0.8% taurocholic acid sodium salt hydrate. The media is filtered through a 22-μm filter.

8. qRT-PCR kit: qScript™ One-Step SYBR® Green qRT-PCR Kit, Low ROXTM (Quanta BioSciences/VWR, Radnor, PA).

9. PCR amplification plate: MicroAmp® Fast 96-well (Applied Biosystems, Foster City, CA).

10. PCR machine: qPCR in a 7500 Fast Real-Time PCR System (Applied Biosystems).

11. PCR optical adhesive film for 96-well PCR plates.

12. RNA purification kit: RNeasy plus mini kit (Qiagen, Germantown, MD).

## 3   Methods

### 3.1   Preparation and Design of Single-Stranded RNA (ssRNA)

1. We design the ssRNA using computational software and synthetize the ssRNA from a commercial vendor (*see* **Note 1**).

2. Resuspend ssRNA at 100 mM with RNAse-free water (*see* **Note 2**).

3. Dilute the sample 1:10 with RNAse-free water and store at −20 °C until use.

*3.2 Complex Assembling (ssRNA–hAgo2)*

1. Linearize the ssRNA: Incubate the tube (with the diluted ssRNA) for 1 min at 95 °C.

2. Assembling ssRNA–hAgo2 complexes (Table 1): Mix in a microcentrifuge tube 250 ng (2.5 μL) of recombinant hAgo2 (*see* **Note 3**) and 2.5 μL of the respective ssRNA previously diluted (*see* Subheading 3.1), add 15 μL of the assembling buffer and mix well with pipette (triturate).

3. Incubate the sample at room temperature for 1 h.

*3.3 ssRNA–hAgo2 Complex Encapsulation With Protein Transfection Reagent (PTR)*

1. Add 15 μL of PTR into the 1.5 mL tube that contains the assembling reaction (prepared as above in Subheading 3.2) and mix the encapsulation reaction (Table 2) by triturating.

2. Incubate the sample for 30 min at room temperature.

**Table 1**
**Assembling reaction**

| Components | Positive control (μL) | Negative control (μL) | Sample (μL) |
|---|---|---|---|
| ssRNA (1:10) | 2.5 (*see* Table 5) | 2.5 (*see* Table 5) | 2.5 |
| Human Argonaute protein 2 | 2.5 (250 ng) | 2.5 (250 ng) | 2.5 (250 ng) |
| Assembling buffer | 15 | 15 | 15 |
| Total volume | 20 | 20 | 20 |

**Table 2**
**Encapsulation reaction**

| Components | Positive control (μL) | Negative control (μL) | Sample (μL) |
|---|---|---|---|
| Assembling reaction components | 20 | 20 | 20 |
| Protein transfection reagent | 15 | 15 | 15 |
| Adjust to 35 μL with RNAse-free water | 0 | 0 | 0 |
| Total volume | 35 | 35 | 35 |

| | |
|---|---|
| *3.4 Parasite Transfection* | 1. Transfer $5 \times 10^5$ *Cryptosporidium* oocysts (*see* **Note 4**) to a 1.5-mL tube, add equal volumes of water (RNAse free), and triturate. |

2. Centrifuge the sample at $500 \times g$ for 10 min. Discard the supernatant in a plastic container containing ~250 mL of water containing 6% bleach.

3. Resuspend the pellet in the required amount of RNAse-free water. For each tested sample, use the ratio of $5 \times 10^5$ oocysts resuspended in 5 µL, then multiply this volume for the total number of samples (*see* **Note 4**).

4. Prepare transfection reaction (Table 3) as follows: Transfer $5 \times 10^5$ oocysts (5 µL) to a reaction tube (1.5 tube) that contains the assembled complex and the protein transfection reagent described above. Note: After transferring the parasites, mix the sample by triturating to resuspend parasites settled at the bottom of the tube.

5. To allow transfection, incubate the sample for 1 h at room temperature.

6. To activate the silencing, incubate the sample for 2 h at 37 °C.

7. In preparation for RNA extraction or excystation assays, centrifuge parasites for 1 min at maximum speed, and discard the supernatant.

8. Silencing analysis is performed by extracting the total RNA. Lyse the parasites for RNA extraction by adding 350 µL of RLT buffer contained in the RNeasy Plus Mini Kit as described in Subheading 3.6 (*see* **Note 5**).

*3.5 Parasite Excystation*

1. Resuspend the pellet of treated parasites in 25 µL of acidic water (pH 2.5) and incubate on ice for 10 min.

2. Add 250 µL of excystation media to the tube with the parasites.

3. Activate the excystation by incubating the samples for 1 h at 37 °C.

**Table 3**
**Transfection reaction**

| Components | Positive control (µL) | Negative control (µL) | Sample (µL) |
|---|---|---|---|
| Parasites | $5 \times 10^5$ (5 µL) | $5 \times 10^5$ (5 µL) | $5 \times 10^5$ (5 µL) |
| Acidic water, pH 2.5 | 25 | 25 | 25 |
| Excystation media | 250 | 250 | 250 |
| Total volume | 280 | 280 | 280 |

*3.6   RNA Extraction*

1. Resuspend transfected oocysts in 350 μL of RLT buffer included in the RNA isolation kit (*see* **Note 6**).

2. Before starting RNA extraction, heat the sample for 2 min at 95 °C.

3. Remove genomic DNA by transferring the heated 350 μL to the genomic DNA (gDNA) separator columns provided in the RNA extraction kit.

4. Centrifuge the samples for 30 s at >8000 × *g* and recover the elution in a collection tube. Discard the genomic DNA column and save the flow-through in collection tube for further processing.

5. Add 350 μL ethanol (70%) to the flow-through in the collection tube and mix well (triturate).

6. After mixing, transfer a maximum of 700 μL to the RNeasy column, and centrifuge the sample at >8000 × *g* for 30 s. Discard the elution from collection tube (provided in the kit).

7. Place column in elution tube.

8. Wash the column with 700 μL of RWI buffer and centrifuge as before. Discard the elution.

9. Wash the column with 500 μL of RPE buffer and centrifuge as before. Discard the elution.

10. Wash the column a final time with 500 μL RPE buffer, but then centrifuge at >8000 × *g* for 2 min. Discard the elution.

11. Transfer the empty RNeasy column to a new collection tube and centrifuge for 1 min at maximum speed to remove ethanol traces.

12. Transfer the RNeasy column to a new 1.5-mL microcentrifuge tube, and add 100 μL of RNAse-free water to detach and elute the RNA from the silica membrane.

13. Measure the RNA concentration of the sample by spectrophotometry or using a Nanodrop.

14. Dilute RNA samples at 5 ng/μL (*see* **Note 7**).

*3.7   Primer Design for mRNA Target Amplification by RT-PCR*

1. For designing primers, we use computational software (*see* **Note 8**). Target and primers sequences of ssRNAs used as controls for silencing are indicated in Table 6.

*3.8   Evaluation of Silencing by RT-PCR (See Table 4)*

1. Add a total of 2 μL of purified RNA template to a 96-well reaction plate (0.1 mL).

2. Prepare RT-PCR mix as follow: 5 μL of the One-Step SYBR Green Master Mix, Low Rox (2×), 0.25 μL of each primer at a 10 μM concentration, 0.25 μL of the reverse transcriptase,

**Table 4**
**RT-PCR silencing reaction**

| Components | Positive control (μL) | No RNA control (μL) | Sample (μL) |
|---|---|---|---|
| RNA sample | 2 (Table 5) | 2 (Table 5) | 2 |
| One-Step SYBR Green Mix, Low ROX (2×) | 5 | 5 | 5 |
| qScript One-Step reverse transcriptase | 0.25 | 0.25 | 0.25 |
| Forward primer | 0.25 | 0.25 | 0.25 |
| Reverse primer | 0.25 | 0.25 | 0.25 |
| Nuclease-free water | 4.25 | 4.25 | 4.25 |
| Total volume | 12 | 12 | 12 |

and 4.25 μL of nuclease-free water for a total of 10 μL of mix per sample.

3. After RNA is loaded, add 10 μL of the reaction mix to each well containing 2 μL of RNA sample, for a total of 12 μL per well.

4. Seal the plate with PCR optical adhesive film.

5. Run the reactions on a real-time PCR system with the following cycling conditions:

   · 50 °C for 15 min.

   · 95 °C for 5 min.

   · 95 °C for 15 s, 63 °C for 1 min for 50 cycles.

   · Melting point analysis (95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s and 60 °C for 15 s).

6. Normalize the results for each target with a housekeeping gene (e.g., GAPDH, *see* Table 6) to probe for parasite counts.

7. For analysis, use the ddCt calculation method to compute the relative gene expression in all samples. Results are shown by the average for each target with the respective standard deviation. The calculation steps are as follows:

   · Determine the averages and standard deviation from the PCR replicates for all samples.

   · Normalize all targets to the housekeeping gene Ct value of the control:

   $$dCt_{GAPDH} = Ct_{GAPDH}\ Control - Ct_{GAPDH}\ Gene$$

   · Normalize the Gene Ct values by adding the $dCt_{GAPDH}$ value to the corresponding Gene $Ct_{Target}$ values. This will be the $dCt_{Target}$:

$$dCt_{Target} = Ct_{Target} \; Gene + dCt_{GAPDH} \; Gene$$

- Calculate the ddCt by subtracting the $dCt_{Target}$ Control from the $dCt_{Target}$ Gene:

$$ddCt_{Target} \; Gene = dCt_{Target} \; Gene - dCt_{Target} \; Control$$

- Calculate the fold change:

$$FC = 2 \char`\^ -ddCt_{Target}$$

## 4   Notes

1. We used computational software to design ssRNA of 17–23 nt complementary to *Cryptosporidium* mRNA targets. We synthesize the ssRNA with the following modifications: 5′prime is phosphorylated and 3′prime is tailed with dTdT. Negative controls may include (17–22 nt) unrelated ssRNA sequences, sense RNA, or scrambled RNA. Positive controls are listed in Table 5.

2. To prepare ssRNA stock at 100 mM, centrifuge the tube with the lyophilized ssRNA for 5 min at maximum speed. To calculate the amount of water to resuspend ssRNA at 100 mM, first we identify the number of nmol indicated in the tube, then we use the following formula: number of mM = μL of RNAse-free water to be added.

**Table 5**
**ssRNA sequences**

| Single-Strand antisense RNA | Sequence |
|---|---|
| ssCDPK1[a,g] | 5′ rCrArCrUrUrCrCrUrCrUrCrUrCrUrCrCrUrCrCrC TT 3′ |
| ssActin[b,g] | 5′ rCrArUrArUrGrArArCrUrArCrCrArGrArUrGrGrC 3′ |
| ssAp2[c,g] | 5′ rGrCrUrArGrUrGrGrArUrArUrArGrGrGrArUrGrC 3′ |
| ssNDK[d,g] | 5′ rArArArCrUrArGrCrCrCrArCrArArUrCrCrArCrG 3′ |
| ssRom1[e] | 5′ rUrArArCrGrArCrUrArCrUrUrGrArArUrGrGrG TT 3′ |
| Scrambled (negative control)[f] | 5′ rGrGrGrUrCrArCrGrUrGrCrGrArArCrArUrGrA 3′ |

[a]ssCDPK1: ssRNA for calcium-dependent kinase 1
[b]ssActin: ssRNA for actin
[c]ssAp2: ssRNA for Apicomplexan transcription factor 2
[d]ssNDK: ssRNA for nucleoside diphosphate kinase
[e]ssROM1: ssRNA for Rhomboid protein 1
[f]ssRNA scr: scrambled RNA used as negative control
[g]ssRNA sequences that induce silencing >70% by RT-PCR

**Table 6**
**Target genes and primer sequences used for RT-PCR analysis**

| Target | Accession number | Primer sequence |
|---|---|---|
| GAPDH[a] | XM_627718 | Forward: 5′-TGA CCC ATC AAA ATT CAA CG-3′<br>Reverse: 5′-ACG AAA ACA TCT GGA GTT GG-3′ |
| CP23[a] | XM_625938 | Forward: 5′-CAA TCA GCA ACC AAG CTC AA-3′<br>Reverse: 5′-TTG TTG AGC AGC AGG TTC AG-3′ |
| CDPK1[b] | XM_001388059 | Forward: 5′-GCA GTA GGG AAT ACA GGA ACA A-3′<br>Reverse: 5′-CCG CTA CCA CTC TGA AGA AA-3′ |
| Actin[b] | FX115681 | Forward: 5′-CAT TGC TTT AGA TTA CGA GGA AGA-3′<br>Reverse: 5′-TTG AAA TAA AGC CTC AGG ACA-3′ |
| Ap2[b] | XM_627219 | Forward: 5′-TAA ACA AGC CTG AGC CAA CC-3′<br>Reverse: 5′-TCG GTT CCA ATT TCA TGT CC-3′ |
| NDK[b] | FX115659 | Forward: 5′-GCA ACT CGA CTG AAT CGT CA-3′<br>Reverse: 5′-CTT TCC TGC ATG ATC GGA AT-3′ |
| GP900[c] | AF068065 | Forward: 5′-GCA CCATTT GAG TTG GAT GTT AG-3′<br>Reverse: 5′-AGT TGG GAG ACC AGT GAT AGA-3′ |

[a]Primers for housekeeping genes (nonsilenced) used for normalization
[b]Primers for positive ssRNA controls used for silencing
[c]Primers for nontargeted gene during silencing (i.e., silencing with ssNDK does not affect GP900 expression in treated parasites vs. WT)

3. For assembling protocol, resuspend the lyophilized hAgo2 at 125 mg/μL with 160 μL ultrapure RNAse-free water. Aliquot the protein and store at −80 °C until use.

4. For silencing experiments, we recommend using fresh *C. parvum* oocysts (up to 1 month old). On the day of the experiments, the viability of the oocyst is tested by the excystation assay as follows. We only use oocysts with >80% excystation rate determined by microscopy. For the transfection experiments, the parasites can be prepared during the encapsulation time (30 min) described in the encapsulation section. Initially, *Cryptosporidium* oocysts ($1 \times 10^8$) are diluted in 1 mL of PBS/Tween 20 solution (stock solution). We calculate the total number of parasites for our experiment as follows: For each tested sample, we use $5 \times 10^5$ oocysts, then we multiply this number by the total number of treated samples (including duplicates, untreated samples, and negative controls). For example, the total number of oocysts needed for 20 samples would be calculated as follows: ($5 \times 10^5$ oocysts) $\times 20 = 100 \times 10^5 = 1 \times 10^7$ total oocysts.

5. For silencing experiments, the effect can be evaluated before the excystation and/or after excystation. If the RNA is isolated after the excystation, then skip to the excystation section.

6. RNA extraction is conducted with the Qiagen's RNeasy Plus Mini Kit following the instructions listed in the quick protocol included in the kit.

7. After extraction, the sample can be stored at −20 °C until qPCR analysis.

8. For designing, we analyze full *Cryptosporidium* mRNA sequences obtained from GenBank and CryptoDB. For primer positions, we select regions that flank a 17- to 22-nt splicing region. Primers for qPCR of positive controls are indicated in Table 6.

## Acknowledgments

## References

1. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391(6669):806–811. https://doi.org/10.1038/35888

2. Agrawal N, Dasaradhi PV, Mohmmed A, Malhotra P, Bhatnagar RK, Mukherjee SK (2003) RNA interference: biology, mechanism, and applications. Microbiol Mol Biol Rev 67(4):657–685

3. Rivas FV, Tolia NH, Song JJ, Aragon JP, Liu J, Hannon GJ, Joshua-Tor L (2005) Purified Argonaute2 and an siRNA form recombinant human RISC. Nat Struct Mol Biol 12(4):340–349. https://doi.org/10.1038/nsmb918

4. Batista TM, Marques JT (2011) RNAi pathways in parasitic protists and worms. J Proteome 74(9):1504–1514. https://doi.org/10.1016/j.jprot.2011.02.032

5. Kolev NG, Tschudi C, Ullu E (2011) RNA interference in protozoan parasites: achievements and challenges. Eukaryot Cell 10(9):1156–1163. https://doi.org/10.1128/EC.05114-11

6. Lye LF, Owens K, Shi H, Murta SM, Vieira AC, Turco SJ, Tschudi C, Ullu E, Beverley SM (2010) Retention and loss of RNA interference pathways in trypanosomatid protozoans. PLoS Pathog 6(10):e1001161. https://doi.org/10.1371/journal.ppat.1001161

7. Xu P, Widmer G, Wang Y, Ozaki LS, Alves JM, Serrano MG, Puiu D, Manque P, Akiyoshi D, Mackey AJ, Pearson WR, Dear PH, Bankier AT, Peterson DL, Abrahamsen MS, Kapur V, Tzipori S, Buck GA (2004) The genome of *Cryptosporidium hominis*. Nature 431(7012):1107–1112. https://doi.org/10.1038/nature02977

8. Abrahamsen MS, Templeton TJ, Enomoto S, Abrahante JE, Zhu G, Lancto CA, Deng M, Liu C, Widmer G, Tzipori S, Buck GA, Xu P, Bankier AT, Dear PH, Konfortov BA, Spriggs HF, Iyer L, Anantharaman V, Aravind L, Kapur V (2004) Complete genome sequence of the Apicomplexan, *Cryptosporidium parvum*. Science 304(5669):441–445. https://doi.org/10.1126/science.1094786

9. Castellanos-Gonzalez A, Perry N, Nava S, White AC Jr (2016) Preassembled single-stranded RNA-Argonaute complexes: a novel method to silence genes in *Cryptosporidium*. J Infect Dis 213(8):1307–1314. https://doi.org/10.1093/infdis/jiv588

10. Castellanos-Gonzalez A, Sparks H, Nava S, Huang W, Zhang Z, Rivas K, Hulverson MA, Barrett LK, Ojo KK, Fan E, Van Voorhis WC, White AC Jr (2016) A novel calcium-dependent kinase inhibitor, bumped kinase inhibitor 1517, cures cryptosporidiosis in immunosuppressed mice. J Infect Dis 214(12):1850–1855. https://doi.org/10.1093/infdis/jiw481

11. Tilley M, Upton SJ (1994) Both CP15 and CP25 are left as trails behind gliding sporozoites of *Cryptosporidium parvum* (Apicomplexa). FEMS Microbiol Lett 120(3):275–278

# Chapter 12

## Use of miRNAs to Study Host Cell–Parasite Interactions

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### Abstract

MicroRNAs (miRNAs) represent a subclass of endogenous small noncoding RNAs that have been identified in both mammalian and nonmammalian cells. miRNAs are an essential part of the complex regulatory networks that control numerous biological processes and may play an important role in host defense and/or microbial offense during host–parasite interactions. Here, several methodologies to explore the role for miRNAs in host–parasite interactions are briefly summarized, including the detection, quantification, and intracellular localization of miRNAs, identification and validation of miRNA targets, and functional manipulation of specific miRNAs.

**Key words** miRNA, RNA interference, Host defense, Parasite, PCR, In situ hybridization, Luciferase reporter assay, siRNA, Northern blot, Western blot

## 1  Introduction

MicroRNAs (miRNAs) refer to a subclass of endogenous small noncoding RNAs (~24 nt) [1, 2]. Most miRNAs are initially transcribed as primary transcripts (termed pri-miRNAs) by Poly II and processed by the RNase III Drosha (in the nucleus to produce pre-miRNAs) and a second RNase III Dicer (in the cytoplasm) to generate mature miRNA molecules. In the cytoplasm, miRNAs function via base pairing with complementary sequences within messenger RNA molecules (mRNAs), mostly at the 3′ untranslated regions (UTR) of mRNAs, resulting in mRNA cleavage or translational suppression [1, 2]. Therefore, miRNAs resemble the small interfering RNAs (siRNAs) of the RNA interference (RNAi) pathway through the Argonaute-containing RNA-induced silencing complex (RISC). It has been proposed that a single miRNA can repress hundreds of target transcripts, and multiple miRNAs may target the same RNA transcript [1, 2].

miRNAs have been identified in both mammalian and nonmammalian cells, including plants, animals, and some

viruses [3, 4]. Not all parasites encode miRNA genes. The lack of coding genes for Dicer and Argonaute proteins (key factors of RISC) in the genome of a number of protozoan parasites, such as *Cryptosporidium parvum*, implies the absence of miRNA-induced regulation of gene expression [5]. Nevertheless, alignment analysis of Argonaute showed that the expression modulation by small RNAs is extensively present across parasitic cestodes and nematodes [6].

Accumulating data indicate that miRNAs are an essential part of the complex regulatory networks that control numerous biological processes, including host antimicrobial immune responses and microbial offense. The human genome may encode thousands of miRNAs which appear to target about 60% of the mRNAs in humans. Host miRNAs in mammalian cells have been shown to regulate cellular responses to infection by diverse pathogens, including viruses, parasites, and bacteria [7–9]. On the other hand, pathogens, in particular viruses, may deliver their miRNAs into infected host cells to modulate host cell functions to benefit pathogen replication [8]. Unraveling the regulatory functions of miRNAs in host–parasite interactions will not only yield new insights into our understanding of the pathogenesis of parasite diseases, but also provide a basis for the development of new therapeutic interventions.

Here, we briefly summarize several methodologies which have recently been used to explore miRNAs in host–parasite interactions, including the detection, quantification, and intracellular localization of miRNAs (via PCR, in situ hybridization, northern blot), identification and validation of targeted mRNAs by specific miRNAs (luciferase assay and western blot validation), and functional manipulation of each miRNA (loss- and gain-of-function methods).

## 2 Materials

### 2.1 miRNA Extraction and Quantification Kits

1. *mir*Vana miRNA Isolation Kit (Thermo Fisher Scientific, Inc., Waltham, MA).
2. Ethanol: 100% ACS grade.
3. TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, Inc.).
4. TaqMan Universal PCR Master Mix (Thermo Fisher Scientific, Inc.).

### 2.2 Buffers and Kits for In Situ Hybridization

1. Fixation buffer: 4% (w/v) formaldehyde, 5% (v/v) acetic acid, 0.9% (w/v) NaCl.
2. 20× SSC: NaCl 3 M, trisodium citrate 0.3 M; adjust the pH to 7.0 with NaOH or HCl. Dilute to 2×, 1×, and 0.1× SSC with ddH$_2$O.

3. RNA dilution buffer: (DEPC $H_2O$: 20× SSC: 37% formaldehyde) = ratio of 5:3:1, respectively.

4. DEPC-PBS, DEPC-$H_2O$.

5. BM purple alkaline phosphatase (AP) substrate (Roche Holding AG, Basel, Switzerland).

6. 4′,6-Diamidino-2-phenylindole (DAPI) for staining cell nuclei on slides.

| | |
|---|---|
| **2.3 Buffers and Kits for Northern Blot** | 1. Northern blot urea gel: 15% polyacrylamide, Tris/Borate/EDTA (90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3) (TBE-Urea Gels, Thermo Fisher Scientific, Inc.). |

2. 2× RNA gel loading dye (Thermo Fisher Scientific, Inc.).

3. Electrophoretic transfer buffer: trisodium citrate 120 mM, sodium phosphate, dibasic ($Na_2HPO_4$) 160 mM, pH 8.3.

4. Whatman 3MM paper; nylon transfer membrane.

5. Hybridization: 0.1% Triton X-100, 0.1% proteinase K, 0.5% Triton X-100, RNase inhibitor, DIG Easy Hyb (Roche Holding AG), blocking buffer, 1× maleic acid buffer.

6. Hybridization buffer: ULTRAhyb™ ultrasensitive hybridization buffer (Thermo Fisher Scientific, Inc.).

**2.4 Luciferase Reporter Assay**

1. Luciferase construct establishment and transfection: pGL3 Basic Vector, T4 DNA ligase, Lipofectamine 2000.

2. 5× Luciferase lysis buffer: dilute to 1× luciferase lysis buffer with dd$H_2O$ (Promega Corp. Fitchburg, WI).

3. Luciferase assay reagent (Promega Corp.).

4. β-Gal assay: 2× β-Gal assay buffer—200 mM sodium phosphate buffer, pH 7.3, 2 mM $MgCl_2$, 100 mM β-mercaptoethanol, 4 mg/mL ONPG.

**2.5 Western Blot [10]**

1. Cell lysis buffer: mammalian protein extraction reagent (MPER) with 1 mM PMSF, 10 mg/mL leupeptin, and 2 mg/mL pepstatin (Thermo Fisher Scientific, Inc.).

2. Running buffer: 25 mM Tris base, 192 mM glycine, 0.1% SDS, pH 8.3.

3. Transfer buffer: 0.025 M Tris-HCL, 0.192 M glycine, 20% methanol (*see* **Note 1**).

4. PVDF membrane.

5. Tris-buffered saline (TBS, 10×): 1.5 M NaCl, 0.1 M Tris–HCl, pH 7.4.

6. TBS-T: TBS containing 0.05% Tween 20, 5% bovine serum albumin (BSA) (*see* **Note 2**).

7. Clarity Western ECL Substrate (Bio-Rad Laboratories, Inc., Hercules, CA).

8. Selected primary antibody, corresponding secondary antibody.

# 3    Methods

### 3.1 miRNA Extraction [11] and Quantification by Real-Time PCR (qRT-PCR) (See Note 3)

While some pri-miRNAs or pre-miRNAs can be up to thousands of nt long, the mature miRNAs are quite small (~24 nt). Therefore, it is critical to retain the small RNA fraction in the RNA extraction, particularly for mature miRNA detection from either the host or parasites. Total RNA extraction is usually adequate as it can retain both long and small RNA transcripts. Accordingly, qRT-PCR can quantify the amount of miRNA transcripts, pri-miRNA, pre-miRNA, or mature miRNAs, depending on the specificity of the designed PCR primers [12]. For example, the TaqMan™ microRNA Assays are for qRT-PCR assays and are designed to detect and accurately quantify mature miRNAs by annealing a short RT-priming sequence to the 3′ portion of the miRNA [13]. Many of such PCR primers are commercially available.

### 3.1.1 Cell Lysis and Tissue Disruption

1. Collect $10^2$–$10^7$ cells or 0.5–250 mg tissue; wash cells in cold PBS.

2. Disrupt samples in 300–600 μL lysis/binding buffer.

### 3.1.2 Organic Extraction

1. Add 1/10 vol of miRNA homogenate additive, incubate 10 min on ice.

2. Extract with a volume of acid–phenol:chloroform equal to the initial lysate volume.

3. Recover the aqueous phase; transfer the aqueous phase to a fresh tube.

### 3.1.3 Final RNA Isolation (See Note 4)

1. Add 1.25 volumes 100% ethanol and mix thoroughly.

2. Pass the lysate/ethanol mixture through a *mir*Vana glass fiber filter cartridge.

3. Wash the filter with 700 μL miRNA wash solution 1.

4. Wash the filter twice with 500 μL wash solution 2/3 vol.

5. Elute RNA with 100 μL 95 °C elution solution or nuclease-free water.

### 3.1.4 Enrichment Procedure for Small RNAs

1. Add 1/3 vol 100% ethanol and mix thoroughly.

2. Pass the sample through a *mir*Vana glass fiber filter cartridge, and collect the filtrate.

3. Add 2/3 vol 100% ethanol and mix thoroughly.

4. Pass the mixture through a second *mir*Vana glass fiber filter cartridge, and discard the flow-through.

5. Wash the filter with 700 μL miRNA wash solution 1.

6. Wash the filter twice with 500 μL wash solution 2/3.

7. Elute RNA with 100 μL 95 °C elution solution or nuclease-free water.

*3.1.5   qRT-PCR*

1. An amount of 0.05 mg total RNA is reverse transcribed using the TaqMan MicroRNA Reverse Transcription Kit.

2. Perform comparative qRT-PCR in triplicate using the TaqMan Universal PCR Master Mix on the qRT-PCR System. Normalization can be performed using RNU6B primers and probes.

3. Relative expression is then calculated using the comparative CT method [12, 14] (Fig. 1).

**3.2   In Situ Hybridization for Detection of miRNAs**

In situ hybridization has been used to characterize the intracellular distribution of miRNAs, either the pre-miRNAs or mature form, depending on the specificity of designed probes. Of note, probes for the mature forms should also be able to detect the corresponding pri-miRNAs and pre-miRNAs. In addition, hybridization signals may be weak for these miRNAs if they are expressed at a very low level.

*3.2.1   Cleaning Slides*

1. Wash slides with 10% HCl, then 70% ethanol at room temperature for 2 min.



**Fig. 1** *Cryptosporidium parvum*-induced alterations in mature miRNA profile in host epithelial cells as assessed by qRT-PCR. Alterations of selected miRNA expression in cultured human biliary epithelial (H69) cells after exposure to *C. parvum* for various periods of time as assessed by qRT-PCR. The amounts of mature miRNAs were obtained by normalizing to the level of a snRNA (RNU6B) in the samples. Data are expressed as the amounts of mature miRNAs in the infected samples relative to the control uninfected samples and representative of three independent experiments. $*p < 0.05$ versus the noninfected control (Reproduced from ref. 12 with permission from *PLoS Pathogens*)

2. Wash slides with ddH$_2$O.

3. Immerse in 95% ethanol, then dry in oven at 150 °C for 5 min.

4. Cool to room temperature.

5. Immerse with poly-l-lysine (1:10) at room temperature for 5 min.

6. Drain slides and dry in 60 °C oven for 1 h.

*3.2.2 Fixation and Hybridization*

1. Seed cells on 8-well chamber:

   (a) $4 \times 10^4$/well or 10-well black slides.

   (b) $2 \times 10^5$/mL, 40 μL.

   (c) $2 \times 10^5$/mL, 20 μL.

2. Wash the cells with PBS.

3. Fix cells with fixation buffer at room temperature for 30 min.

4. Wash the fixed cells with PBS at room temperature.

5. Immerse with 70% ethanol (precold on ice) at 4 °C for 10 min.

6. Wash with DEPC-PBS.

7. Digestion:

   (a) 0.1% Triton X-100, 10 min at room temperature.

   (b) 0.1% proteinase K, 10 min at 50 °C.

   (c) 0.5% Triton X-100 3 min at room temperature. Then add RNase inhibitor.

8. Wash with PBS for 2 min, twice.

9. Postfix with 1% formaldehyde, 10 min room temperature (27 μL 37% formaldehyde + 973 μL DEPC-H$_2$O).

10. Wash with DEPC-PBS, can be stored for 5 days.

11. Preheat DIG Easy Hyb at 50 °C.

12. Prehybridization at 50 °C for 30 min.

13. Prepare RNA dilution buffer with RNase inhibitor added, 20 μL/tube + 2 μL probe (200 ng), incubate at 95 °C for 5 min. Then, mix 1 mL DIG Easy Hyb solution, 200 ng probe, and 100 U RNase inhibitor. Incubate at 50 °C overnight.

14. Incubate at 37 °C for 10 min.

15. Posthybridization wash:

    (a) Wash with 2× SSC + 0.1% SDS twice for 5 min at room temperature.

    (b) Wash with 0.5× SSC + 0.1%SDS twice for 3 min at 50 °C.

    (c) Wash with 1× DEPC-PBS for 5 min at room temperature.

16. Blocking: 1 mL 10× blocking buffer + 9 mL 1× maleic acid buffer at room temperature for 30 min.
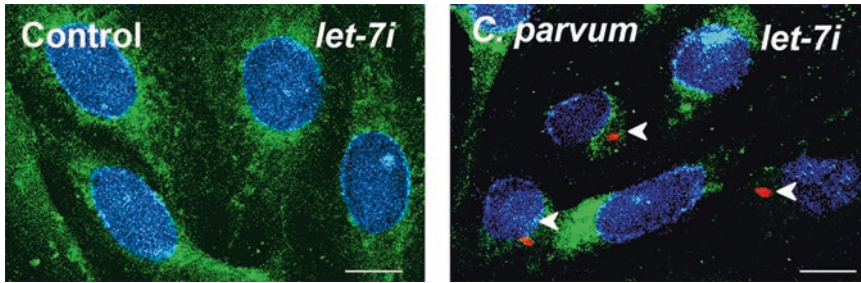
**Fig. 2** *Cryptosporidium parvum* infection decreased let-7 expression in host epithelial cells as assessed by in situ hybridization. Suppression of let-7 expression in cultured human biliary epithelial (H69) cells after exposure to *C. parvum* for 12 h as assessed by in situ hybridization analysis. An FITC-tagged antisense probe complementary to let-7 (Ambion) was used to detect let-7 family miRNAs. Cells were also stained with DAPI to label the nuclei in blue. Representative confocal images are shown with *C. parvum* stained red using a specific antibody (*arrowheads*). Bars = 5 μm (Reproduced from ref. 15 with permission from *JBC*)

17. Incubate with anti-DIG-FITC antibody (diluted to 1:200 and 1:100 with 0.5% BSA-PBS) at room temperature for 2 h.

18. Wash with DEPC-PBS.

19. Wash with DEPC-H$_2$O.

20. DAPI stain and mount slides with coverslip.

21. For anti-DIG-AP group: wash with PBS-T (0.2% Tween20) twice, wash with DEPC-H$_2$O; pre-warm BM purple AP substrate to room temperature, invert to mix before use, room temperature 30 min for color to expose. Rinse with DEPC-H$_2$O and air dry for observation (Fig. 2).

*3.3 Northern Blot*

Northern blot can detect the amounts of pri-miRNAs, pre-miRNAs, or mature miRNAs depending on the specificity of designed probes. In addition, it can detect the ratio of pre-miRNA versus its mature form, reflecting the mature process of a given miRNA [12].

*3.3.1 Denaturing Polyacrylamide–Urea Gel and Electrophoretic Transfer [16]*

1. Resuspend 10–15 μg total RNA in 8–12 μL of RNA gel loading dye. Heat the samples for 2 min at 90 °C.

2. Load the samples and the RNA marker ladder onto a 15% TBE–urea gel. Run the gel between 15 and 20 W on constant power, usually for 3–4 h, or until the bromophenol blue (in the formamide gel-loading buffer) is near the bottom of the gel.

3. Remove the gel from the tank and separate the plates. Wet two sheets of Whatman 3MM paper with 20× SSC and place them on top of the gel.

4. Soak a sponge in electrophoretic transfer buffer. Transfer the gel with the paper onto the sponge. Ensure that the paper is on top of the sponge.

5. Float the nylon transfer membrane in $H_2O$, then immerse it in 20× SSC.

6. Place the nylon membrane on top of the gel and avoid any bubbles.

7. Wet two more sheets of Whatman 3MM paper with 20× SSC and place them on top of the membrane.

8. Add the second sponge on top of the Whatman paper sheets and clamp the cassette closed (*see* **Note 5**).

9. Place the assembled gel cassette into the electroblot chamber and fill the chamber with electrophoretic transfer buffer, pre-cooled to 4 °C. Carry out the transfer at 250 mA for 2 h at 4 °C and then at 350 mA for a further 2 h at 4 °C.

*3.3.2 Cross-Linking, Prehybridization, and Hybridization*

1. Disassemble the apparatus. Carefully remove any gel fragments from the membrane.

2. Wrap the membrane in plastic wrap and place it on a sheet of Whatman 3MM paper.

3. To cross-link the RNA to the nylon transfer membrane, place the membrane in an ultraviolet (UV) cross-linker device with the RNA surface facing up. Expose it to the short-wave UV light (254 nm; the 1200 mJ autocross-linking setting) for 1 min.

4. Preheat ULTRAhyb to 68 °C (*see* **Note 6**).

5. Prehybridize the blot for 30 min at 68 °C in enough buffer to keep the membrane uniformly wet.

6. Add LNA DIG probes for selected miRNAs to the prehybridized blot.

7. Hybridize overnight (14–24 h) at 68 °C.

8. Discard the hybridization buffer and wash the blot 2 × 5 min in 2× SSC, 0.1% SDS at 68 °C.

9. Wash the blot 2 × 15 min in 0.1× SSC, 0.1% SDS at 68 °C.

10. Detect the probe (Fig. 3).

*3.4 Knockdown miRNAs and miRNA Mimic*

Several technologies have been developed to silence miRNAs, including anti-miRNA antisense inhibitor oligoribonucleotide (AMO) technology, multiple-target AMO technology (MT-AMO technology), miRNA sponge technology, miRNA-masking antisense oligonucleotides technology, sponge miR-mask technology, and miRNA knockout technology [17]. Moreover, synthetic siRNA targeting pre-miRNA loop has been reported to knockdown pre-miRNA transcript [18]. The following technologies have been used for achieving gain of function of miRNAs by forced expression or overexpression: Synthetic canonical miRNA technology, miRNA mimic technology, multi-miRNA hairpins technology, multi-miRNA mimics technology, and miRNA transgene
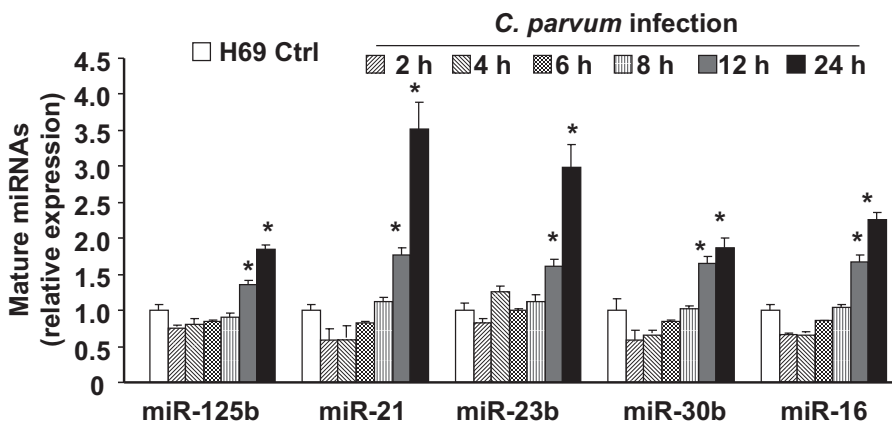
**Northern blot**
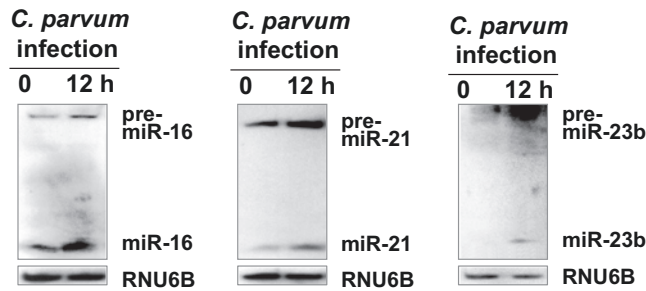


**Fig. 3** *Cryptosporidium parvum*-induced alterations in mature miRNA profile in host epithelial cells as assessed by northern blot. Alterations of selected miRNA expression in cultured human biliary epithelial (H69) cells after exposure to *C. parvum* for 12 h as determined by northern blot. Positive signals show both the pre-miRNA and mature forms of selected miRNAs. snRNA RNU6B was used as a control to ensure equal loading. Representative northern blots (*C. parvum*-infected cells vs. noninfected control) from three independent experiments are shown (Reproduced from ref. 12 with permission from *PLoS Pathogens*)

technology [17]. Most miRNA mimics are small, double-stranded RNA molecules, designed to mimic endogenous mature miRNA molecules when transfected into cells. Antagomirs are powerful tools to silence specific miRNAs in vivo [18]. Here, we list the approach to use AMOs to knockdown miRNAs and miRNA mimics to overexpress miRNAs in cultured cells.

1. Query the NCBI Gene database (Entrez Gene) to obtain miRNA sequence (http://www.rnaiweb.com/RNAi/siRNA_Design/).

2. Use BLOCK-iT™ RNAi Designer to design specific siRNA (https://rnaidesigner.invitrogen.com/rnaiexpress).

3. Use NCBI BLAST tools for homology search (*see* **Note** 7).

*3.5 Identification and Validation of miRNA Targets*

Considerable effort has gone into developing different computational algorithms that identify miRNA targets [19]. Currently there are more than a dozen algorithms which claim to predict miRNA targets, including miRanda, PicTar, MicroTar, RNA22, TargetScanS, and RNAhybrid [20]. These algorithms are based on several parameters of which homology between the 5′ seed sequence of the miRNA and a complementary region in the 3′ UTR of the target mRNA is an important component. However, a major drawback of these prediction models is a substantial false-positive rate and an inevitable bias due to reliance on the few known miRNA/target mRNA interactions. Perfectly matched miRNA seeds are neither necessary nor sufficient for all functional miRNA–target mRNA interactions [19]. There is a clear need for methodologies that identify and validate the functional targets of

specific miRNAs. Experimental methods for target validations are difficult, expensive, and time-consuming. Here, we briefly describe the luciferase reporter assay to identify miRNA targets based on computational algorithm prediction, followed by validation using real-time PCR (at RNA level) and western blot (at the protein level) for the targets. There are many other technologies for miRNA target identification and validation, such as a functional assay based on a 3′ UTR-enriched library and a positive/negative selection strategy [21].

*3.5.1  Luciferase Reporter Constructs*

Predicted region of the target mRNA, for example, based on the above computational algorithm prediction, will be ligated into the pMIR-REPORT Luciferase vector (Ambion). The basic pMIR-REPORT Luciferase vector (empty vector) and the pMIR-REPORT Luciferase construct containing a mutant at the putative seed region of the target mRNA are often used as control. β-Gal is commonly used as a control for transfection efficiency. Constructs will be confirmed by sequencing.

*3.5.2  Luciferase Reporter Assay [22]*

1. Grow cells to 70–90% confluence in a cell culture plate.
2. Transfect cells in culture with each reporter construct, as well as miRNA antisense oligonucleotide or precursor by Lipofectamine 2000.
3. After transfection for 24 h, wash cultured cells with PBS.
4. Add 100 μL 5× lysis buffer, shake 10 min at 4 °C.
5. Freeze at −20 °C for 10 min.
6. Thaw at room temperature.
7. Scrape cell and collect sample in a 1.5-mL microcentrifuge tube.
8. Centrifuge at 13,000 rpm (15,700 g) for 10 min at 4 °C.
9. Transfer 25 μL lysate for luciferase assay and 25 μL lysate for β-gal assay.

*3.5.3  Luciferase and β-Gal Measurement*

1. Add 25 μL cell lysate into 96-well plate. Assess the luciferase activity with luciferase assay reagent.
2. Add 25 μL cell lysate and 25 μL 2× β-gal reaction reagent into a new 96-well plate.
3. Incubate β-gal reaction reagent at 37 °C for 1 h.
4. Detect β-gal activity which is presented as OD 420 nm value.
5. Calculate luciferase/β-gal ratio.

*3.5.4  Validation of miRNA Targets*

The miRNA targets can be validated through the following processes.

1. Establish the specific siRNAs, siRNA mimics, and target sequence plasmids. Verify the transcriptional suppression on target genes via luciferase reporter assay.

2. Confirm the targeted mRNA expression level after the transfection of siRNA or siRNA mimics (Fig. 4).

3. Ascertain the modulation effect of siRNA or siRNA mimic on targeted protein. Whole cell proteins were obtained from cells with cell lysis buffer. Samples were separated by SDS-PAGE and electrotransfer of the resulting protein bands to a PVDF membrane. Incubate with the selected primary antibody against the candidate protein and the corresponding secondary antibody. Detect the protein using ECL substrate (Fig. 4).

# 4    Notes

1. Dilute 100 mL of 10× native buffer to 800 mL with water and add 200 mL of methanol. Avoid adding methanol directly to the 10× buffer, since it precipitates its ingredients. Even in such a scenario the precipitate can be redissolved by the addition of 800 mL water.

2. Add 100 mL of 10× TBS to a 1-L graduated cylinder and fill it to about 800 mL with water. Transfer 50 g skim milk powder into the cylinder and stir until dissolved. Dilute to 1 L with water. Separate 500 mL as the blocking solution. To the remaining 500 mL, add 250 μL of Tween 20 (cut end off 100–1000 μL pipette tip to aspirate Tween 20 easily), dissolve, and use it as the diluent.

3. To globally assess miRNA expression in host cells following parasite infection, a microarray analysis or whole genomic RNA-Seq (e.g., The Exiqon miRCURY LNA microRNA arrays) of mature miRNA expression in cells could be applied.

4. It is extremely important to inactivate RNases as quickly as possible after sample collection to avoid RNA degradation. It is also important to limit the time between death and sample processing for the best yield of high-quality RNA.

5. The layers of the sandwich are as follows: starting with the negative (−) pole—sponge, two sheets of Whatman 3MM paper, gel, nylon membrane, two sheets of Whatman 3MM paper, sponge. The RNA will migrate to the positive (+) pole.

6. This is ~6–10 mL depending on the size of the membrane and the size of the hybridization bottle or bag.

7. The siRNA targets on the miRNA sequence should be specific. Homology with other genes or sequences will result in off-target effects.

**A**

SNAP23 mRNA

let-7/miR-98

5'  5'UTR   Coding sequence   Potential binding site   3'UTR   3'

let-7i     UUGUCGUGUUUGAUGAUGGAGU
let-7d     UGAUACGUUGGAUGAUGGAGA
let-7f     UGAUAUGUUAGAUGAUGGAGU
let-7e     UUGAUAUGUUGGUGGAUGGAGU
miR-98  3'  UUGUUAUGUUGAAUGAUGGAGU  5'

hsa-SNAP23  3'UTR  1116   5'  AUGUGAAUUCAGAUUUACCUCAA   3'  1138
mmu-SNAP23  3'UTR  766    5'  ACAUGAAUUCAGAUUUACCUCAA   3'  788

5'   3'UTR of SNAP23 mRNA   3'

Human   ACATGAATTCAGATTTACCTCAATGCTAAGAATTA   -- WT
        ACATGAATTCAGATTTAGGAGAATGCTAAGAATTA   -- Mutant
Mouse   CATGTGAATTCAGATTTACCTCAATACTA   -- WT
        CATGTGAATTCAGATTTTGGACAATACTA   -- Mutant

**B**

Luciferase activity (pMIR report/β-gal)

(bar chart, y-axis 0–120)

Categories: Empty Vector, SNAP23-3'UTR-Mut, SNAP23-3'UTR, SNAP23-3'UTR+Precursor-Ctrl, SNAP23-3'UTR+miR-98 precursor, SNAP23-3'UTR+Anti-miR Ctrl, SNAP23-3'UTR+Anti-miR-98

**C**

SNAP23 (36 kDa)   Actin (42 kDa)

SNAP23 mRNA (Fold changes) 0–1.2

Precursor Ctrl   30   25   15   0 nM
let-7i precursor  0    5   15   30 nM

SNAP23 (36 kDa)   Actin (42 kDa)

SNAP23 mRNA (Fold changes) 0–1.2

Anti-miR-ctrl    30   25   15   0 nM
Anti-let-7i       0    5   15   30 nM

**D**

SNAP23 (36 kDa)   Actin (42 kDa)

SNAP23 mRNA (Fold changes) 0–1.4

C. parvum          -   +   +   +   +   +
Precursor Ctrl     -   -  30  25  15   0 nM
let-7i precursor   -   -   0   5  15  30 nM

**Fig. 4** The let-7/miR-98 miRNAs target the synaptosome-associated protein 23 (SNAP23), resulting in translational suppression (but not mRNA degradation), and are involved in the induction of SNAP23 expression in epithelial cells following *Cryptosporidium parvum* infection. (**a**) The schematic of SNAP23 mRNA showed a potential binding site in its 3′ UTR for the let-7/miR-98 miRNA family in humans and in mice. The SNAP23 3′

## Acknowledgments

## References

1. Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116:281–297

2. Lim LP, Lau NC, Garrett-Engele P, Grimson A, Schelter JM, Castle J, Bartel DP, Linsley PS, Johnson JM (2005) Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature 433:769–773

3. Winter J, Jung S, Keller S, Gregory RI, Diederichs S (2009) Many roads to maturity: microRNA biogenesis pathways and their regulation. Nat Cell Biol 11:228–234

4. Scaria V, Hariharan M, Maiti S, Pillai B, Brahmachari SK (2006) Host-virus interaction: a new role for microRNAs. Retrovirology 3:68

5. Militello KT, Refour P, Comeaux CA, Duraisingh MT (2008) Antisense RNA and RNAi in protozoan parasites: working hard or hardly working? Mol Biochem Parasitol 157:117–126

6. Zheng Y, Cai X, Bradley JE (2013) microRNAs in parasites and parasite infection. RNA Biol 10:371–379

7. Maudet C, Mano M, Eulalio A (2014) MicroRNAs in the interaction between host and bacterial pathogens. FEBS Lett 588:4140–4147

8. Piedade D, Azevedo-Pereira JM (2016) The role of microRNAs in the pathogenesis of Herpesvirus infection. Viruses 8:pii: E156

9. Staedel C, Darfeuille F (2013) MicroRNAs and bacterial infection. Cell Microbiol 15:1496–1507

10. Kurien BT, Scofield RH (2015) Multiple immunoblots by passive diffusion of proteins from a single SDS-PAGE gel. Methods Mol Biol 1312:77–86

11. https://www.thermofisher.com

12. Zhou R, Hu G, Liu J, Gong AY, Drescher KM, Chen XM (2009) NF-kappaB p65-dependent transactivation of miRNA genes following *Cryptosporidium parvum* infection stimulates epithelial cell immune responses. PLoS Pathog 5:e1000681

13. Schmittgen TD, Lee EJ, Jiang J, Sarkar A, Yang L, Elton TS, Chen C (2008) Real-time PCR

**Fig. 4** (continued) UTR sequence covering the potential binding sites for the let-7 miRNA family was inserted into the pMIR-REPORT luciferase plasmid. A control plasmid with the mutant 3′ UTR sequence was also generated for control. (**b**) Binding of let-7/miR-98 miRNAs to the potential binding site in the SNAP23 3′ UTR results in translational suppression. Cells were transfected with the pMIR-REPORTER luciferase constructs and treated with anti-miR AMOs or precursors (or mimics) to miR-98, or nonspecific oligo control, for 24 h, followed by luciferase analysis. (**c**) Functional manipulation of let-7/miR-98 causes reciprocal alterations in SNAP23 expression at the protein level (but not the RNA level). H69 cells were treated with various doses of let-7i precursor or anti-let-7i for 24 h, followed by using real-time PCR and western blot for SNAP23. (**d**) The let-7/miR-98 miRNAs are involved in the induction of SNAP23 expression in epithelial cells following *C. parvum* infection. The let-7/miR-98 miRNAs are downregulated in H69 cells following *C. parvum* infection [12, 15]. Overexpression of let-7i blocks *C. parvum*-induced upregulation of SNAP23. H69 cells were transfected with let-7i precursor for 48 h and then exposed to *C. parvum* infection for 24 h. Expression of SNAP23 was measured by using real-time PCR and western blot. Data are averages of three independent experiments. $*p < 0.05$ ANOVA versus the empty vector control; $\#p < 0.05$ ANOVA versus the control precursor or control anti-miR (Reproduced from ref. 23 with permission from *PLoS Pathogens*)

quantification of precursor and mature microRNA. Methods 44:31–38

14. Zhou R, Hu G, Gong AY, Chen XM (2010) Binding of NF-kappaB p65 subunit to the promoter elements is involved in LPS-induced transactivation of miRNA genes in human biliary epithelial cells. Nucleic Acids Res 38:3222–3232

15. Chen XM, Splinter PL, O'Hara SP, LaRusso NF (2007) A cellular micro-RNA, let-7i, regulates toll-like receptor 4 expression and contributes to cholangiocyte immune responses against *Cryptosporidium parvum* infection. J Biol Chem 282:28929–28938

16. Rio DC (2014) Northern blots for small RNAs and microRNAs. Cold Spring Harb Protoc 2014:793–797

17. Wang Z (2009) MicroRNA interference technologies: an overview. Springer-Verlag, Berlin

18. Lee YS, Kim HK, Chung S, Kim KS, Dutta A (2005) Depletion of human micro-RNA miR-125b reveals that it is critical for the proliferation of differentiated cells but not for the down-regulation of putative targets during differentiation. J Biol Chem 280:16635–16641

19. Bentwich I (2005) Prediction and validation of microRNAs and their targets. FEBS Lett 579:5904–5910

20. Hamzeiy H, Allmer J, Yousef M (2014) Computational methods for microRNA target prediction. Methods Mol Biol 1107: 207–221

21. Gäken J, Mohamedali AM, Jiang J, Malik F, Stangl D, Smith AE, Chronis C, Kulasekararaj AG, Thomas NS, Farzaneh F, Tavassoli M, Mufti GJ (2012) A functional assay for microRNA target identification and validation. Nucleic Acids Res 40:e75

22. Gong AY, Zhou R, Hu G, Li X, Splinter PL, O'Hara SP, LaRusso NF, Soukup GA, Dong H, Chen XM (2009) MicroRNA-513 regulates B7-H1 translation and is involved in IFN-gamma-induced B7-H1 expression in cholangiocytes. J Immunol 182:1325–1333

23. Hu G, Gong AY, Roth AL, Huang BQ, Ward HD, Zhu G, LaRusso NF, Hanson ND, Chen XM (2013) Release of luminal exosomes contributes to TLR4-mediated epithelial antimicrobial defense. PLoS Pathog 9:e1003261

# Chapter 13

## Genetic Manipulation of *Cryptosporidium parvum* with CRISPR/Cas9

**Adam Sateriale, Mattie Pawlowic, Sumiti Vinayak, Carrie Brooks, and Boris Striepen**

### Abstract

*Cryptosporidium parvum* can be reliably genetically manipulated using CRISPR/Cas9-driven homologous repair coupled to in vivo propagation within immunodeficient mice. Recent modifications have simplified the initial protocol significantly. This chapter will guide through procedures for excystation, transfection, infection, collection, and purification of transgenic *Cryptosporidium parvum*.

**Key words** Transfection, Transgenics, CRISPR, Cas9, Genetics

### 1 Introduction

Cas9 is an RNA-guided endonuclease and part of the bacterial immunity pathway CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) [1]. When provided with an RNA guide sequence, the Cas9 protein will scan DNA for a precise match prior to cutting, allowing to deliver directed double-strand breaks in the target genome. In many eukaryotic cells, double-strand breaks in genomic DNA are quickly repaired by nonhomologous end joining; DNA is trimmed back from the break site and then re-ligated [2]. *Cryptosporidium* appears to lack the essential machinery for nonhomologous end joining; therefore, any break in the DNA must be repaired by homologous recombination [3]. This can be exploited by providing homologous DNA stretches flanking a selection cassette. The combination of a targeted cut by Cas9 with matching homologous flanks will direct recombination of the desired locus with high fidelity, and transgenic parasites can be drug selected for during an in vivo infection [4]. To enrich and propagate these transgenic *C. parvum* strains, we use immuno-compromised mice (Fig. 1). We prefer IFNγ-deficient mice for creating transgenic strains as they yield the highest level of infection;
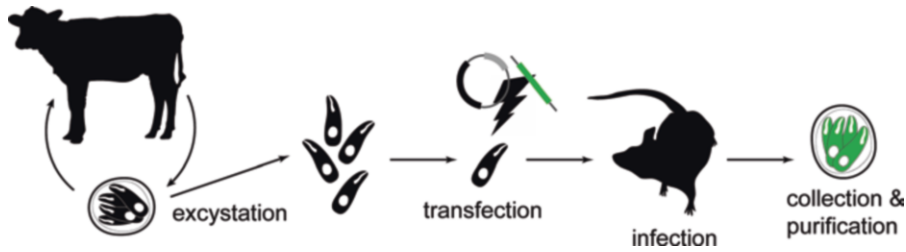
**Fig. 1** Overview of *Cryptosporidium parvum* genetic manipulation. *Cryptosporidium* sporozoites are induced to excyst and then transfected by electroporation. Next, exogenous DNA inserts into the parasite genome using Cas9-driven homologous recombination. Transgenic parasites are selected for using drug treatment and propagated in immunodeficient mice

however, other models such as NOD-SCID-gamma (NSG) may also be suitable.

The success of this protocol depends heavily on the transfection efficiency achieved with *Cryptosporidium*. For this reason, we recommend optimization of transfection in vitro prior to attempting to make stable transgenics. Transfection of *Cryptosporidium* was first pioneered using a BTX electroporator (Harvard Apparatus, MA, USA), and then optimized using an AMAXA 4D-Nucleofector (Lonza, Basel, Switzerland). We have included the steps for transfection using the AMAXA 4D-Nucleofector, as this delivers superior efficiency (please *see* Pawlowic et al. [5] for detail on other instruments). To pilot transfection, we recommend using a simple *Cryptosporidium* Nanoluciferase expression vector [4]. The sensitivity of the Nanoluciferase assay allows for the detection of even low levels of transfection. The transfection efficiency of *Cryptosporidium* declines as the parasites age, so we recommend using oocysts that were recently isolated from infected animals, ideally within the past 4 months.

## 2    Materials

***2.1    Optimizing Transfection with Transient Expression of Nanoluciferase***

*2.1.1    Excystation*

1. $1 \times 10^7$ viable *Cryptosporidium parvum* oocysts.
2. Phosphate buffered saline (PBS), pH 7.4.
3. Household bleach, diluted 1:4 in deionized water.
4. Sodium taurodeoxycholate: 0.2 mM dissolved in PBS.
5. Water bath or incubator set to 37 °C.
6. Microcentrifuge.
7. 1.5 mL microcentrifuge tubes.
8. Hemocytometer.

*2.1.2    Transfection*

1. AMAXA 4D-Nucleofector (Lonza Group AG, Basel, Switzerland).

2. SF Cell Line 4D-Nucleofector X Kit S (Lonza).

3. 10 μg *Cryptosporidium* Nanoluciferase expression vector [4].

*2.1.3 Cell Monolayer Infection*

1. 24-well tissue culture plate with HCT-8 cells (ATCC) grown to 70% confluency.

2. Infection media: RPMI or DMEM with 1% fetal bovine serum.

*2.1.4 Measuring Nanoluciferase*

1. Nano-Glo Luciferase Assay System (Promega Corp., Fitchburg, WI).

2. White 96-well polystyrene plates (medium binding) for reading luminescence (Corning Inc., Corning, NY).

3. Plate reader capable of measuring luminescence.

**2.2 Creation of Stable Transgenic *Cryptosporidium parvum***

1. $2.5 \times 10^7$ viable *Cryptosporidium parvum* oocysts.

2. Phosphate buffered saline (PBS), pH 7.4.

3. Household bleach, diluted 1:4 in deionized water.

*2.2.1 Excystation*

4. Sodium taurodeoxycholate: 0.2 mM dissolved in PBS.

5. Water bath or incubator set to 37 °C.

6. Microcentrifuge and 1.5 mL microcentrifuge tubes.

7. Hemocytometer.

*2.2.2 Transfection*

1. AMAXA 4D-Nucleofector (Lonza).

2. SF Cell Line 4D-Nucleofector X Kit L (Lonza).

3. 50 μg Cas9/gRNA expression vector [4] (*see* **Notes 1 & 2**).

4. 50 μg PCR segment for homologous repair (*see* **Notes 1 & 3**).

*2.2.3 Mouse Infection*

1. 1 mL syringes with needle tips for gavage.

2. Saturated sodium bicarbonate solution (8% w/v).

3. One cage of four adult IFNγ KO mice (B6.129S7-*Ifng*$^{tm1Ts}$/J)) (Jackson Labs, Bar Harbor, ME).

4. 16 mg/mL paromomycin sulfate salt dissolved in sterile water.

5. Optional: ampicillin, streptomycin, and vancomycin.

*2.2.4 Collection*

1. DietGel Boost, calorie supplement for mice (Clear $H_2O$, Portland, ME).

2. Dedicated forceps for fecal collection.

3. Optional: Raised wire mesh flooring for cage collection.

*2.2.5 Purification*

1. Tap water.

2. LabGen 125 homogenizer (Cole-Parmer, Vernon Hills, IL).

3. Saturated sucrose flotation solution: 1.33 specific gravity (756 g sucrose dissolved in 483 mL deionized water produces 1 L).

4. Saline solution: 0.85% NaCl in deionized water.

5. CsCl solution: 1.25 M (21.75 g dissolved in 103.25 mL deionized water).

6. 50 mL conical tubes.

7. 500 mL centrifuge tubes.

8. 2 mL low-binding microcentrifuge tubes.

9. 1.5 mL microcentrifuge tubes.

10. Tissue cell scrapers.

11. 250 μm wire mesh Micro Sieve Set (Bel-Art, Wayne, NJ).

12. Tabletop centrifuge.

13. Microcentrifuge.

14. Hemocytometer.

## 3   Methods

### 3.1   Optimizing Transfection with Transient Expression of Nanoluciferase

#### 3.1.1   Excystation

To transfect *Cryptosporidium*, it is necessary to rupture the protective oocyst wall and release the motile sporozoites. We use the well-established combination of sodium taurodeoxycholate and elevation of temperature to 37 °C to induce excystation [6]. To test your electroporation efficiency, begin by transfecting $1 \times 10^7$ sporozoites with a simple Nanoluciferase expression vector. We recommend starting with $1 \times 10^7$ oocysts for this protocol because even with lower excystation rates, 50%, for example, sporozoite numbers should suffice to observe transfection: $1 \times 10^7$ oocysts $\times 0.5$ excystation rate $\times 4$ sporozoites per oocyst $= 2 \times 10^7$ excysted sporozoites.

1. Resuspend $1 \times 10^7$ oocysts in 500 μL of cold diluted household bleach [1, 4]. Incubate on ice for 10 min. Oocysts are naturally resistant to chlorine treatment, and this incubation is intended to kill any bacterial contaminants. Bleach treatment also enhances wall permeability and can enhance excystation.

2. Centrifuge at $16,000 \times g$ for 3 min, then resuspend in 1 mL cold PBS.

3. Repeat **step 2** to remove residual bleach.

4. Centrifuge at $16,000 \times g$ for 3 min. Then, resuspend oocysts in 400 μL of 0.2 mM sodium taurodeoxycholate.

5. Incubate on ice for 10 min.

6. Add 1 mL PBS and incubate at 37 °C for 1 h.

7. Use a disposable cell counting chamber/hemocytometer to monitor excystation. Excystation rates vary depending on the viability of the oocysts, and we normally see excystation rates between 50% and 90%. Incubation at 37 °C can be prolonged up to 2 h to achieve a higher excystation rates if needed. If you are experiencing low excystation rates, consider making a fresh stock of sodium taurodeoxycholate.

8. Count sporozoites and aliquot 2 new 1.5 mL tubes with $1 \times 10^7$ each.

*3.1.2   Transfection*

As discussed in the introduction, we recommend optimizing transfection with a simple *Cryptosporidium* Nanoluciferase expression vector. The protocol below is specific to the AMAXA 4D-Nucleofector.

1. Resuspend $1 \times 10^7$ sporozoites in 15 μL complete SF buffer (12.3 μL Cell Line Solution + 2.7 μL Supplement 1).

2. Add 10 μg of *Cryptosporidium* Nanoluciferase expression vector resuspended in 5 μL of nuclease free water.

3. Transfer sporozoites and DNA into one well of the 16-well cuvette strip and electroporate using the program "EH100."

4. Move transfected sporozoites into a new 1.5 mL tube and keep on ice until infection.

*3.1.3   Cell Monolayer Infection*

1. Replace growth medium of a 70% confluent 24-well HCT-8 culture with infection medium.

2. Distribute sporozoites equally between three wells of the 24-well plate.

3. Incubate for 24–48 h at 37 °C.

*3.1.4   Measuring Nanoluciferase*

1. Combine Nanoglo substrate and lysis buffer at a 1:50 ratio.

2. Remove media from wells and replace with 500 μL substrate and lysis buffer.

3. Mix thoroughly with a 1 mL pipette.

4. Aliquot 200 μL to the 96-well luciferase plate. You should have enough to measure each well from the 24-well plate in duplicate.

5. Measure luminescence.

**3.2   Establish Stable Transgenic *Cryptosporidium parvum* Parasites**

*3.2.1   Excystation*

To create a stable transgenic strain of *Cryptosporidium* we typically infect a cage of four mice. Each mouse should be infected with $1 \times 10^7$ transfected sporozoites, therefore, we recommend starting with $2.5 \times 10^7$ oocysts. Again, even with a low excystation rate of 50%, you will still have enough sporozoites to attempt transfection ($2.5 \times 10^7$ oocysts × 0.5 excystation rate × 4 sporozoites per oocyst = $5 \times 10^7$ excysted sporozoites).

1. Resuspend $2 \times 10^7$ oocysts in 500 μL of cold diluted household bleach. Incubate on ice for 10 min.

2. Centrifuge at $16{,}000 \times g$ for 3 min, then resuspend in 1 mL cold PBS.

3. Repeat **step 2** to remove any residual bleach.

4. Centrifuge at $16{,}000 \times g$ for 3 min, then resuspend oocysts in 400 μL sodium taurodeoxycholate.

5. Incubate on ice for 10 min.

6. Add 1 mL PBS and incubate a 37 °C for 1 h.

7. Use a hemocytometer to monitor excystation. Excystation rates vary depending on the viability of the oocysts, and we normally see excystation rates between 50 and 90%. Incubation at 37 °C can be prolonged up to 2 h to achieve a higher excystation percentage. If you are experiencing low excystation rates, consider making a fresh stock of sodium taurodeoxycholate.

8. Count sporozoites and aliquot $5 \times 10^7$ to a new 1.5 mL tube on ice.

*3.2.2  Transfection*

This protocol is specific to the AMAXA 4D-Nucleofector.

1. Resuspend sporozoites in 80 μL complete SF buffer (65.6 μL Cell Line Solution +14.4 μL Supplement 1).

2. Add 20 μL of concentrated DNA (50 μg Cas9/gRNA expression plasmid +50 μg PCR segment for homologous repair).

3. Transfer to cuvette and electroporate using the program "EH100."

4. Transfer to a new 1.5 mL tube and add 300 μL cold PBS. Keep transfected sporozoites on ice until infection.

*3.2.3  Mouse Infection*

Once out of the protective oocyst, sporozoites lose their ability to survive the harsh environment and acidity of the host stomach. The most effective way to bypass this problem is to surgically inject transfected sporozoites directly into the small intestine, a method explained in detail here [5]. However, sporozoites can also be successfully delivered to the small intestine by simpler oral gavage if the stomach acid is neutralized with sodium bicarbonate prior to infection.

*Optional: Pretreat mice with antibiotics prior to infection. In our hands, pretreatment with an antibiotic cocktail for 1 week increases* Cryptosporidium parvum *infection. Mice are given ampicillin (1 g/L), streptomycin (1 g/L), and vancomycin (0.5 g/L)* ad libitum *in their drinking water for 1 week. Mice are then provided normal drinking water upon infection.*

1. Gavage each mouse with 200 μL of saturated sodium bicarbonate and wait 5 min.

2. Gavage each mouse with 100 μL of transfected sporozoites.

3. After allowing the sporozoites to infect overnight, change the cage drinking water to start in vivo selection with 16 mg/mL paromomycin. We recommend that mice be treated with paromomycin for no longer than 4 weeks to avoid toxicity from chronic exposure.

*3.2.4 Collection*

Mice will typically begin to shed transgenic parasites between the 7th and 14th day post infection. Parasite shedding can be monitored from collected fecal samples by qPCR using a *Cryptosporidium*-specific set of probe and primers [7]. More conveniently, the Nanoluciferase reporter component of our standard selection cassette can be monitored in collected fecal samples without need for DNA isolation, thus greatly reducing the workload associated with establishing parasite shedding from multiple cages. Mice should be monitored daily for signs of distress, including hunching, lethargy, and significant weight loss. To mitigate weight loss during infection, we supplement mouse diet with DietGel Boost at the onset of parasite shedding.

Once mice begin to shed transgenic parasites, start daily fecal collections. Take extra precautions to avoid cross-contamination of transgenic strains. Each strain should have its own collection cage and forceps, and mouse handlers should change gloves before and after handling infected mouse cages. Moving mice to cages with wire mesh floors during the day is a simple way to maximize collections. Store all collected fecal samples at 4 °C until purification and avoid freezing.

*3.2.5 Purification*

To purify oocysts, we use sucrose flotation followed by CsCl gradient centrifugation. There are many other purification methods for *Cryptosporidium* oocysts, including sucrose gradients, Nycodenz gradients, ether treatment, and sodium chloride flotation, and you may want to optimize this for your specific requirements [8]. Keep in mind the need to balance oocyst yield, purity, and viability. To maximize viability, all purifications should be done on ice with cold reagents.

1. Gather collected fecal samples (approximately 5–10 days of collection per purification) into a 50 mL conical tube and add 35 mL cold tap water.

2. Thoroughly blend samples. For this, we recommend using a handheld homogenizer, but this can be done manually.

3. Filter samples through a 250 μm wire mesh and into a 500 mL centrifuge tube. Pour 450 mL cold water over the sample and stir with a cell scraper to dislodge oocysts.

4. Centrifuge at $1000 \times g$ for 10 min at 4 °C.

5. Resuspend pellet in 50 mL cold tap water and split into two 50 mL conical tubes.

6. Add 25 mL (1:1) cold saturated sucrose solution to conical tubes and invert several times to mix.

7. Centrifuge at $1000 \times g$ for 5 min at 4 °C.

8. Carefully decant supernatant into a new 500 mL centrifuge tube and add 450 mL cold tap water.

9. Centrifuge at $1500 \times g$ for 15 min at 4 °C.

10. Resuspend pellet in 5 mL cold saline solution.

11. Slowly overlay 1 mL of sample onto 1 mL of cold CsCl solution. For this, we use low-binding 2 mL centrifuge tubes.

12. Centrifuge at $16,000 \times g$ for 3 min at 4 °C.

13. Oocysts should be visible as a white layer at the saline–CsCl interface. Slowly pipette 0.75 mL from the interface and transfer into a new 1.5 mL tube. Add 0.75 mL saline solution.

14. Centrifuge at $16,000 \times g$ for 3 min at 4 °C.

15. Discard supernatant and combine pellets from all tubes using 1 mL cold saline solution.

16. Count oocysts using a hemocytometer to determine yield.

## 4    Notes

1. *Concentration of DNA for transfection:* The protocols for transient and stable transfection call for highly concentrated DNA. We recommend using ethanol precipitation to concentrate plasmid DNA and/or PCR products prior to transfection.

2. *Design of Cas9 guide RNA:* There are a number of resources to help design an appropriate guide RNA. Eukaryotic Pathogen CRISPR guide RNA/DNA Design Tool (EuPaGDT) has the genome of *Cryptosporidium parvum* preloaded and allows for adjustable on-target and off-target search parameters [9]. For a walk-through of cloning guide RNA into a *Cryptosporidium* Cas9/U6 vector, *see* ref. 5. Keep in mind that recombination efficiency increases when the Cas9 cut site is located in close proximity to either of the homologous flanks.

3. *Design of PCR segments for homologous repair:* The amount of homologous DNA required for recombination in *Cryptosporidium* is surprisingly low, around 50 bp. With this low requirement, you can create homologous regions simply by adding them to the 3′ end of primers used to amplify the selection cassette (Fig. 2). When designing your selection

a) Basic Elements of Selection Cassette



b) Knockout of Gene X
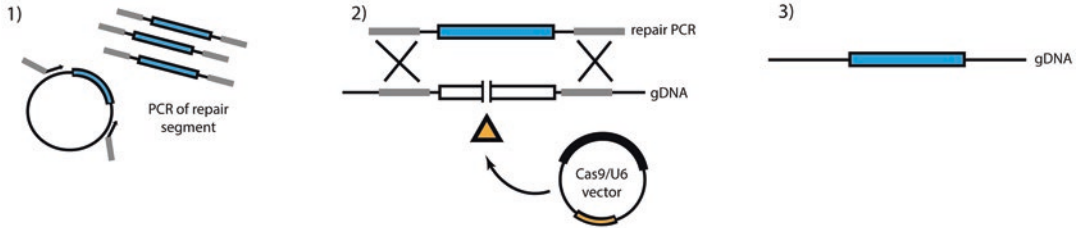


c) C-terminal Tagging of Gene X
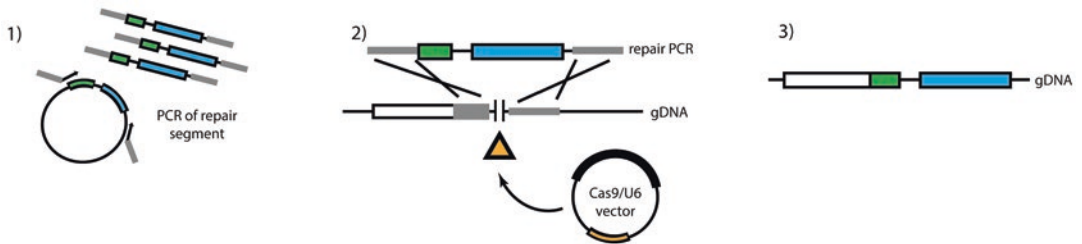


**Fig. 2** Designing a selection cassette for *Cryptosporidium parvum.* (**a**) The basic elements of a selection cassette are a reporter (Nanoluciferase) and selection marker (Neomycin phosphotransferase). To ensure continuous expression, these genes should be flanked by 5′ UTR and 3′ UTR DNA from a constitutively active *Cryptosporidium* gene (e.g., actin). (**b**) To target a gene for KO, design your Cas9 guide RNA to target a sequence within your gene of interest. Then, amplify your selection cassette with PCR primers containing homologous regions flanking this gene of interest. (**c**) To C-terminally tag a gene of interest, first you will need a tagging sequence (e.g., HA, FLAG, MYC) and 3′ UTR upstream of your selection cassette. When designing your left homology primer to amplify this tagging cassette, remember to make sure that your gene is in frame with the coding sequence of this new tag. Next, design your Cas9 guide RNA to target an area in the 3′ UTR of your gene of interest. Your right homology primer can then be designed using genomic DNA located 3′ to this Cas9 recognition site. Pay close attention to the proximity of nearby genes because *Cryptosporidium* has a very compact genome

cassette, remember that you must include neomycin phosphotransferase, which provides resistance to paromomycin. Your parasites will be constantly exposed to paromomycin during mouse infection, so drive the expression of this resistance marker with a constitutive *Cryptosporidium* promoter. We have successfully used the promoters for actin, tubulin, enolase, and aldolase for constitutive expression. Adding a luminescent reporter, such as Nanoluciferase, to your selection cassette will make it easier and faster to measure shedding of transgenic parasites. We commonly use a translational fusion of these two genes for our selection cassettes.

## Acknowledgments

## References

1. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337(6096):816–821. https://doi.org/10.1126/science.1225829

2. van Gent DC, Hoeijmakers JH, Kanaar R (2001) Chromosomal stability and the DNA double-stranded break connection. Nat Rev Genet 2(3):196–206. https://doi.org/10.1038/35056049

3. Abrahamsen MS, Templeton TJ, Enomoto S, Abrahante JE, Zhu G, Lancto CA, Deng M, Liu C, Widmer G, Tzipori S, Buck GA, Xu P, Bankier AT, Dear PH, Konfortov BA, Spriggs HF, Iyer L, Anantharaman V, Aravind L, Kapur V (2004) Complete genome sequence of the apicomplexan, *Cryptosporidium parvum*. Science 304(5669):441–445. https://doi.org/10.1126/science.1094786

4. Vinayak S, Pawlowic MC, Sateriale A, Brooks CF, Studstill CJ, Bar-Peled Y, Cipriano MJ, Striepen B (2015) Genetic modification of the diarrhoeal pathogen *Cryptosporidium parvum*. Nature 523(7561):477–480. https://doi.org/10.1038/nature14651

5. Pawlowic MC, Vinayak S, Sateriale A, Brooks CF, Striepen B (2017) Generating and maintaining transgenic *Cryptosporidium parvum* parasites. Curr Protoc Microbiol 46:20B 22 21–20B 22 32. https://doi.org/10.1002/cpmc.33

6. Robertson LJ, Campbell AT, Smith HV (1993) In vitro excystation of *Cryptosporidium parvum*. Parasitology 106:13–19

7. Jothikumar N, da Cilva AJ, Moura I, Qvarnstrom Y, Hill VR (2008) Detection and differentiation of *Cryptosporidium hominis* and *Cryptosporidium parvum* by dual TaqMan assays. J Med Microbiol 57(Pt 9):1099–1105. https://doi.org/10.1099/jmm.0.2008/001461-0

8. Arrowood MJ, Donaldson K (1996) Improved purification methods for calf-derived *Cryptosporidium parvum* oocysts using discontinuous sucrose and cesium chloride gradients. J Eukaryot Microbiol 43(5):89S

9. Peng D, Tarleton R (2015) EuPaGDT: a web tool tailored to design CRISPR guide RNAs for eukaryotic pathogens. Microb Genom 1(4):e000033. https://doi.org/10.1099/mgen.0.000033

# Mouse Models for Use in *Cryptosporidium* Infection Studies and Quantification of Parasite Burden Using Flow Cytometry, qPCR, and Histopathology

## Karine Sonzogni-Desautels, Jan R. Mead, and Momar Ndao

## Abstract

Cryptosporidiosis threatens life of young children in developing countries and newborn calves around the world. No vaccine or therapy can prevent or cure this diarrhea-inducing enteric disease caused by *Cryptosporidium* spp. protozoan parasites. There is an essential need to discover new therapeutic drugs efficient in reducing parasite burden in infected individuals. Research therefore relies on reliable small animal models of cryptosporidiosis. Here, we present excellent mouse models which can efficiently mimic pathogenesis of human and bovine cryptosporidiosis. We also describe methods to purify *C. parvum* oocysts from stool and intestine of infected mice to facilitate oocyst quantification. Moreover, we present protocols using flow cytometry, quantitative polymerase chain reaction, and histopathology to accurately quantify parasite burden in stool or intestine samples.

**Key words** *Cryptosporidium parvum*, Oocysts, Interferon gamma knock-out mice, Interferon gamma receptor knock-out mice, IL-12 knock-out mice, SCID mice, Flow cytometry, Quantitative polymerase chain reaction, Histopathology

## 1 Introduction

Cryptosporidiosis causes transient diarrhea in immunocompetent individuals and life-threatening diarrhea and dehydration in immunocompromised individuals [1]. This enteric disease affects hundreds of thousands of infants and toddlers in sub-Saharan Africa and South Asia [2]. From these *Cryptosporidium*-positive cases of moderate-to-severe diarrhea, 78% were associated to *C. hominis* and 10% to *C. parvum*, the zoonotic species [3]. No mouse model can sustain persistent *C. hominis* infection [1]. However, several mouse models of *C. parvum* infection mimic clinical signs and pathogeny of human and bovine cryptosporidiosis [4–8].

While there is no ideal animal model to study cryptosporidiosis, small rodent models provide a mechanism for studying infection dynamics, host-parasite interactions, and for evaluating drugs

and other therapeutics [1]. Neonatal mice, less than 3 weeks old are very susceptible to infection and have been used to study immune responses and for some drug testing and may provide some insight to diseases in child [9, 10]. However, these mice are more difficult to dose due to their size and rapidly changing weight and samples could be more limiting [11]. Adult wild type mice are not usually suitable to evaluate drugs due to the fact that infections with *C. parvum* are transient and generate low oocyst output [1].

For drug testing, mice that are genetically immunocompromised provide a consistent model to study host–parasite interactions and for drug evaluation. Acute models include interferon gamma receptor knock-out (IFNγR-KO) mice [6, 5, 8], interferon gamma knock-out (IFNγ-KO) mice [4, 7, 12, 13], and IL-12 knockout (IL-12 KO) mice [4, 7, 14, 15]. IFNγR-KO and IFNγ-KO mice on a C57 black/6 background are highly susceptible to infection [5, 6, 8, 13] mimicking the high susceptibility to infection of infants and newborn calves [9]. Effectively, IFNγ is the most important cytokine to fight cryptosporidiosis via an innate immune response involving natural killer cells and an adaptive $T_H1$ cell-mediated immune response [16]. In our hands, these mice died or had to be euthanized between 8 and 11 days postinfection [6, 13]. However, other studies have observed that infections in these mice are not lethal [17] which may reflect differences in *C. parvum* strains or intestinal flora. In fact, virulence of *C. parvum* strains can differ and pathogenicity needs to be determined for each strain. For example, the Iowa strain is highly virulent and 4000 oocysts are a lethal dose threatening life of IFNγR-KO mice in 9–11 days postinfection [6]. However, other *C. parvum* strains such as oocysts purified from naturally infected calves [18, 19] may induce a less severe infection in mice.

Likewise, infections in IL-12 KO mice are acute, increasing several logs over a couple of days, eventually recovering by 10–14 days postinfection (*see* Fig. 1) [14].

In contrast, *C. parvum* infections are chronic in severely immunodeficient mouse models. Several chronic infection models exist which include nude mice [20, 21], SCID mice [21, 22], and NOD SCID mice [23]. As in wild type mice, neonatal immunodeficient mouse models develop rapidly intensifying infections, but differ in the frequently lethal outcome [21]. Even with high inoculum doses, infections in the adult immunodeficient models are initially lower (*see* Fig. 2) than in the acute models due to intact innate immune system responses. Over time (8–12 weeks), infections can become disseminated and weight loss is observed in the late stages; mice eventually succumb to infection [21]. Depending on the desired infection level and whether extraintestinal sites are involved, timing of treatment or experimental procedure is important [21].

To sum up, genetically immunocompromised mice (such as IFNγR-KO, IFNγ-KO, and IL-12KO mice) can provide consistency

**Infection pattern of IL-12 Knockout mice following challenge with 1,000 C. parvum oocysts**
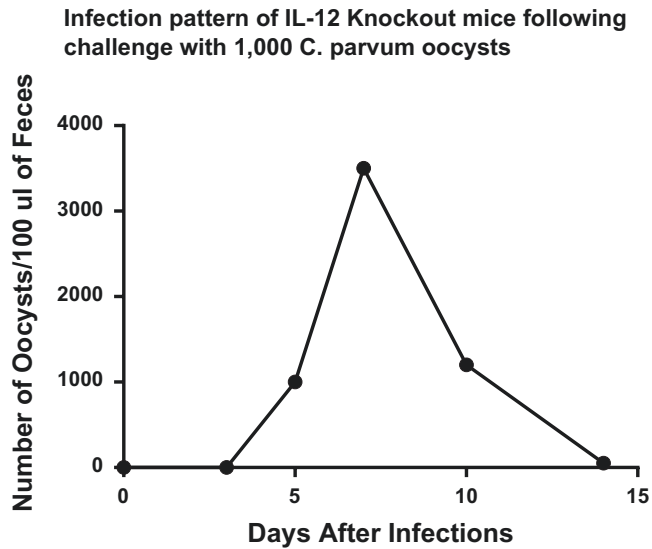


**Fig. 1** Infection pattern of IL-12 KO mice infected with $10^3$ oocysts. Note that infections are not detectable before 4 days postinfection. Oocyst numbers increase rapidly, peaking around 7 days postinfection. Infection levels decrease and oocysts are usually not detectable beyond 12–14 days postinfection

**Infection pattern of SCID mice after challenge with $10^6$ C. parvum oocyts**
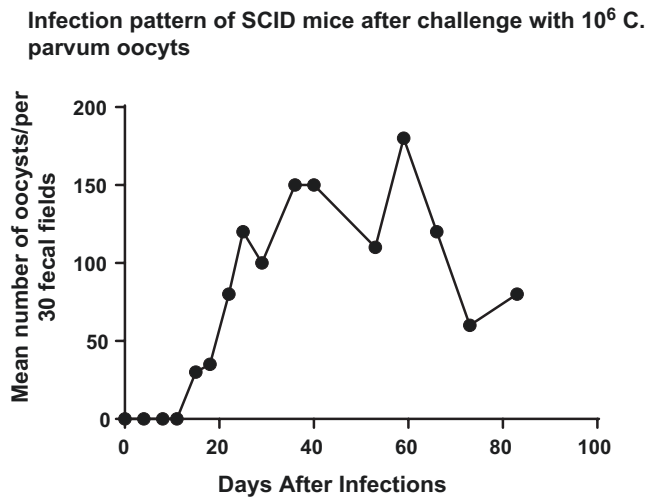


**Fig. 2** Infection pattern of SCID mice infected with $10^6$ oocysts. Note that infections are not detectable before 10–12 days postinfection. Oocyst numbers increase slowly over 8–10 weeks, waxing and waning. Decline in infection levels is usually the result of morbidity or mortality late in infection

in a model of cryptosporidiosis. While chronic models may be useful for certain objectives (e.g., disseminated disease and eradication confirmation), the acute models are a challenging and rapid model for drug testing. Candidate compounds proven efficient to inhibit *C. parvum* infection in mammalian cell culture can be tested in vivo

in these mouse models [6]. Accurate methods of detection allowing quantification of oocysts in stool and intestine samples are then essential to compare parasite burden of treated versus control mice. For detection of oocysts, we describe flow cytometric methods, using staining antibodies or a two-step gating strategy relying on the inherent morphological properties of oocysts, for the quantification of *Cryptosporidium parvum* oocysts in stool and intestine specimens. These flow cytometric methods were originally developed to replace conventional microscopic immunofluorescent assays [24] and constitute a relatively rapid and simple alternative to microscopy. We also present quantitative polymerase chain reaction (qPCR) and histopathology as protocols to obtain quantitative or semiquantitative, respectively, evaluation of parasite burden in mouse stool during the curse of the study and intestine at time of necropsy. But, first we describe methods of oocyst purification from stool and intestine samples using either discontinuous sucrose gradients or a saturated NaCl/distilled water layer. Using these protocols to purify *C. parvum* oocysts, techniques of flow cytometry, qPCR, and histopathology allow investigating the potential effect of candidate compounds on oocyst shedding in stool and parasite burden in intestine in acute mouse models of infection [5, 6].

## 2   Materials

*C. parvum* is a zoonotic parasite and precautions (lab coat, gloves, washing hands, etc.) should be taken to avoid infection of laboratory personnel (*see* **Note 1**). All experiments using *C. parvum* oocysts or infected samples should be carried out inside Biological Safety Cabinets by trained personnel. Disposable material that was in contact with stool or intestine of infected mice or purified oocysts (specimen containers, microcentrifuge tubes, pipet tips, etc.) should be autoclaved before disposal. Nondisposable material and equipment potentially contaminated (hood, homogenizer, hemocytometer, etc.) should be disinfected using hydrogen peroxide [25] or treated with heat to inactivate oocysts [26]. Oocyst-containing liquids (e.g., supernatant from stool and intestine oocyst purification protocols) should be treated with heat to inactivate oocysts (this is the preferred alternative to chemical disinfection which can be challenging in the presence of significant organic loads). Infected mice should be kept in filter top cages and housed in Biological Level 2 facilities. Mice should be handled in Biological Safety Cabinets and bedding should be autoclaved before disposal.

*2.1   Kinyoun Stain*

1. Carbol fuchsin.
2. 50% ethanol.

3. 2% sulfuric acid.

4. Methylene blue.

| | |
|---|---|
| ***2.2   Infection with C. parvum Oocysts*** | 1. PBS 1×: 0.01 M, pH 7.2 (or equivalent). |

*2.2   Infection with C. parvum Oocysts*

1. PBS 1×: 0.01 M, pH 7.2 (or equivalent).

2. Hemocytometer.

3. Luer lock 1 mL syringes.

4. 20G or 22G, disposable or autoclavable, bulb gavage needles.

*2.3   Collection of Stool Pellet and Intestine Samples*

1. 1.7 mL low binding microcentrifuge tubes.

2. 60 mL specimen container.

3. 2.5% (w/v) potassium dichromate in sterile cold distilled water.

4. 0.02% (v/v) Tween 20 in PBS (phosphate-buffered saline) 1×.

*2.4   Purification of Oocysts from Stool*

1. 1.7- and 2.2-mL low-binding microcentrifuge tubes.

2. 1:2 and 1:4 sucrose solutions (*see* **Note 2**).

3. Solution of saturated NaCl.

*2.5   Purification of Oocysts from Intestine*

1. Stir bars and magnetic stirrers.

2. 50 mL conical polypropylene centrifuge tubes and 2.2 mL microcentrifuge tubes.

3. Tissue cell scrapers.

4. 150 W handheld tissue homogenizer.

5. 0.02% (v/v) Tween 20 in distilled water.

6. Sputasol (dry mixture of 10% DTT (dithiothreitol), 76% NaCl, 2% KCl, 10% $Na_2HPO_4$ and 2% $KHPO_4$).

7. Diethylether (or substitute ethyl acetate, where required).

8. Solution of saturated NaCl.

*2.6   Flow Cytometry*

1. Microtiter tubes and flow tubes.

2. 96-well plate.

3. 8% paraformaldehyde.

4. Counting beads.

5. Oocyst-specific monoclonal antibody labeled with fluorescein isothiocyanate (OW50-FITC) (also available in the MERIFLUOR *Cryptosporidium/Giardia* kit (Meridian Bioscience, Inc., Cincinnati, OH).

6. Anti-*Cryptosporidium* Alexa Fluor® 488 monoclonal antibody.

7. Flow cytometer such as BD LSRFortessa or BD LSRFortessa X-20 flow cytometer (BD Biosciences, San Jose, CA).

8. Flow cytometry analysis software such as FlowJo v.10 (FlowJo LLC, Ashland, OR).

*2.7  qPCR Analysis*

1. 1.7 mL microcentrifuge tubes.

2. Liquid nitrogen.

3. PCR-grade water.

4. 10 μM forward primer (5′-AACTTTAGCTCCAGTT GAGAAAGTACTC-3′).

5. 10 μM reverse primer (5′-CATGGCTCTTTACCGTT AAAGAATTCC-3′).

6. 2 μM 5′ 6-carboxyfluorescein reporter dye (FAM) probe with a 3′ tetramethylrhodamine (TMR) quencher dye (5′-AATACG TGTAGAACCACCAACCAATACAACATC-3′).

7. QIAamp DNA Mini Kit qPCR (Qiagen, Germantown, MD).

8. LightCycler TaqMan Master kit (Roche Molecular Systems, Inc., Pleasanton, CA).

9. LightCycler capillaries (Roche Molecular Systems).

10. LightCycler 2.0 system (Roche Molecular Systems).

*2.8  Histopathology*

1. Histology cassettes.

2. 10% formalin.

3. 100% ethanol, 80% ethanol, 70% ethanol.

4. Xylene.

5. Hematoxylin.

6. Eosin–phloxine (eosin Y, phloxine B, 95% ethanol, acetic acid).

7. Mounting media.

8. Microscope slides and coverslips.

9. Microtome.

10. Tissue processor such as Leica ASP300S tissue processor (Leica Biosystems Inc., Buffalo Grove, IL).

11. Paraffin-embedding station such as Leica EG1150 station (Leica Biosystems).

# 3  Methods

*3.1  Kinyoun Staining to Identify Sporozoites-Containing C. parvum Oocysts*

Proportion of sporozoites-containing oocysts versus "empty shells" affects virulence of *C. parvum* parasites (*see* Fig. 3). Sporozoites being the infectious form of the parasite, "empty shells," which correspond to oocysts that released their four
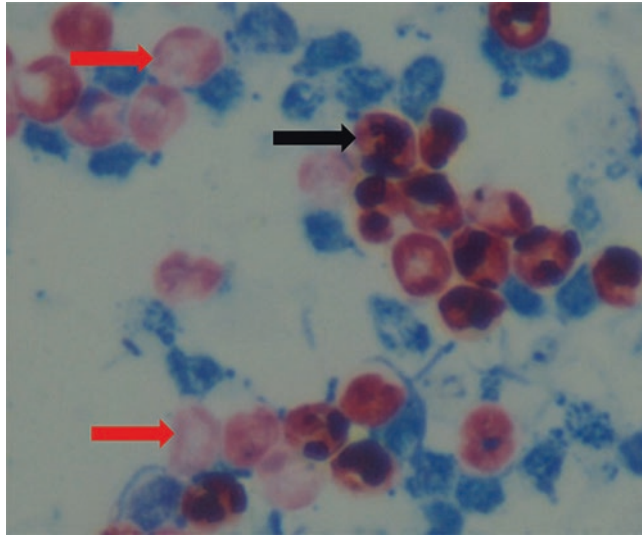
**Fig. 3** Kinyoun stain of *C. parvum* oocysts showing sporozoite-containing oocysts (black arrow) and "empty shells" (red arrows) corresponding to oocysts that released their four sporozoites

sporozoites, are noninfectious. More than 80% sporozoites-containing oocysts, as determined by a Kinyoun stain, characterize a stock of good quality (*see* **Note 3**).

1. If oocyst were stored in 2.5% potassium dichromate, wash oocysts with 1 mL of PBS 1×.

2. Centrifuge microcentrifuge tube at $10,000 \times g$ for 5 min at 4 °C.

3. Discard supernatant and repeat **steps 1** and **2** for a total of three washes.

4. Put one drop of oocyst solution on a microscope slide.

5. Fix with methanol for 1 min and air dry.

6. To stain, pour carbol fuchsin for 5 min.

7. To decolorize, pour 50% ethanol for 1 min and, then, 2% sulfuric acid for 30 s.

8. Rinse with tap water for 1 min.

9. To counterstain, pour methylene blue for 1 min.

10. Rinse with tap water, drain, and air dry.

*3.2 Infection of Mice with C. parvum Oocysts*

*Cryptosporidium parvum* oocysts can be kept in 2.5% potassium dichromate or water for up to 6 months with reasonable viability [27, 28] but viability decreases with age. However, in our laboratory, we experienced that *C. parvum* oocysts kept in 2.5% potassium dichromate maintained viability longer (*see* **Note 4**). Also, oocysts less than 3 months old should have more than 80% viability.

1. In a sterile microcentrifuge tube, transfer from the oocyst stock a volume containing a number of oocysts that exceeds the minimum required to infect all mice.

    *If oocysts were kept in 2.5% potassium dichromate, perform* **steps 2–4**.

    *If oocysts were kept in water, directly proceed with* **step 5**.

2. Wash oocysts with 1 mL of PBS 1×.

3. Centrifuge microcentrifuge tube at $10,000 \times g$ for 5 min at 4 °C.

4. Repeat **steps 2** and **3** for a total of three washes. If oocysts are still orange-colored due to trace of potassium dichromate, perform one supplemental wash.

5. Add PBS 1× for a total volume of 1 mL.

6. In a separate tube, mix 10 µL of oocysts with 10 µL of Trypan blue and load a chamber of a hemocytometer.

7. Count intact oocysts in each of the four big squares (containing 4 × 4 small squares) at the four corners of the grid of the hemocytometer (*see* Fig. 4) using a light microscope.

8. Calculate the mean of the counts of the four big squares and multiply by the factor of dilution with Trypan blue (×2) and by the factor of the hemocytometer according to manufacturer protocol (×10,000) to obtain the concentration of oocysts/mL.

9. Dilute oocysts with PBS 1× to a final concentration of 30,000 oocysts/mL in a final volume exceeding the volume needed to infect all mice (3000 oocysts in 100 µL per mouse).
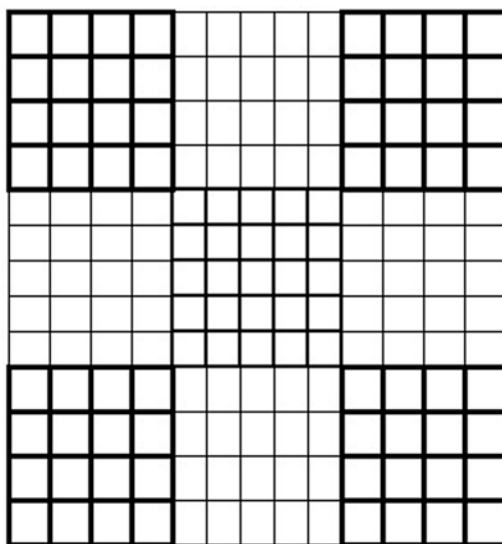


**Fig. 4** Hemocytometer grid presenting four big squares (containing 4 × 4 small squares) at the four corners of the grid

10. Keep oocysts on ice for the transport to the animal facility.

11. Fill 1-mL syringe with oocyst solution using 20G bulb gavage needle for mice weighing between 20 and 25 g or 22G bulb gavage needle for mice weighing 15–20 g.

12. Eliminate any bubble and set plunger to a 100 µL increment.

13. Pick up the mouse to be gavaged by the scruff of the neck with index finger and thumb tightly grasping the skin around the neck (*see* **Note 5**).

14. Tuck the tail under the small finger to prevent movement of the lower body.

15. Gently insert the bulb gavage needle into the mouse esophagus by angling the needle along the roof of the mouth and back of the throat, keeping slightly to the left of center.

16. Wait for the mouse to swallow the bulb of the needle and allow the needle to descend slightly further in the esophagus (*see* **Note 6**).

17. Gently depress the syringe plunger to administer 100 µL of oocysts (*see* **Note 7**).

18. Carefully remove the gavage needle and return the mouse to the cage.

19. Check that the mouse is breathing and moving normally after gavage to ensure that the procedure was done properly and that no solution was aspirated.

*3.3 Treatment of Mice*

Usually, compounds are resuspended/diluted in distilled water, PBS, saline or DMSO (dimethyl sulfoxide) according to chemical properties of each compound. Then, compounds are administered orally using feeding needle as mentioned above or via parenteral route (often intraperitoneally). Dose, route of administration, and treatment regimen have to be optimized for each compound. We routinely prepare compounds fresh daily and administer them by oral gavage for 10 days starting at 3 days postinfection [6].

*3.4 Monitoring of Infected Mice*

Apart from signs of compound toxicity which are specific to each compound, mice should also be monitored for any sign of *C. parvum* infection adverse effects. Each institution will have their own endpoint criteria. Typical endpoints which may lead to euthanasia include weight loss (>20% weight lost from initial body weight), reluctance to move, hunched posture, and/or severe sign of dehydration by pinching the skin or drop in temperature. Clinical signs of cryptosporidiosis such as reduced activity and soft stool may be observed in more susceptible models after 7 days postinfection.

*3.5 Evaluation of Treatment Efficacy*

First, clinical signs of infection should be monitored closely to compare treated infected mice with untreated infected mice (positive controls). Weight curves and survival curves can also provide

significant insights on the capacity of the compound to prevent frailty and lethality, respectively. Moreover, both oocyst shedding during the course of infection and intestinal parasite burden at time of necropsy illustrate the effect of compounds on parasite survival and replication. Mice can be sacrificed either at peak of oocyst shedding, on Day 10 or Day 11 postinfection [8], or when they reach an interventional point requiring euthanasia as mentioned above. Oocysts from stool or intestine samples can be quantified by visual counting using a hemocytometer, by flow cytometry or by qPCR. In addition, histopathology presents a semiquantitative representation of oocyst burden in ileum of infected mice.

### 3.6 Collection of Mouse Fecal Pellet

1. Collect fresh stool in preweighed 1.7 mL microcentrifuge tube (*see* **Note 8**).

2. Weigh microcentrifuge tube containing fecal pellet to calculate weight of stool sample for normalization of count of oocysts.

3. Add 1 mL 2.5% potassium dichromate or sterile cold distilled water into the tube.

4. Store stool samples at 4 °C until processed.

### 3.7 Collection of Mouse Intestine Sample

1. Collect intestine (except ileum which is kept for histology) at time of necropsy.

2. Cut intestine into 0.5 cm pieces in a preweighed 60 mL specimen container containing 10 mL 0.02% Tween 20 in PBS.

3. Weigh specimen container containing intestine for normalization of count of oocysts.

4. Store specimen container until oocyst purification at 4 °C (maximum 48 h).

### 3.8 Purification of Oocysts from Stool Sample Using Discontinuous Sucrose Gradients

1. Add 400 µL 2.5% potassium dichromate to each pellet of approximately 20 g.

2. Incubate pellet overnight at 4 °C.

3. Homogenize the fecal mixture using a wooden application stick and vortex.

4. In a separate 2.2 mL microcentrifuge tube, add 750 µL of 1:4 sucrose solution using a glass pipe, then add 750 µL of 1:2 sucrose solution beneath the first solution (*see* **Note 9**).

5. Vortex the fecal sample again and let large debris settle for approximatively 30 s.

6. Add 200 µL of the supernatant at the top layer to the microscale sucrose gradients tube and store the remaining original fecal solution in 4 °C.

7. Centrifuge the microscale sucrose gradients tube for 20 min at $1000 \times g$.

8. Aspirate the supernatant down to the 1.25 mL mark and transfer into a new tube.

9. Add PBS 1× to the 2 mL mark and centrifuge tube 16,000 × *g* for 3 min at 4 °C.

10. Aspirate down to the 0.5 mL mark and add PBS 1× to the 2 mL mark.

11. Centrifuge tube 16,000 × *g* for 3 min at 4 °C.

12. Repeat **steps 10** and **11** for four washes or until the sample is completely clear.

13. Aspirate down to the 0.25 mL mark and adjust volume to 1000 μL with PBS 1× or water.

*3.9   Purification of Oocysts from Stool Sample Using NaCl/Distilled Water Layer*

1. Add 1 mL of sterile cold distilled water to fecal pellet and store at 4 °C until process.

2. Homogenize stool sample by vortexing and doing up and down using pipet tips.

3. Centrifuge microcentrifuge tube 10,000 × *g* for 5 min at 4 °C and discard supernatant.

4. Wash stool pellet with sterile cold distilled water and discard supernatant.

5. Resuspend stool pellet in 1 mL of saturated NaCl.

6. Vortex thoroughly stool sample and overlay with 250 μL of sterile cold distilled water.

7. Centrifuge microcentrifuge tube 2000 × *g* for 10 min at 4 °C.

8. Collect supernatant and transfer into a separate 1.7-mL micro-centrifuge tube.

9. Repeat **steps 5–8** a second time.

10. Pool two supernatants together in a 2.2-mL microcentrifuge tube.

11. Centrifuge the 2.2-mL microcentrifuge tube 10,000 × *g* for 5 min at 4 °C.

12. Discard supernatant and resuspend oocysts in 1000 μL of sterile cold distilled water.

13. Store oocyst-containing sample at 4 °C for short-term storage until analysis.

*3.10   Purification of Oocysts from Intestine Sample Using NaCl/Distilled Water Layer*

1. Homogenize intestine sample using 150 W handheld tissue homogenizer.

2. Incubate intestine sample with 0.05 g sputasol (dry mixture of 10% DTT, 76% NaCl, 2% KCl, 10% $Na_2HPO_4$ and 2% $KHPO_4$) and a stir bar for 90 min at 4 °C on a magnetic stirrer.

3. Transfer intestine sample into 50-mL centrifuge tube using cell scraper.

4. Centrifuge 50 mL centrifuge tube at $2000 \times g$ for 10 min at 4 °C and discard the supernatant.

5. Resuspend pellet in 8 mL 0.02% (v/v) Tween 20 in distilled water and 4 mL of diethylether (or ethyl acetate, where required by local safety guidelines).

6. Centrifuge 50-mL centrifuge tube at $2000 \times g$ for 10 min at 4 °C.

7. Discard supernatant and fatty layer and wash pellet with 20 mL sterile cold distilled water.

8. Centrifuge 50 mL centrifuge tube at $2000 \times g$ for 10 min at 4 °C.

9. Discard supernatant and resuspend pellet in 20 mL saturated NaCl.

10. Vortex thoroughly intestine sample and overlay with 5 mL sterile cold distilled water.

11. Centrifuge 50 mL centrifuge tube at $2000 \times g$ for 10 min at 4 °C.

12. Collect 1 mL of the interphase in each of four 2.2 mL micro-centrifuge tubes prefilled with 1 mL sterile cold distilled water (total 1 mL sample + 1 mL water in each of four tubes/mouse).

13. Invert several times 2.2 mL microcentrifuge tubes to mix sample/water to dilute traces of NaCl.

14. Centrifuge tubes at $16,400 \times g$ for 30 min at 4 °C.

15. Discard 1.8 mL of supernatant of each 2.2-mL microcentrifuge tube.

16. Resuspend oocysts in remaining 200 μL in each 2.2-mL microcentrifuge tube.

17. Pool oocyst solutions of the four tubes from the same mouse.

18. Adjust volume of combined oocyst sample to 1000 μL with sterile cold distilled water.

19. Store oocyst-containing sample at 4 °C for short-term storage until analysis.

*3.11 Quantification of Oocysts by Flow Cytometry Using OW50-FITC Monoclonal Antibody*

The oocyst-specific monoclonal antibody labeled with fluorescein isothiocyanate (e.g., OW50-FITC), same as in MERIFLUOR *Cryptosporidium/Giardia* test, can be used to count *C. parvum* oocysts by flow cytometry [14].

1. Prepare 2 mL of 1:10 antibody solution composed of 1.8 mL PBS 1× and 200 μL OW50-FITC.

2. Keep the antibody solution and samples covered with foil and work with the hood light off because OW50-FITC is light-sensitive.

3. Transfer 250 μL of stool sample into 1.7-mL microcentrifuge tube.

4. Add 20 μL of OW50-FITC antibody solution to each sample and vortex.

5. Incubate for at least 20 min.

6. Wash 2× with PBS and add 4% paraformaldehyde to fix sample while bringing the sample volume up to 1 mL (PBS wash steps are not absolutely necessary, paraformaldehyde can be added directly to oocyst/antibody suspension and then analyzed).

7. Vortex and let the sample fix ideally overnight, or at least for 1 h.

8. Transfer 250 μL of each sample to an appropriate 96-well plate or to flow tubes.

9. Perform flow cytometry using any basic flow cytometer according to manufacturer's recommendations and standard operating procedures.

10. Acquire samples in 96-well plate or in flow tubes using a set volume (100 μL) at a particular flow rate or a set amount of events to acquire.

11. Run positive controls with either unlabeled or labeled oocysts to calibrate the region settings necessary to discriminate the labeled oocyst population from the background.

12. Run positive and negative controls to draw "regions" to define the oocyst-containing population ("oocyst region") in the forward scatter (FSC) versus side scatter (SSC) plot (*see* Fig. 5, panel 1).

13. Draw a "fluorescent oocyst region" in the fluorescence (FL1) plot to identify the antibody-labeled subset (*see* Fig. 5, panel 2, ≥2 log).

14. Create a logical "oocyst gate" by combining the "oocyst region" defined in the FSC/SSC plot with the "fluorescent oocyst region" in the FL1 plot.

*3.12 Quantification of Oocysts by Flow Cytometry Using an Anti-Cryptosporidium Alexa Fluor® 488 Monoclonal Antibody or a Two-Step Gating Strategy Without Staining*

*C. parvum* oocysts can also be counted by flow cytometry using other staining antibodies such as an anti-*Cryptosporidium* Alexa Fluor® 488 monoclonal antibody [29] or a novel method using a two-step gating strategy without staining. This novel protocol uses the inherent property of *C. parvum* oocysts (i.e., oocysts show no auto-fluorescence unlike intestinal debris). To describe the relevance of the unstained method, the protocol using the anti-*Cryptosporidium* Alexa Fluor® 488 monoclonal antibody and the one using the two-step gating strategy without staining will be presented side-by-side. Using the above protocols to purify oocysts from stool and intestine samples, oocysts purified are a homogenous population of organisms corresponding to a clear population
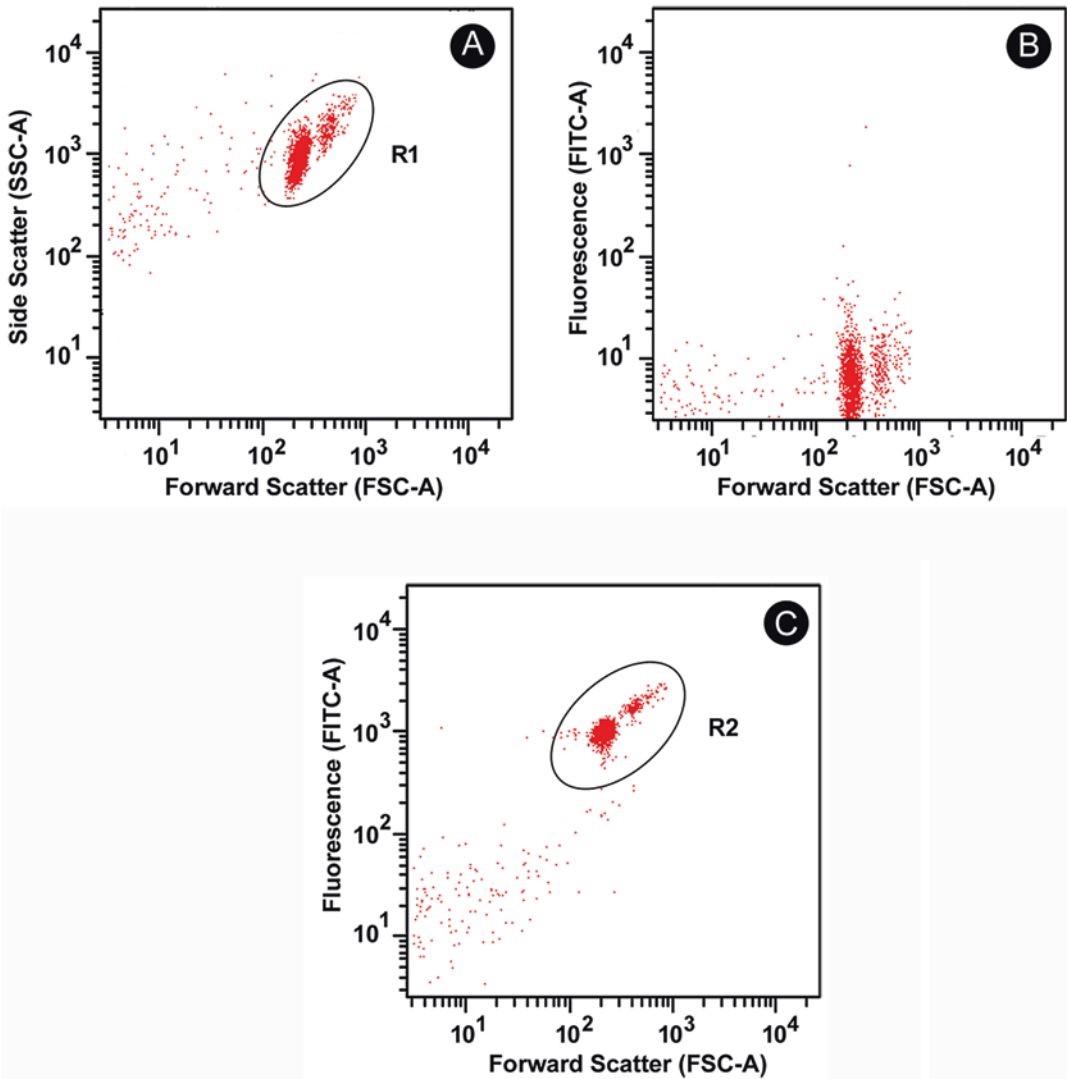
**Fig. 5** Panel **a** illustrates a purified oocyst sample plotted in two dimensions including forward scatter (size) and side scatter (internal complexity). Axes are presented in log scale and oocysts are clearly evident in region 1 (R1). Panel **b** illustrates the same population of particles seen in Panel **a** using forward scatter versus log fluorescence with the 488 nm laser and the FITC-A filter. Here, the sample has not been labeled with OW50-FITC reagent (oocyst-specific monoclonal antibody labeled with fluorescein isothiocyanate) and all particles show background fluorescence (nonfluorescent). Panel **c** illustrates the same sample labeled with OW50-FITC, clearly showing the intense fluorescence of the labeled oocysts and a slight increase in the background fluorescence of the nonoocyst debris. Coupling regions 1 and 2 (R1 and R2) into a "logical gate" enables the quantification of antibody-labeled oocysts by the flow cytometer instrument and software. Oocyst labeling is effective with specific antibodies and with appropriate fluorescent lectins. Fluorescent labeling is particularly important if excessive debris remains in the sample after the oocyst purification step

of events in a SSC-A vs FSC-A plot. One gate surrounding this population defines oocyst-like events which can then be further studied to determine fluorescent events. With the unstained method, the channel usually used for PerCP allow adequate gating between oocysts and debris because intestinal debris (e.g., vegetal fibers) are autofluorescent in the PerCP channel and they can easily be differentiated from oocysts which are not autofluorescent (*see* **Note 10**).

1. Vortex thoroughly oocyst sample kept in sterile cold distilled water at 4 °C.

2. Transfer into microtiter tube 4 µL of oocysts purified from stool with 16 µL of sterile cold distilled water or 20 µL of oocysts purified from intestine.

   *If using the protocol without staining antibody, directly proceed with* **step 8**.

3. Incubate oocyst sample with 1 µL mouse anti-*Cryptosporidium* Alexa Fluor® 488 monoclonal antibody (final concentration 0.5 µg/mL) for 30 min at room temperature.

4. Wash oocyst sample with 900 µL of 1% w/v bovine serum albumin in PBS 1×.

5. Centrifuge oocyst samples at 10,000 × *g* for 5 min at room temperature.

6. Discard supernatant.

7. Resuspend oocysts in 20 µL PBS 1×.

8. Add 60 µL sterile cold distilled water in microtiter tubes.

9. Add 100 µL 8% paraformaldehyde in PBS in microtiter tubes.

10. Incubate samples overnight at room temperature or at 4 °C (*see* **Note 11**).

11. Add 20 µL counting beads (total volume of 200 µL).

12. Vortex samples immediately before acquisition.

13. Acquire samples using a flow cytometer such as BD LSRFortessa or BD LSRFortessa X-20.

14. Use a flow cytometry analysis software such as FlowJo v.10 for data analysis.

15. Define a "bead gate" surrounding counting beads in the SSC-A vs FSC-A plot.

16. Define an "oocyst gate" surrounding the oocyst-like events in the SSC-A vs FSC-A plot.

17. From events in the "oocyst gate" (*see* Fig. 6):

    *Top panel*: Method <u>with</u> staining antibody: define a gate around Alexa Fluor-488-positive events.

    *Bottom panel*: Method <u>without</u> staining antibody: define a gate around PerCP-negative events
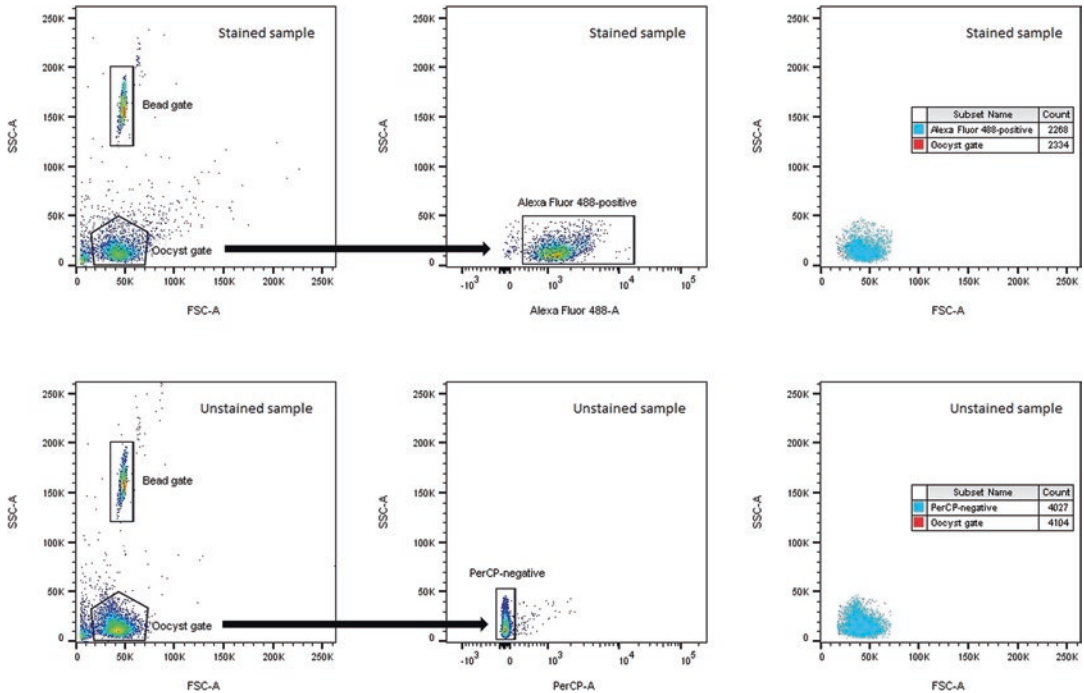
**Fig. 6** Stained and unstained oocysts from intestine sample of the same infected mouse. Top panels from left to right: bead gate and oocyst gate, Alexa Fluor-488-positive events from oocyst gate and overlay of Alexa Fluor-488-positive events from oocyst gate over all events in oocyst gate (visible extra red events being debris). Bottom panels from left to right: bead gate and oocyst gate, PerCP-negative events from oocyst gate and overlay of PerCP-negative events from oocyst gate over all events in oocyst gate (visible extra red events being debris). These two methods provide similar result, but stained method presents lower counts of oocysts because some were lost during washes

18. Determine counts of oocysts in acquired sample by applying appropriate formula:

    *Method <u>with</u> staining antibody*: (Count of Alexa Fluor-488-positive events in "oocyst gate" × quantity of beads per 20 μL of counting beads ÷ count of events in "bead gate").

    *Method <u>without</u> staining antibody*: (Count of PerCP-negative events in "oocyst gate" × quantity of beads per 20 μL of counting beads ÷ count of events in "bead gate").

19. Subtract count of uninfected control (debris) from count of sample as a baseline *(optional)*.

20. Multiply count of oocysts in acquired sample ×250 for sample purified from stool (4 μL acquired vs 1000 μL total volume of sample purified from stool) or ×50 for sample purified from intestine (20 μL acquired vs 1000 μL total volume of sample purified from intestine).

21. Normalize final count of oocysts using the weight of the stool or the intestine sample (e.g., count of oocysts/g of stool or intestine).

*3.13 Quantification of Oocysts by qPCR Analysis (see* Note 12*)*

1. Vortex thoroughly oocyst sample kept in sterile cold distilled water at 4 °C.

2. Transfer 200 µL of oocysts purified from stool or intestine into 1.7-mL microcentrifuge tubes.

3. Freeze oocyst sample in liquid nitrogen for 2 min.

4. Briefly dip bottom of microcentrifuge tube in room temperature water to warm up sample.

5. Thaw oocyst sample in 56 °C water bath during 2 min.

6. Briefly dip bottom of microcentrifuge tube in room temperature water to cool down sample.

7. Repeat **steps 3–6** for a total of 10 freeze/thaw cycles.

8. Extract DNA from oocyst sample using the QIAamp DNA Mini Kit qPCR.

9. Prepare mastermix for qPCR reaction (multiply volume of reagents × number of samples):

    5 µL of PCR-grade water.

    2 µL of 10 µM forward primer (5′-AACTTTAGCTCCAG TTGAGAAAGTACTC-3′).

    2 µL of 10 µM reverse primer (5′-CATGGCTCTTTACCG TTAAAGAATTCC-3′).

    2 µL of 2 µM 5′ 6-carboxyfluorescein reporter dye (FAM) probe with a 3′ TMR quencher dye (5′-AATACGTGT AGAACCACCAACCAATACAACATC-3′).

    4 µL of LightCycler TaqMan Master buffer including dNTPs, $MgCl_2$ and Taq-polymerase.

10. Transfer 15 µL of mastermix into a LightCycler capillary tube.

11. Add 5 µL of DNA extracted from oocyst sample to the mastermix in capillary tube.

12. Briefly centrifuge the capillary tube to move the solution at the bottom of the capillary tube.

13. Run qPCR in LightCycler 2.0 system using reported settings [6, 30]: 95 °C for 15 min, 50 cycles at 94 °C for 15 s, and 60 °C for 1 min.

14. In parallel, establish standard curve from known concentrations of oocysts (*see* Fig. 7):

    As mentioned above in Subheading 3.2, Infection of mice with *C. parvum* oocysts, wash oocysts from stock kept in 2.5% potassium dichromate at 4 °C with PBS 1×, count oocysts using a hemocytometer and calculate concentration of oocysts.

    Do serial dilution of oocysts → series of samples with known concentrations of oocysts.

    Freeze/thaw, extract DNA, and perform qPCR on series of samples with known concentrations of oocysts and determine their threshold cycle values (Ct values).
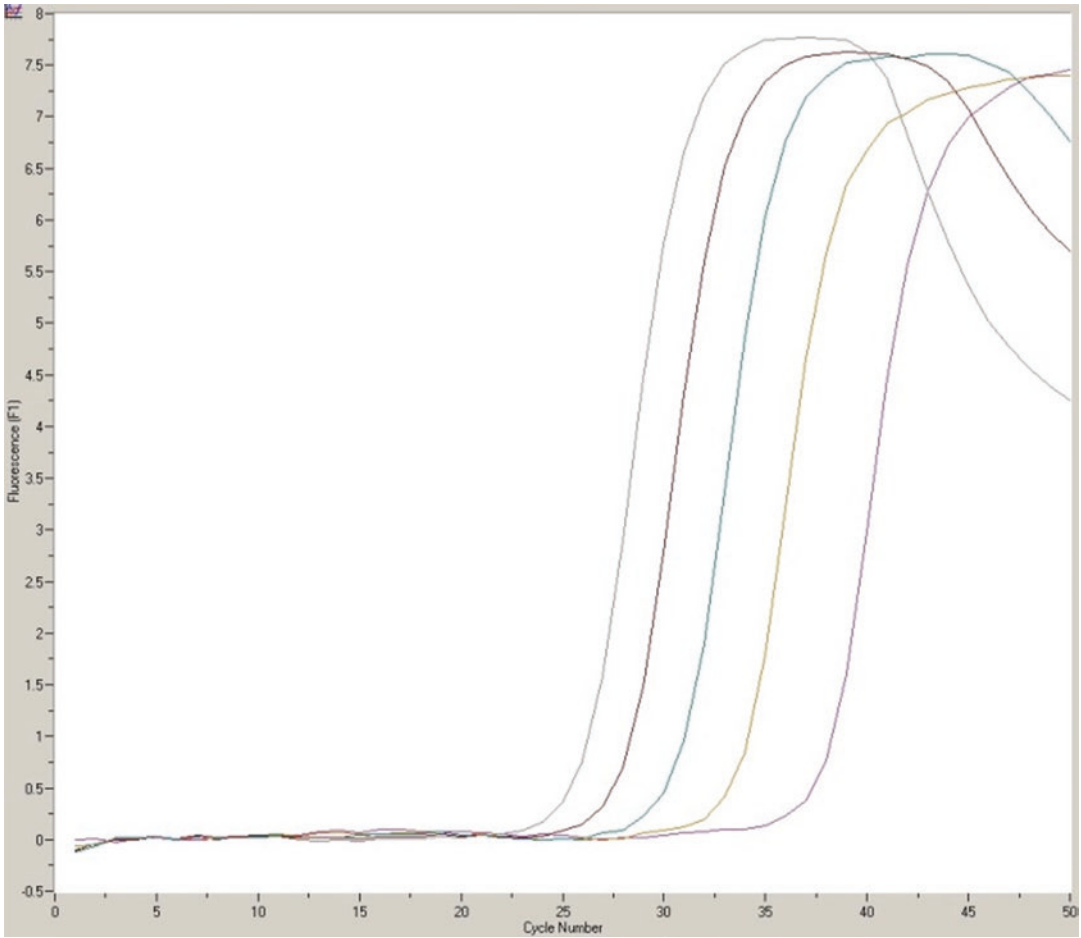
**Fig. 7** Visual representation of Ct values of known concentrations of *C. parvum* oocysts. From right to left, lines present sample of $10^3$ oocysts in purple, Ct value = 37.33; $10^4$ oocysts in yellow, Ct value = 33.15; $10^5$ oocysts in blue, Ct value = 30.16; $10^6$ oocysts in red, Ct value = 27.57 and $10^7$ oocysts in grey, Ct value = 25.54

15. Extrapolate parasite burden of mouse sample by comparing Ct values with the standard curve made from Ct values of samples with known oocyst concentrations.

*3.14  Quantification of Oocysts by Histopathologic Analysis of Ileum*

The histopathologic method offers a visual representation of the parasite burden (life cycle stages) in the ileum while providing only a semiquantitative value. We routinely use a custom scoring system in which grade 1 = absence of stages, grade 2 = occasional presence of stages, grade 3 = abundance of stages without clusters, grade 4 = abundance of stages with occasional clusters and grade 5 = abundance of stages with presence of multiple clusters. If a sample exceeded the preceding grade without meeting the criteria of the next grade, 0.5 is added to its score.

1. Collect 1.5 cm of ileum (distal part of the small intestine) at time of necropsy.

2. Store ileum in a histology cassette in 10% formalin for a minimum of 72 h.

3. Transfer histology cassettes to 70% ethanol just before processing to wash 10% formalin.

4. Transfer histology cassettes to a tissue processor such as Leica ASP300S tissue processor.

5. Paraffin-embed ileum samples using a paraffin-embedding station such as Leica EG1150 station.

6. Cut ileum samples into 4 μm thick sections using a microtome and transfer sections on slides.

7. Air-dry microscope slides for at least 48 h.

8. Heat microscope slides for 30 min at 60 °C.

9. Deparaffinize microscope slides by 10 min incubation in two successive xylene baths.

10. Transfer slides to successive ethanol baths (5 min in first 100% ethanol, 5 min in second 100% ethanol, 3 min in 80% ethanol, and 3 min in 70% ethanol).

11. Wash slides by 5 min incubation in bath with water.

12. Stain in hematoxylin for 15 s.

13. Rinse for 5 min under soft running tap water.

14. Dip 10 times in 80% ethanol bath.

15. Stain in eosin–phloxine for 1 min.

16. Wash for 5 min in bath containing eosine–phloxine:100% ethanol in a 1:50 ratio.

17. Rinse slides with squeeze bottle filled with 100% ethanol.

18. Transfer slides for 5 min into 100% ethanol bath, then for 5 min into first xylene bath, and then for 5 min into second xylene bath.

19. Wipe excess of xylene and apply two drops of mounting media and a coverslip.

20. Squeeze coverslip and microscope slide together to remove bubbles.

21. Use a light microscope to identify *C. parvum* life cycle stages in ileum sections (*see* Fig. 8) (*see* **Note 13**).

## 4   Notes

1. Due to the zoonotic potential of this parasite, it would be advisable that immunocompromised people and pregnant women avoid contact with infectious *C. parvum* oocysts.
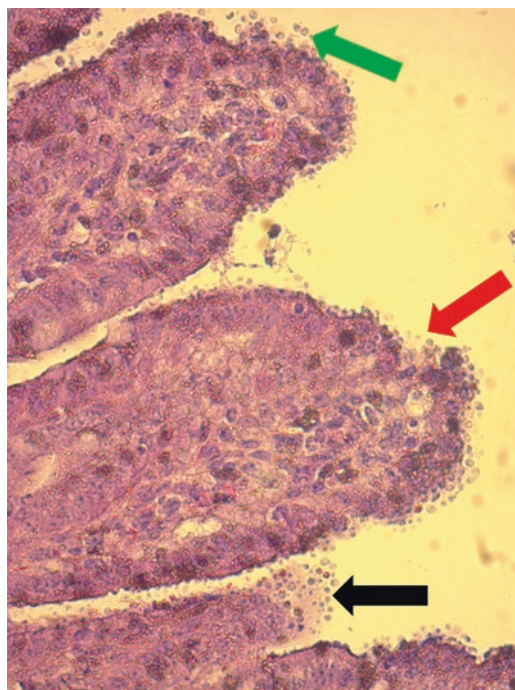
**Fig. 8** Histopathologic representation at 400× magnification of ileum of infected mouse showing *C. parvum* life cycle stages (green arrow), cellular damage/loss of membrane integrity (red arrow) and clumps of *C. parvum* life cycle stages (black arrow)

2. For Sheather's solution preparation, add 454 g of sucrose to 355 mL of distilled water. Dissolve slowly over gentle heat. Cool and add 6.0 mL full-strength phenol as a preservative. Store at room temperature.

3. To reduce the number of "empty shells" and improve the quality of a stock of parasites, *C. parvum* oocysts should be stored at 4 °C, and during experiments, reagents should be cold and centrifugations performed at 4 °C to avoid excystation which releases sporozoites from oocysts.

4. To maintain *C. parvum* oocysts, we infect 6- to 8-week old IFNγR-KO mice with 3000 oocysts stored in 2.5% potassium dichromate at 4 °C. Oocysts are then purified from intestines of infected mice as per the protocol presented above and stored in 2.5% potassium dichromate at 4 °C as we experienced a loss of infectivity of attenuated stains of *C. parvum* oocysts when kept in sterile cold distilled water at 4 °C. However, when conducting a study with mice to evaluate treatment efficacy of a compound, oocysts purified from stool or intestine of infected mice can be kept in sterile cold distilled water at 4 °C instead of 2.5% potassium dichromate. If stored in potassium dichromate, wash

the potassium dichromate from the samples or it may interfere with read-out technique (i.e., reduce sensitivity of the protocol of flow cytometry using staining antibody) to quantify oocysts in stool or intestine samples of mice of different groups.

5. When inoculating mice, be sure that the grip is secure and prohibits the mouse from moving its head in order to prevent damage to internal tissues during gavage. The mouse should be immobilized, but not so much that it shows signs of distress.

6. During oral gavage using a 20G bulb gavage needle, no resistance should be felt in mice older than 6 weeks old. If any resistance is felt during insertion of the gavage needle or if the mouse at any time begins to gasp, gently remove the needle and again try to find the esophagus.

7. It is possible for reflux to occur during oral gavage with proper insertion of the gavage needle, as well as adjusting the volume and viscosity of the solution can help to limit reflux and ensure accurate dosing.

8. Fecal pellets can be collected by placing mice in cages or other containers for a brief period of time. Another way is to pick the mice up (usually defecate instantly) and stool can be collected into a tube.

9. To prepare gradients microcentrifuge tubes, put the tip of the glass pipet at the very bottom of the tube when adding the 1:2 sucrose solution, beneath the 1:4 sucrose solution, and make sure that the interface rises.

10. This unstained method to count oocysts by flow cytometry was proven efficient to differentiate oocysts from debris in numerous experiments in our laboratory and to be as effective as the protocol using staining antibodies with the additional advantage of being less expensive. Obviously, the *sine qua non* condition is to start with a purified population of oocysts containing rare intestinal debris which is an objective easily achievable with protocols presented above.

11. On the one hand, paraformaldehyde fully inactivates oocysts at room temperature; however, if oocysts are incubated overnight at room temperature, some excystation may occur. One can argue that all samples are exposed to same conditions, thus samples can be compared between one another. On the other hand, incubation of oocysts overnight at 4 °C prevents excystation, but paraformaldehyde loses some efficacy at 4 °C and does not inactivate 100% of oocysts. Therefore, some bleach should be passed in the flow cytometer for cleaning after acquisition and some bleach will need to be added to the waste tank of the flow cytometer.

12. The proposed qPCR technique targeting the hsp70 gene of *C. parvum* (GenBank: U69698.2) is a more sensitive method of quantification, but detects DNA of both infectious and premature parasite stages instead of only typical oocysts.

13. *C. parvum* oocysts from different strains are indistinguishable by microscopy, but more virulent strains such as the Iowa strain can induce more tissue damage as well as melena.

## References

1. Fayer R, Xiao L (eds) (2007) *Cryptosporidium* and cryptosporidiosis, 2nd edn. CRC Press, Boca Raton, FL

2. Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, Panchalingam S, Wu Y, Sow SO, Sur D, Breiman RF, Faruque AS, Zaidi AK, Saha D, Alonso PL, Tamboura B, Sanogo D, Onwuchekwa U, Manna B, Ramamurthy T, Kanungo S, Ochieng JB, Omore R, Oundo JO, Hossain A, Das SK, Ahmed S, Qureshi S, Quadri F, Adegbola RA, Antonio M, Hossain MJ, Akinsola A, Mandomando I, Nhampossa T, Acacio S, Biswas K, O'Reilly CE, Mintz ED, Berkeley LY, Muhsen K, Sommerfelt H, Robins-Browne RM, Levine MM (2013) Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. Lancet 382(9888):209–222. https://doi.org/10.1016/S0140-6736(13)60844-2

3. Sow SO, Muhsen K, Nasrin D, Blackwelder WC, Wu Y, Farag TH, Panchalingam S, Sur D, Zaidi AK, Faruque AS, Saha D, Adegbola R, Alonso PL, Breiman RF, Bassat Q, Tamboura B, Sanogo D, Onwuchekwa U, Manna B, Ramamurthy T, Kanungo S, Ahmed S, Qureshi S, Quadri F, Hossain A, Das SK, Antonio M, Hossain MJ, Mandomando I, Nhampossa T, Acacio S, Omore R, Oundo JO, Ochieng JB, Mintz ED, O'Reilly CE, Berkeley LY, Livio S, Tennant SM, Sommerfelt H, Nataro JP, Ziv-Baran T, Robins-Browne RM, Mishcherkin V, Zhang J, Liu J, Houpt ER, Kotloff KL, Levine MM (2016) The burden of *Cryptosporidium* diarrheal disease among children <24 months of age in moderate/high mortality regions of Sub-Saharan Africa and South Asia, utilizing data from the Global Enteric Multicenter Study (GEMS). PLoS Negl Trop Dis 10(5):e0004729. https://doi.org/10.1371/journal.pntd.0004729

4. Jakobi V, Petry F (2008) Humoral immune response in IL-12 and IFN-gamma deficient mice after infection with *Cryptosporidium parvum*. Parasite Immunol 30(3):151–161

5. Ndao M, Nath-Chowdhury M, Sajid M, Marcus V, Mashiyama ST, Sakanari J, Chow E, Mackey Z, Land KM, Jacobson MP, Kalyanaraman C, McKerrow JH, Arrowood MJ, Caffrey CR (2013) A cysteine protease inhibitor rescues mice from a lethal *Cryptosporidium parvum* infection. Antimicrob Agents Chemother 57(12):6063–6073. https://doi.org/10.1128/AAC.00734-13

6. Sonzogni-Desautels K, Renteria Flores A, Vasquez Camargo F, Di Lenardo TZ, Mikhail A, Arrowood MJ, Fortin A, Ndao M (2015) Oleylphosphocholine (OlPC) arrests *Cryptosporidium parvum* growth in vitro and prevents lethal infection in interferon gamma receptor knock-out mice. Front Microbiol 6:973. https://doi.org/10.3389/fmicb.2015.00973

7. Tessema TS, Schwamb B, Lochner M, Forster I, Jakobi V, Petry F (2009) Dynamics of gut mucosal and systemic Th1/Th2 cytokine responses in interferon-gamma and interleukin-12p40 knock out mice during primary and challenge *Cryptosporidium parvum* infection. Immunobiology 214(6):454–466

8. von Oettingen J, Nath-Chowdhury M, Ward BJ, Rodloff AC, Arrowood MJ, Ndao M (2008) High-yield amplification of *Cryptosporidium parvum* in interferon gamma receptor knock-out mice. Parasitology 135(10):1151–1156

9. Basha S, Surendran N, Pichichero M (2014) Immune responses in neonates. Expert Rev Clin Immunol 10(9):1171–1184. https://doi.org/10.1586/1744666X.2014.942288

10. Delaunay A, Gargala G, Li X, Favennec L, Ballet JJ (2000) Quantitative flow cytometric evaluation of maximal *Cryptosporidium parvum* oocyst infectivity in a neonate mouse model. Appl Environ Microbiol 66(10):4315–4317

11. Blagburn BL, Sundermann CA, Lindsay DS, Hall JE, Tidwell RR (1991) Inhibition of *Cryptosporidium parvum* in neonatal Hsd:(ICR)BR Swiss mice by polyether ionophores and aromatic amidines. Antimicrob Agents Chemother 35:1520–1523

12. Theodos CM, Sullivan KL, Griffiths JK, Tzipori S (1997) Profiles of healing and non-healing *Cryptosporidium parvum* infection in C57BL/6 mice with functional B and T lymphocytes: the extent of gamma interferon modulation determines the outcome of infection. Infect Immun 65(11):4761–4769

13. You X, Mead JR (1998) Characterization of experimental *Cryptosporidium parvum* infection in IFN-gamma knockout mice. Parasitology 117(6):525–531

14. Campbell LD, Stewart JN, Mead JR (2002) Susceptibility to *Cryptosporidium parvum* infections in cytokine- and chemokine-receptor knockout mice. J Parasitol 88(5):1014–1016

15. Urban JF Jr, Fayer R, Chen SJ, Gause WC, Gately MK, Finkelman FD (1996) IL-12 protects immunocompetent and immuno-deficient neonatal mice against infection with *Cryptosporidium parvum*. J Immunol 156(1):263–268

16. Lemieux MW, Sonzogni-Desautels K, Ndao M (2017) Lessons learned from protective immune responses to optimize vaccines against cryptosporidiosis. Pathogens 7(1):E2. https://doi.org/10.3390/pathogens7010002

17. Love MS, Beasley FC, Jumani RS, Wright TM, Chatterjee AK, Huston CD, Schultz PG, McNamara CW (2017) A high-throughput phenotypic screen identifies clofazimine as a potential treatment for cryptosporidiosis. PLoS Negl Trop Dis 11(2):e0005373. https://doi.org/10.1371/journal.pntd.0005373

18. Zambriski JA, Nydam DV, Bowman DD, Bellosa ML, Burton AJ, Linden TC, Liotta JL, Ollivett TL, Tondello-Martins L, Mohammed HO (2013) Description of fecal shedding of *Cryptosporidium parvum* oocysts in experimentally challenged dairy calves. Parasitol Res 112(3):1247–1254. https://doi.org/10.1007/s00436-012-3258-2

19. Zambriski JA, Nydam DV, Wilcox ZJ, Bowman DD, Mohammed HO, Liotta JL (2013) *Cryptosporidium parvum*: determination of $ID_{50}$ and the dose-response relationship in experimentally challenged dairy calves. Vet Parasitol 197(1–2):104–112. https://doi.org/10.1016/j.vetpar.2013.04.022

20. Heine J, Moon HW, Woodmansee DB (1984) Persistent *Cryptosporidium* infection in congenitally athymic (nude) mice. Infect Immun 43:856–859

21. Mead JR, Arrowood MJ, Sidwell RW, Healey MC (1991) Chronic *Cryptosporidium parvum* infections in congenitally immunode-ficient SCID and nude mice. J Infect Dis 163(6):1297–1304

22. McDonald V, Deer R, Uni S, Iseki M, Bancroft GJ (1992) Immune responses to *Cryptosporidium muris* and *Cryptosporidium parvum* in adult immunocompetent or immunocompromised (nude and SCID) mice. Infect Immun 60(8):3325–3331

23. Jumani RS, Bessoff K, Love MS, Miller P, Stebbins EE, Teixeira JE, Campbell MA, Meyers MJ, Zambriski JA, Nunez V, Woods AK, McNamara CW, Huston CD (2018) A novel piperazine-based drug lead for cryptosporidiosis from the medicines for malaria venture open-access malaria box. Antimicrob Agents Chemother 62(4):e01505-17. https://doi.org/10.1128/AAC.01505-17

24. Arrowood MJ, Hurd MR, Mead JR (1995) A new method for evaluating experimental cryptosporidial parasite loads using immu-nofluorescent flow cytometry. J Parasitol 81(3):404–409

25. Delling C, Holzhausen I, Daugschies A, Lendner M (2016) Inactivation of *Cryptosporidium parvum* under laboratory conditions. Parasitol Res 115(2):863–866. https://doi.org/10.1007/s00436-015-4813-4

26. Fujino T, Matsui T, Kobayashi F, Haruki K, Yoshino Y, Kajima J, Tsuji M (2002) The effect of heating against *Cryptosporidium* oocysts. J Vet Med Sci 64(3):199–200

27. Chen F, Huang K, Qin S, Zhao Y, Pan C (2007) Comparison of viability and infectivity of *Cryptosporidium parvum* oocysts stored in potassium dichromate solution and chlorinated tap water. Vet Parasitol 150(1–2):13–17. https://doi.org/10.1016/j.vetpar.2007.09.001

28. Yang S, Healey MC, Du C (1996) Infectivity of preserved *Cryptosporidium parvum* oocysts for immunosuppressed adult mice. FEMS Immunol Med Microbiol 13(2):141–145. https://doi.org/10.1016/0928-8244(95)00096-8

29. Barbosa JM, Costa-de-Oliveira S, Rodrigues AG, Hanscheid T, Shapiro H, Pina-Vaz C (2008) A flow cytometric protocol for detection of *Cryptosporidium* spp. Cytometry A 73(1):44–47. https://doi.org/10.1002/cyto.a.20502

30. Shahiduzzaman M, Dyachenko V, Keidel J, Schmaschke R, Daugschies A (2010) Combination of cell culture and quantitative PCR (cc-qPCR) to assess disinfectants efficacy on Cryptosporidium oocysts under standardized conditions. Vet Parasitol 167(1):43–49. https://doi.org/10.1016/j.vetpar.2009.09.042

# Chapter 15

# Calf Clinical Model of Cryptosporidiosis for Efficacy Evaluation of Therapeutics

**Michael W. Riggs and Deborah A. Schaefer**

## Abstract

Cryptosporidiosis, caused by the apicomplexan parasite *Cryptosporidium parvum*, is a moderate-to-severe diarrheal disease now recognized as one of the leading causes of morbidity and mortality in livestock globally, and in humans living in resource-limited parts of the world, particularly those with AIDS or malnourished individuals. This recognition has fueled efforts for the discovery of effective therapeutics. While recent progress in drug discovery has been encouraging, there are presently no acceptably effective parasite-specific drugs for the disease. The urgent need for new drug discovery or drug repurposing has also increased the need for refined animal models of clinical disease for therapeutic efficacy evaluation. Here, we describe an acute model of cryptosporidiosis using newborn calves to evaluate well-defined clinical and parasitological parameter outcomes, including the effect on diarrhea severity and duration, oocyst numbers produced, and multiple measures of clinical health. The model is highly reproducible and provides unequivocal direct measures of treatment efficacy on diarrhea severity and parasite replication.

**Key words** *Cryptosporidium*, Cryptosporidiosis, Bovine model, Therapeutic evaluation, Oocyst quantitation, Diarrhea quantitation, Clinical evaluation

## 1 Introduction

*Cryptosporidium* species are among the leading causative agents of diarrhea in humans and agriculturally important livestock species throughout the world, namely calves [1–3]. Despite this, there are currently no approved vaccines and no parasite-specific drugs to treat the disease in either humans or livestock in the US. The only Food and Drug Administration-approved drug for treating cryptosporidiosis in humans is nitazoxanide [4]. However, the efficacy of nitazoxanide against *Cryptosporidium* in immunocompetent humans is marginal and the drug has no treatment benefit against the parasite in AIDS patients [5–7]. The clear need for more effective parasite-specific drugs has increased the need for refined, reliable animal models of cryptosporidiosis for therapeutic efficacy evaluation.

Neonatal calves can provide an excellent acute clinical model for both human and bovine cryptosporidiosis. During the 10–14-day-old age period most often used, neonatal calves are functional monogastrics and remain so up to 4 weeks of age [8, 9]. Clinical symptoms and diarrhea severity and duration following experimental infection in calves closely mirror what is observed in humans [8, 10]. Further, the anatomic distribution of infection in the GI tract and lesions are essentially the same as those observed in humans [8, 10]. Given these observations supporting the validity of calves as a useful clinical model most closely approximating human cryptosporidiosis, multiple methodologies and experimental designs for evaluation of therapeutics in the model have been published. The most commonly used calf models differ mainly in (1) frequency of fecal collection, (2) type of calf housing used (elevated stalls, or box stalls/pens), (3) whether total daily fecal volume excreted is collected, or a ~10–15 mL fecal sample is obtained per rectum once or twice daily, (4) methods used for determining diarrhea severity (quantitative or semi-quantitative), (5) methods used for evaluating oocyst shedding (quantitative, e.g., qPCR or hemocytometer enumeration of the total number of oocysts shed in total daily fecal collections, or various semi-quantitative measures, e.g., acid-fast stain or immunofluorescence assay on fecal sample smears and microscopic scoring, or qPCR on fecal samples collected every 12–24 h), (6) trial duration and (7) *C. parvum* isolate used (field vs characterized) [11–16]. Total daily fecal collection is required to unequivocally determine diarrhea severity and total number of oocysts produced and requires housing in elevated stalls [11–13]; these measurements are not possible with box stall or pen housing in which a ~10–15 mL fecal sample is collected daily per rectum [14–16]. Model designs vary between investigator and institutional factors (e.g., housing facilities and equipment availability, investigator preferences, staffing resources and abilities, budgetary constraints) and can influence the accuracy of results for efficacy parameters. Therefore, the experimental design used in a particular calf model study should be considered when interpreting efficacy results of a given therapeutic and when making comparisons between studies. For example, in one calf model of cryptosporidiosis, treating calves with nitazoxanide reduced the duration of *C. parvum* oocyst shedding and diarrhea severity [14], while in another calf model, nitazoxanide had no therapeutic or prophylactic efficacy against clinical disease caused by *C. parvum*, and in some *C. parvum*-infected calves nitazoxanide treatment actually prolonged and exacerbated diarrhea [15]. In a calf model similar to the preceding, paromomycin demonstrated prophylactic efficacy against *C. parvum* challenge and proof of concept, but the drug dosage required was determined to be cost prohibitive for veterinary use [16]. While there is no universally applicable design for the calf model given geographic, institutional,

and laboratory constraints, we have used the acute model described here to consistently replicate data demonstrating therapeutic efficacy for multiple bumped kinase inhibitor (BKI) drugs for further study, as well as to consistently replicate data demonstrating lower, clinically less satisfactory efficacy for other BKIs to eliminate from further study [11, 17] (M.W. Riggs and D.A. Schaefer, unpublished).

The model described here is quite labor-intensive and demanding on personnel compared to some other calf models for evaluation of therapeutics and requires housing calves in elevated stalls for total fecal collection. However, the increased labor and demands are justified because the method yields the most accurate efficacy data using defined, quantitative outcome parameters. Determining total daily fecal volume and consistency for each calf provides an objective and unequivocal direct measure of diarrhea severity. Total daily fecal collection is also the preferred practice in human challenge studies to evaluate the efficacy of candidate anti-cryptosporidial preparations [18]. In addition, total daily fecal collection allows accurate determination of total number of oocysts produced per day as an unequivocal measure of treatment effect on parasite replication, and also avoids potential error resulting from diurnal variations in oocyst shedding we have observed in this model [11] (*see* **Note 1**). Reduction of oocyst shedding is an important treatment outcome when considering the epidemiologic importance of the environmental parasite burden and exposure risk to both humans and livestock [19]. By 8 or 9 days post infection (DPI), most untreated control calves are beginning to resolve diarrhea in this model [11, 17]. Therefore, we end the experiment at 10 DPI to conclude initial efficacy determinations.

We previously attempted total fecal collection from calves using strap-on commercial fecal collection bags designed for small ruminant metabolic studies [20]. This allowed individual calves to be housed in larger box stalls. However, fecal collection bags invariably leaked or became detached and failed completely, despite emptying up to three times daily. Emptying the bags also required manual restraint of calves and resulted in visible distress. Given all of the above, housing of calves in elevated stalls in this acute model design is thus scientifically and ethically justified and is approved by the Institutional Animal Care and Use Committee. The method presented here is completed in the following major sequential steps or procedures to evaluate therapeutics in this calf model of cryptosporidiosis: (1) procure isolated *Cryptosporidium parvum* oocysts and age-matched (within 12 h) newborn pathogen-free Holstein bull calves for therapeutic efficacy evaluation, (2) manage husbandry of calves housed in elevated stalls, modified to accurately evaluate clinical health parameters, diarrhea severity, parasite production and urine production, (3) infect calves with *C. parvum* at 36–48 h of age and administer candidate therapeutic agents per

the appropriate dosage and treatment regimen commencing at 2 DPI, (4) collect and process feces to determine total daily fecal volumes and total daily oocyst counts, (5) complete terminal procedures and euthanize calves at 10 DPI, and (6) analyze data for significant treatment effects on clinical health, total fecal volume (diarrhea severity), total oocysts produced (parasite replication), total urine volume (an additional index of hydration), and body weight gain or loss.

## 2 Materials

### 2.1 Preparation of Cryptosporidium parvum Oocysts

1. Fully equipped BSL-2 laboratory.
2. Peracetic acid.
3. Taurocholic acid.
4. Trypsin.
5. Phosphate-buffered saline (PBS).
6. Phase-contrast and immunofluorescence microscopes.
7. Hemocytometer.

### 2.2 Newborn Calves for Therapeutic Efficacy Evaluation

1. Cargo van with climate control for heating and cooling.
2. Rubber boots and coveralls.
3. Roll of 10 mil plastic sheeting, ~185 cm wide.
4. Disinfectant (quaternary ammonium-based for animal facilities).
5. Weight scale for calves.
6. Wheelbarrow.
7. *Clostridium perfringens* Type C antitoxin and anti-*Escherichia coli* passive antibody for calves (Ecolizer®, Novartis, Larchwood, IA).
8. Attenuated bovine rotavirus and coronavirus vaccine (CalfGuard®, Zoetis, Kalamazoo, MI).
9. Clean blankets and thick terry cloth cotton towels.
10. Newborn calf record sheets.
11. Surgical scissors to trim umbilical cords.
12. Ear tags and applicator.
13. 7% iodine scrub.
14. 10% iodine solution.
15. Colostrum replacer (bovine IgG colostrum replacer, Land O' Lakes, Shoreview, MN).
16. Disinfected 6 L buckets.

17. Whisk.

18. Plastic feeding bottles with rubber nipples (2 L).

19. Esophageal tube feeder.

20. Hand soap and disposable disinfectant wipes.

21. Calf transport crates (crates designed for 90–125 pound dogs work well-one calf per crate).

22. Autoclaved straw.

*2.3 Modified Elevated Calf Stalls, Calf Husbandry*

Coordinate with local Institutional Animal Care and Use Committee (IACUC, or equivalent) on stall requirements for calves.

1. Elevated calf stalls (~152 cm *L* × ~122 cm *H* × ~53.5 cm *W*; Wenke Mfg, Pender NE).

2. Large rectangular plastic bins (~58.5 cm × ~43 cm × ~15 cm; ~26 L) (4 per calf stall—3 for fecal collection and 1 for urine collection).

3. Reinforced waterproof industrial strength adhesive tape.

4. Roll of 6 mil plastic sheeting, ~122 cm wide.

5. Large clamp binder clips to hold fecal collection bins together.

6. Wall mirrors (~100 cm × 100 cm).

7. Serum refractometer.

*2.4 Calf Infection and Treatment*

1. Isolated peracetic acid-disinfected *C. parvum* oocysts.

2. Candidate therapeutic in vehicle and vehicle alone.

3. *C. parvum*-specific monoclonal antibody(ies) and fluorescein-ated secondary antibody for indirect immunofluorescence assay.

4. Disposable 50 cc blunt ended catheter tip dosing syringes.

5. Disposable Tyvek® suits (various sizes) with elastic-cinched ankles and wrists.

6. Disposable plastic booties and plastic boot covers (all to be worn over shoes); alternatively rubber boots.

7. N95 respirators.

8. Goggles and protective glasses.

9. Assorted disposable nitrile gloves.

10. Hair net bonnets.

11. Fecal specimen cups (150 mL urinalysis cups) with lids.

12. Labels for fecal specimen cups.

13. Rectal thermometer.

14. Disinfectant (quaternary ammonium-based for animal facilities).

15. Mop bucket and mop.

16. Plastic scraper.

17. Assorted syringes and needles.

18. Appropriate blood collection tubes.

19. Flunixin meglamine.

20. Injectable selenium and vitamin E supplement (~1 mg selenium, 50 mg (68 USP units) vitamin E per mL).

21. Oral electrolytes for calves (or equivalent).

22. Milk replacer (crude protein ~22%, crude fat ~20%).

*2.5 Fecal Processing*

1. Personal protective equipment listed in Subheading 2.4.

2. Assorted size graduated cylinders (4, 2, 1 L, and 500 mL)

3. Buckets (4 L)

4. Commercial blender (4 L capacity)

5. Paper towels

6. Plastic ladle and spatula

7. Disinfectant (quaternary ammonium-based for animal facilities)

8. Pressure washer for cleaning post-trial

*2.6 Fecal Oocyst Quantitation*

1. Real-time PCR (qPCR) System (ABI StepOne Plus®; Applied Biosystems, Grand Island, NY).

2. Real-time PCR (qPCR) reagents.

3. Fecal oocyst DNA extraction reagents and buffers (QIAamp® Fast DNA stool mini kit; Qiagen, Gaithersburg, MD).

4. DNA quantification method (Nanodrop®; Nanodrop Technologies, Wilmington, DE).

5. Primers (we use the *C. parvum* oocyst wall protein gene (COWP) (Genbank no. AF248743) as the target for primers and selected a 151-bp sequence from the COWP gene).

6. SYBR-green (or equivalent nucleic acid stain).

7. Bovine serum albumin.

*2.7 Terminal Procedures and Calf Euthanasia*

1. Xylazine.

2. Ketamine.

3. Appropriate blood collection tubes.

4. Euthanasia solution (390 mg pentobarbital sodium/mL, 50 mg phenytoin sodium/mL, or equivalent).

5. Weight scale.

## 3    Methods

For use in the following methods for evaluation of therapeutics in a bovine clinical model of cryptosporidiosis, calves must be maintained in climate-controlled Animal Biosafety Level 2 (ABSL-2) biocontainment facilities in accordance with the *Biosafety in Microbiological and Biomedical Laboratories* guidelines and *Guide for the Care and Use of Laboratory Animals.* Approval by The Institutional Animal Care and Use Committee (IACUC) is required and the methods must be performed in compliance with guidelines in the Animal Welfare Act and *Guide for the Care and Use of Agricultural Animals in Research and Teaching* (Federation of Animal Science Societies), preferably in facilities fully accredited by the American Association for Laboratory Animal Care. The model described here has been established and operative in the authors' laboratory for almost two decades [11–13]. Based on the large number of unsolicited outside requests we receive for testing of candidate anti-cryptosporidials in this model from industry and established *Cryptosporidium* investigators at other institutions, we presume that the model is a preferred practice. However, the model should only be used for further evaluation of lead candidate therapeutics or prophylactics which have demonstrated highly significant anti-cryptosporidial activity in vitro and in at least one less sentient species rodent model such as neonatal mice. While neonatal mice are the lowest species suitable for initial in vivo evaluation of therapeutics against *Cryptosporidium parvum*, the infection is asymptomatic. However, reduction of infection levels in the neonatal mouse model has positive predictive value for therapeutic efficacy against *C. parvum* in clinical models of disease including calves [11, 13, 21, 22].

**3.1    Procurement and Preparation of Cryptosporidium parvum Oocysts for Calf Infection**

1. Decide on the *C. parvum* isolate to be used and obtain purified oocysts from a source production laboratory. We routinely use the Iowa *C. parvum* isolate [23, 24] because it is well characterized and is maintained by monthly propagation in newborn *Cryptosporidium*-free Holstein bull calves in our laboratory [25, 26] (*Cryptosporidium* Production Laboratory; https://acbs.cals.arizona.edu/crypto). Other established laboratories and commercial sources exist for obtaining purified *C. parvum* oocysts in the numbers required for calf trials including Bunch Grass Farm (Deary, ID; bunchgrassfarm@gmail.com). Alternatively, use the method described here to propagate *C. parvum* oocysts in calves and isolate oocysts from feces using published methods. Store oocysts in the saline/antibiotic solution the source laboratory ships them in (4 °C) for up to 60 days, but ideally use oocysts within 30 days of isolation for calf infection. All procedures involving *C. parvum* must be

performed at Biosafety Level-2 (BSL-2) in an approved BSL-2 laboratory.

2. Immediately prior to administration to calves, disinfect oocysts with 0.5% peracetic acid (4 °C) to inactivate any possible contaminant bacterial or viral pathogens [23, 27] (*see* **Note 2**). Peracetic acid is the preferred disinfectant as treatment of oocysts with diluted sodium hypochlorite (bleach) has been shown to promote spontaneous excystation at 37 °C [25, 27]. Use of hypochlorite-disinfected oocysts could potentially result in excystation in the abomasum of experimental calves and sporozoite killing by acidic conditions, effectively reducing the intended infectious dose [25]. In contrast, peracetic acid disinfection does not promote spontaneous excystation. All disinfection steps are performed at 4 °C. Centrifuge oocysts previously stored in saline/antibiotic solution (8000 × $g$, 7 min, 4 °C), remove saline/antibiotic solution, resuspend the oocyst pellet in PBS (4 °C) and wash oocysts with PBS twice. Then resuspend the pelleted oocysts <u>thoroughly</u> in 1–10 mL 0.5% peracetic acid (stock peracetic acid diluted in DI water to 0.5% vol/vol). Incubate resuspended oocysts at 4 °C for 3 min, inverting once each min. The 3 min timing period starts immediately upon addition of peracetic acid. After 3 min treatment, immediately pellet oocysts (8000 × $g$, 7 min, 4 °C), remove peracetic acid and wash 4× with PBS as described above. Keep oocysts at 4 °C at all times post-peracetic acid treatment until immediately prior to inoculation of calves.

3. Following disinfection, determine percent oocyst excystation as a viability index and adjust the challenge dose if necessary (*see* **Note 3**). To determine percent excystation, resuspend a disinfected oocyst aliquot (~$10^6$ oocysts) in 250 µL excystation medium (1.5% taurocholic acid, 0.5% trypsin in PBS, pH 7.2–7.4) and place in a shaking water bath (1.5 h, 37 °C). At the end of the incubation, count the number of intact unexcysted oocysts and oocyst shells (fully/partially excysted oocysts) using a hemocytometer and phase microscopy. Divide the number of oocyst shells by the total number of oocyst shells plus intact oocysts and multiply by 100 to determine % excystation. Excystation is typically >95% for oocysts stored properly for up to 30 days following isolation and disinfection.

*3.2 Procurement and Enrollment of Neonatal Calves for Therapeutic Efficacy Evaluation*

1. Trained veterinary staff obtain age-matched (born within a 12 h period) Holstein bull calves from a closed herd USDA-licensed dairy production unit, preferably the same source unit for all studies. Cohort size will vary depending on the investigator's facility capacity for ABSL-2 calf isolation and source dairy average daily calving numbers. The minimum cohort size desired is six calves (three treated, three control) (*see* **Note 4**).

With two such cohorts completed, data for six treated and six control calves are obtained, allowing potential exclusion of up to one calf per group without compromising statistical analyses in most cases. Occasionally such exclusions are required because of inadvertent coinfection with one or more other enteropathogens, need for euthanasia because of disease severity before completion of the study or other confounding variables. Trained laboratory staff manually assist birthing of calves for delivery onto a 10 mil plastic sheet barrier to minimize possible exposure to other enteropathogens or *Cryptosporidium* isolates or species (*see* **Note 5**). Because newborn calves are wet and difficult to securely hold, use clean thick cotton terrycloth towels to grasp calves during delivery. Calves from cows which experience dystocia or prolonged calving, or twin calves are not accepted. Weigh and perform a physical exam on each calf, including careful examination for congenital anomalies such as imperforate anus, cleft palate, and limb abnormalities. Calves weighing ≥38 kg and having normal physical exam findings are the most suitable for use in the method described here; calves weighing <38 kg are excluded (*see* **Note 6**). Place acceptable calves into a previously disinfected wheelbarrow for transport to a pre-disinfected designated concrete area. Disinfect the wheelbarrow between use for each calf. Wash any body parts, especially the muzzle and head region, with 7% iodine scrub and warm water if necessary to remove any exogenous material, then dry with a clean towel. Clean both sides of one ear with 7% iodine scrub and rinse, then apply a numbered ear tag to enrolled calves to serve as the calf's identifying number. Record date and time of birth. Dip the navel in 10% iodine solution (not scrub), again after colostrum replacer is fed, again on arrival at the ABSL-2 facility and again 24 h after arrival. Orally administer to each calf Ecolizer (*Clostridium perfringens* Type C antitoxin and anti-*Escherichia coli* antibody, Novartis, Larchwood, IA) and CalfGuard vaccine (attenuated bovine rotavirus and coronavirus, Zoetis, Kalamazoo, MI) within 20 min of birth, and 1 h prior to feeding colostrum replacer per manufacturer's instructions. Commencing 1 h after administration of the preceding antibodies and vaccine, feed enrolled calves commercial colostrum replacer from a single lot by nipple bottle (*see* **Note 7**), continuing until 6 h after birth at twice the manufacturer's recommended calf dose reconstituted in 2 L of water (*see* **Notes 8 and 9**). Record actual time and volume of colostrum replacer intake hourly to confirm optimal full dosing is achieved within 6 h after birth. If a calf will not voluntarily consume the full amount of colostrum replacer within 6 h of birth, attempt to nipple bottle-feed the remainder within 9 h of birth (*see* **Note 10**). If a calf will not voluntarily consume the full amount of

colostrum replacer within 9 h of birth, carefully feed the remainder using an esophageal tube feeder [28] (*see* **Note 11**).

2. Place calves into disinfected transport crates (crates for 90–125 pound dogs) with autoclaved straw bedding for transport by cargo van to ABSL-2 Facilities. Disinfect calf rooms and allow them to air dry for at least 3 days prior to and after each experiment to inactivate any residual *Cryptosporidium* or other microbial agents. Upon arrival, assign calves to either treatment or control groups using a randomization method such as Microsoft Excel Random Number Generation Tool, then place calves into individual elevated calf stalls (Wenke Manufacturing, Pender, NE), equipped with coated slat flooring material (9 gauge flat expanded metal with rubberized coating and diamond-shaped openings) (*see* **Note 12**). House treatment and control groups in separate ABSL-2 rooms. Facilities will vary between institutions but we house two to three calves per room (~3 m wide by ~5 m long), allowing placement of elevated stalls such that the calves face each other for environmental enrichment but at distances that do not allow direct contact between calves. In instances when only a single calf is housed per room, place a mirror at eye level on the wall in front of the calf for environmental enrichment.

3. Using a nipple bottle, feed calves non-medicated milk replacer (crude protein ~22% [bovine whey and milk-derived], crude fat ~20%) twice daily commencing at 12 h of age (volume equivalent to 10% of birth weight total per day divided into 2 feedings, 12 h apart through 5 days of age, increased by 25% at 6 days of age and increased again by 25% at 8 days of age) until the end of the experiment at 10 DPI (*see* **Note 13**). Allow 25–30 min per feeding for calves to consume the entire volume of milk replacer. Record the actual quantity of milk replacer consumed at each feeding. If a calf does not voluntarily consume any milk replacer, carefully feed the calculated volume of replacer using an esophageal tube feeder [28] (*see* **Note 14**). Clean and disinfect all nipple bottles, buckets, and utensils used for replacer reconstitution and feeding after each use.

4. At 24 h of age administer vitamin E/selenium supplement (3 mL/45 kg) subcutaneously. Collect a blood sample by venipuncture (~10 mL), isolate the serum fraction, and test serum to verify adequate passive transfer of colostral IgG using a rapid method such as serum refractometry to semi-quantitatively assess serum IgG levels [29, 30] (*see* **Note 15**).

5. At 36 h of age, perform another physical examination on each calf prior to final clearance and admittance into the study. Remove any unhealthy calves from the study (exclusion criteria: fever >103.0 °F, diarrhea, respiratory distress, inappetence, or inability to rise on own). Prior to experimental infection,

examine fecal smears from each calf for the potential presence of *Cryptosporidium* oocysts by immunofluorescence assay (IFA) [31]. Remove any calves from the study that are positive for inadvertent field acquired *Cryptosporidium* infection.

*3.3 Infection of Calves with C. parvum and Administration of Candidate Therapeutic Agents*

1. Choose an optimal time for personnel to infect calves such that at the time of inoculation no calves in the cohort are younger than 36 h of age and no calves are older than 48 h of age. Following acclimation, at the chosen time between 36 and 48 h of age (defined as DPI 0), orally administer disinfected oocysts (*see* Subheading 3.1) to calves as follows. While the calf is standing, allow each calf to suckle milk replacer (250 mL) from a nipple bottle to close the esophageal (gastric) groove and thus bypass the rumen for delivery of the inoculum directly into the abomasum [28]. Immediately thereafter, orally administer to each calf $5 \times 10^7$ viable, peracetic acid-disinfected *C. parvum* oocysts previously enumerated using a hemocytometer and prepared as follows. Prior to use, keep the oocyst inoculum for each calf in ~5 mL PBS at 4 °C in a 15-mL centrifuge tube. Immediately prior to inoculation, allow the inoculum to gradually reach room temperature over a ~10 min period to avoid thermal shock, then vortex the tube to resuspend oocysts. Immediately draw up the inoculum into a 50 cc blunt-ended catheter tip dosing syringe, equipped with a 15 cm Nalgene tube (~2 mm inside diameter) to facilitate recovery of the entire inoculum. Rinse/vortex the oocyst tube once with 5 mL PBS and draw up the rinse into the same 50 cc syringe. Finally, draw up ~20 mL milk replacer (at room temperature) into the syringe and mix by inversion immediately prior to calf inoculation. For clarity, this is the final inoculum for one calf. For each additional calf, the inoculum is identically prepared using a new disposable dosing syringe. Administer the inoculum immediately following the closure of the esophageal (gastric) groove as described above by orally inserting the dosing syringe so that the tip is at the base of the tongue, while the calf is still in the suckling response. With the head elevated to optimize the delivery of the entire inoculum, allow natural suckling of the syringe contents, facilitated by gradual manual expulsion. Administer the inoculum at a rate that does not allow any dripping from the calf's mouth (*see* **Note 16**). Reload the syringe once with ~25 mL milk replacer as a rinse and repeat the administration procedure. Immediately thereafter, feed each calf the calculated volume of milk replacer to meet daily dietary requirements as described above (Subheading 3.2, **step 3**) and to ensure that the full inoculum reaches the abomasum. Examine fecal samples for oocysts by IFA daily following infection to determine pre-patent and patent periods.

2. Initial administration of a given therapeutic candidate begins on DPI 2, at which time untreated *C. parvum* infection is established and nearing completion of one round of the life cycle at the infection dose used [19]. The candidate therapeutic must be prepared according to the institution's *Standard Operating Procedures for Preparation of Compounds for Use in Animals* and be approved by the IACUC prior to administration. The dosage and frequency of dosing for most candidate therapeutics in the calf model can be initially based on mg/kg extrapolation from the most efficacious dosage regimens identified in *C. parvum*-infected mice, allometric modeling in mice and rats, and ultimately pharmacokinetic/pharmacodynamic studies in uninfected and infected newborn calves [11]. Preliminary Pharmacokinetics/Absorption-Distribution-Metabolism-Elimination studies may be performed on treated calves in a given trial to evaluate a candidate therapeutic, if additional stress can be minimized, and control (vehicle only) calves are identically sampled. Of potential relevance to desirable pharmacokinetic/pharmacodynamic properties of candidate chemotherapeutics, it was recently shown that intestinal (vs systemic) exposure is essential for the efficacy of bumped kinase inhibitor drugs against *C. parvum* in mouse models [32]. Treat control calves identically for the regimen selected with vehicle alone. Code by label the therapeutic (in vehicle) being evaluated, and vehicle alone, to blind the treatment and control preparations. Just prior to each treatment, allow each calf to suckle milk replacer (~475 mL) from a nipple bottle to close the esophageal (gastric) groove. Then feed each calf by nipple bottle the candidate therapeutic in vehicle, or vehicle alone, mixed well with milk replacer in a final volume of ~475 mL. Following each treatment, feed each calf the calculated volume of milk replacer to meet daily dietary requirements as described above (Subheading 3.2, **step 3**). Record all treatments with the therapeutic or vehicle alone. Make potable water available to all calves by bucket ad libitum for the entire experiment. At the earliest onset of diarrhea, make an oral electrolyte solution available to each calf by bucket for the remainder of the experiment. At each feeding, record the quantity of water and oral electrolytes consumed from buckets since the previous feeding, clean both buckets, and fill each with fresh water or electrolytes.

**3.4 Stall Modifications for Accurate Evaluation of Diarrhea Severity, Parasite Shedding, and Urine Production in Infected Calves**

1. To ensure collection of all feces from individual calves for accurate total daily fecal volume quantitation and daily fecal oocyst count determinations, place three plastic fecal collection bins beneath the rear of each stall, held together by binder clips. Position fecal bins between the room wall and rear part of stall such that broad coverage is provided for all likely directions diarrhea may project (Fig. 1).

**Fig. 1** (**a**) Elevated calf stall depicting urine collection bin and plastic urine diversion sheet placement, viewed from front. (**b**) Stall depicting fecal collection bins and plastic urine diversion sheet placement, viewed from rear. (**c**, **d**) Newborn 41 kg Holstein bull calf in stall ~1 h following arrival at ABSL-2 facility, prior to infection

**Fig. 1** (continued)

**Fig. 1** (continued)

**Fig. 1** (continued)

2. Prevent contamination of feces with urine by using only bull calves (birth weight ≥38 kg); and a downward angled 6 mil plastic sheet spanning the underside of the full width of the stall floor, extending forward from the front of fecal collection bins, to divert urine into a separate bin positioned beneath the front of each stall (Fig. 1). Adjust the position of the plastic sheet if needed based on calf size so that urine is diverted but feces are allowed to pass into fecal collection bins. Tightly secure the plastic sheet to avoid leakage with reinforced water-proof industrial strength adhesive tape (Fig. 1) (*see* **Notes 6** and **17**). All collection bins and urine diversion shields are most conveniently set up the day prior to calf arrival.

3. Loose stools or diarrhea typically commence between 48 and 72 h PI in this model. Determine total fecal volume and fecal oocyst counts daily for each calf from 3 DPI until the end of the experiment as described in Subheading 3.6. Use extreme care and adequate personal protective equipment to prevent zoonotic infection (*see* **Notes 18** and **19**).

*3.5 Evaluation of Clinical Health, Diarrhea, and Oocyst Shedding in Infected Calves*

1. Once the study has commenced, collect diarrheic fecal samples from each calf (~30 mL/calf) and have them examined for the presence of enteropathogenic bacteria (K99+ *E. coli*, *Clostridium* spp. and *Salmonella* spp.). Perform these tests on fecal samples collected after two consecutive episodes of diarrhea. Immediately remove from the study and exclude from analyses any calves found positive for other enteropathogens which would confound interpretation of the experimental results. Collect additional fecal samples from all calves at the completion of the experiment and reexamine for presence of the enteropathogens above. Exclude any positive calves from analyses (*see* **Note 20**).

2. Commencing 3 DPI, for the duration of the experiment, have trained veterinary clinical staff assess clinical health parameters on each calf twice daily (every 12 h) and assign an objective numerical score for each parameter. Blind personnel involved in the study as to the identity (treatment or control) of calves (single-blind study). Assign and record individual scores to each calf for (1) fecal consistency, (2) willingness to rise, (3) stance when up in stall, (4) appetite, (5) attitude, and (6) hydration status [11, 12, 33] (*see* **Note 21**). Determine the mean daily clinical health score using individual parameter scores for each calf. Determine rectal temperature once daily. Contact the attending veterinarian on call if any of the following are observed: (1) pain and/or distress (grinding teeth, grunting, kicking at abdomen, abnormal vocalizations), (2) severe watery profuse diarrhea or bloody diarrhea, (3) moderate or severe dehydration, (4) high fever (>103.5 °F) or

hypothermia (<96 °F), (5) weight loss exceeding 10%, (6) depressed mental state, minimally responsive or otherwise appearing *in extremis*, (7) inability to stand, or (8) inability to suckle. In such cases, the attending veterinarian makes all decisions on appropriate supportive treatment(s) or whether euthanasia is required. Treat fever (≥103.5 °F) and/or symptoms of pain with palliative therapy such as injectable flunixin meglumine under direction of the attending veterinarian (*see* **Note 22**). Treat moderate dehydration at its very earliest onset with parenteral rehydration under direction of the attending veterinarian to prevent progression to severe dehydration (Table 1). Record the use of any concomitant medications and parenteral fluid therapy in the records for each calf.

3. As an additional index of hydration status, measure total daily urine output by each calf 3–10 DPI. Based on estimated volume in the urine collection bin for each calf, pour all urine into the appropriate size graduated cylinder to determine the actual volume of both AM and PM collections separately. Combine the volumes to determine the total daily urine volume for each calf and record the values. Disinfect and rinse the urine collection bin, then dry and place the bin back under the stall, positioned as previously.

4. On 3–10 DPI, measure and evaluate total daily fecal volume (mL) and consistency for each calf to accurately determine diarrhea severity and oocyst numbers produced daily (*see* **Note 23**). When caring for calves at AM and PM time points, complete fecal collection and assessment of clinical health parameters prior to feeding (*see* **Note 24**). Calves typically rise and stand prior to feeding, which facilitates fecal collection and clinical assessment (*see* **Note 25**). Measure the total daily fecal volume (combined AM and PM collections) just prior to the PM feeding as described in Subheading 3.6. Thoroughly clean and mop calf room floors with quaternary ammonium-based disinfectant after procedures are completed each AM and PM.

5. Accurately quantify the total number of oocysts shed per day (3–10 DPI) from each calf as follows. Using a commercial grade blender, thoroughly blend the total daily fecal collection from each calf to homogeneity and to ensure the release of oocysts trapped in any clumps of excess mucus (detailed in Subheading 3.6, **step 3**). Once homogenized, collect a 50 mL sample, representative of the total volume, and store at 4 °C for oocyst quantitation as described below (Subheading 3.8). Transport fecal samples from the ABSL-2 facility to the BSL-2 laboratory for analyses in a closed container labeled with a biohazard sticker.

**Table 1**
**Clinical health parameter evaluation and scoring**

| Score [a] | Fecal consistency | Willingness to rise | Stance when up | Appetite | Attitude | Hydration status[b] |
|---|---|---|---|---|---|---|
| 1 | Normal-formed stool | Normal-eager to rise or already up in stall | Normal-stable | Ravenous, consumes full ration, wants more | Normal-bright, alert, responsive | Normal-euhydrated, asymptomatic |
| 2 | Loose but not diarrheic | Willing to rise on own but needs encouragement or prodding | Somewhat unstable | Normal-consumes full ration, reaches satiety | Somewhat depressed, dull | Mild dehydration (~5%). Enophthalmos <3 mm. Skin tent duration <5 s. CRT 3–4 s. |
| 3 | Moderately diarrheic | Reluctant to rise but able with assistance | Clearly unstable, loses balance or sways | Decreased, but if coaxed will suckle | Clearly depressed, minimally responsive | Moderate dehydration (~6–8%). Skin tent duration 5–6 s. Enophthalmos 3–4 mm. CRT >4 s. |
| 4 | Severely diarrheic-watery and profuse | Unwilling or unable to rise on own, requires lifting | Unable to stand on own if lifted | Anorectic, will not suckle | | Severe dehydration (~9–12%). Enophthalmos 5–7 mm. Skin tent duration >6–>8 s. CRT >5 s. |

[a]Use consensus assessment by two trained veterinary clinical staff to determine the score for each parameter
[b]Determine hydration status based on lateral mid-cervical skin turgor (duration of skin tenting), severity of enophthalmos (distance between globe and palpebral conjunctiva of lower eyelid) [33] and gingival capillary refill time (CRT)

*3.6 Collection and Processing of Feces*

1. Using a scraper, collect any feces on the floor that may have missed the collection bins and place into the corresponding calf fecal bins. Use a plastic spatula to gently scrape any feces on the plastic urine diversion sheets taking care that no tears are created.

2. Based on estimated volume in the three collection pans for each calf, pour all feces into the appropriate size graduated cylinder to determine the actual volume of both AM and PM collections, separately. Just prior to the PM feeding, combine the AM and PM fecal collections to determine the total daily fecal volume for each calf and record this value. Disinfect and

rinse out fecal collection bins. Dry the bins and place them back under the stall, positioned as previously, or adjusted if needed.

3. Blend to homogeneity all feces collected over successive 24 h periods from each calf in a commercial blender. Blend on the low speed setting for 5 min with the blender lid locked on securely. Place the blender on the floor undisturbed for ~5 min before unlocking and removing the lid to reduce aerosol risks. If the total daily fecal volume for any calf is near or greater than the 4 L blender capacity, split the volume in two and blend each separately as described above. After blending, then thoroughly mix the two using two 4 L size buckets and pouring back and forth at least five times. Immediately following completion of blending/mixing steps, collect a representative ~50 mL fecal sample from the mid-point of the container using a ladle, and place the sample into a fecal specimen container (urinalysis cup) pre-labeled with date and calf #. The blending and mixing processes create aerosols. Take extreme precautions to avoid zoonotic infection.

4. Wash the blender thoroughly with hot water and dish detergent followed by disinfection and rinse well between processing feces from each calf.

*3.7 Terminal Procedures and Euthanasia (see Note 26)*

1. At 10 DPI, following the AM clinical health, diarrhea and oocyst shedding evaluations described above (Subheading 3.5), sedate each calf by intramuscular injection of xylazine (3 mg/kg) combined with ketamine (100 mg/calf). Following sedation, collect blood samples from each calf into appropriate tubes for performance of CBCs and serum chemistry panels to evaluate clinical pathology and screen for potential toxicity of the test therapeutic.

2. Euthanize each sedated calf by intracardiac injection of euthanasia solution (1 mL/10 lb; 1 mL/4.5 kg). This method (Subheading 3.7, **steps 1** and **2**) is in accordance with the AVMA Guidelines on Euthanasia and euthanasia recommendations approved by the local IACUC. If clinically indicated before 10 DPI, as determined by the attending veterinarian, use the same euthanasia procedure.

3. Following euthanasia, weigh each calf for comparison to birth weight to compute average body weight gain or loss.

*3.8 Determination of Daily Fecal Oocyst Counts from Infected Calves*

Determine total daily fecal oocyst counts by real-time PCR (qPCR) as described previously [11, 34]. This step is typically performed following completion of a calf trial.

1. For each fecal sample, extract oocyst DNA using the QIAamp® Fast DNA stool mini kit (Qiagen, Gaithersburg, MD). Add

InhibitEx buffer, incubate at 95 °C (5 min), then perform 5 freeze-thaw cycles. Quantify total DNA in the sample by Nanodrop® (Nanodrop Technologies, Wilmington, DE). We use the *C. parvum* oocyst wall protein gene (COWP) (Genbank no. AF248743) as the target for primers (COWPF, 5′-CAAATTGATACCGTTTGTCCTTCTG-3′; COWPR, 5′-AGCTGAATTAGAATCGACATGCC-3′) and selected a 151 bp sequence from the COWP gene. For each qPCR, prepare a 25 μL reaction containing 400 nM (final concentration) for each primer, 12.5 μL SYBR-green or similar nucleic acid stain and 0.1 ng/μL bovine serum albumin. Add genomic DNA (2 μL) and perform the PCR in a Real-Time PCR System. Use cycling conditions of 10 min incubation at 95 °C followed by 50 cycles at 95 °C for 15 s and 60 °C for 1 min and a final extension at 72 °C for 1 min. Run each sample in triplicate. Concurrently run a control with no template and verify it to be negative. To determine the total number of oocysts shed per day, multiply the oocyst count/mL derived by comparison to standards run concurrently during the qPCR, times the total daily fecal volume (mL).

*3.9 Statistical Analyses*

For statistical analyses, examine all calves given the same treatment (therapeutic candidate or vehicle alone control) as one group for evaluation of all outcome parameter means. Examine clinical health scores, total fecal volumes, total oocyst counts, total urine production, and body weight gain/loss for statistically significant differences using Student's *t* test or another preferred statistically valid method. Additional or more powerful statistical analyses are usually not necessary to validate clinically acceptable efficacy of candidate therapeutics.

# 4   Notes

1. Diurnal variations were observed in ten neonatal Holstein bull calves we used previously for oocyst propagation/isolation, obtained, maintained, and housed as described above and identically infected with $5 \times 10^7$ *C. parvum* oocysts (Iowa) at 36–48 h of age [11]. In these propagation calves, total AM and total PM feces excreted by each calf were collected separately each day, to prioritize and exclude any potential low count collections from laborious oocyst purification procedures. Using the oocyst quantitation methods described in Subheading 3, but before we adopted the use of a blender to homogenize feces, we observed that oocyst counts per gram of feces in AM and PM collections from an individual calf could vary by ten-fold or more on some days, up to 46-fold in one calf (Fig. 2a, b). We also observed grossly that calves
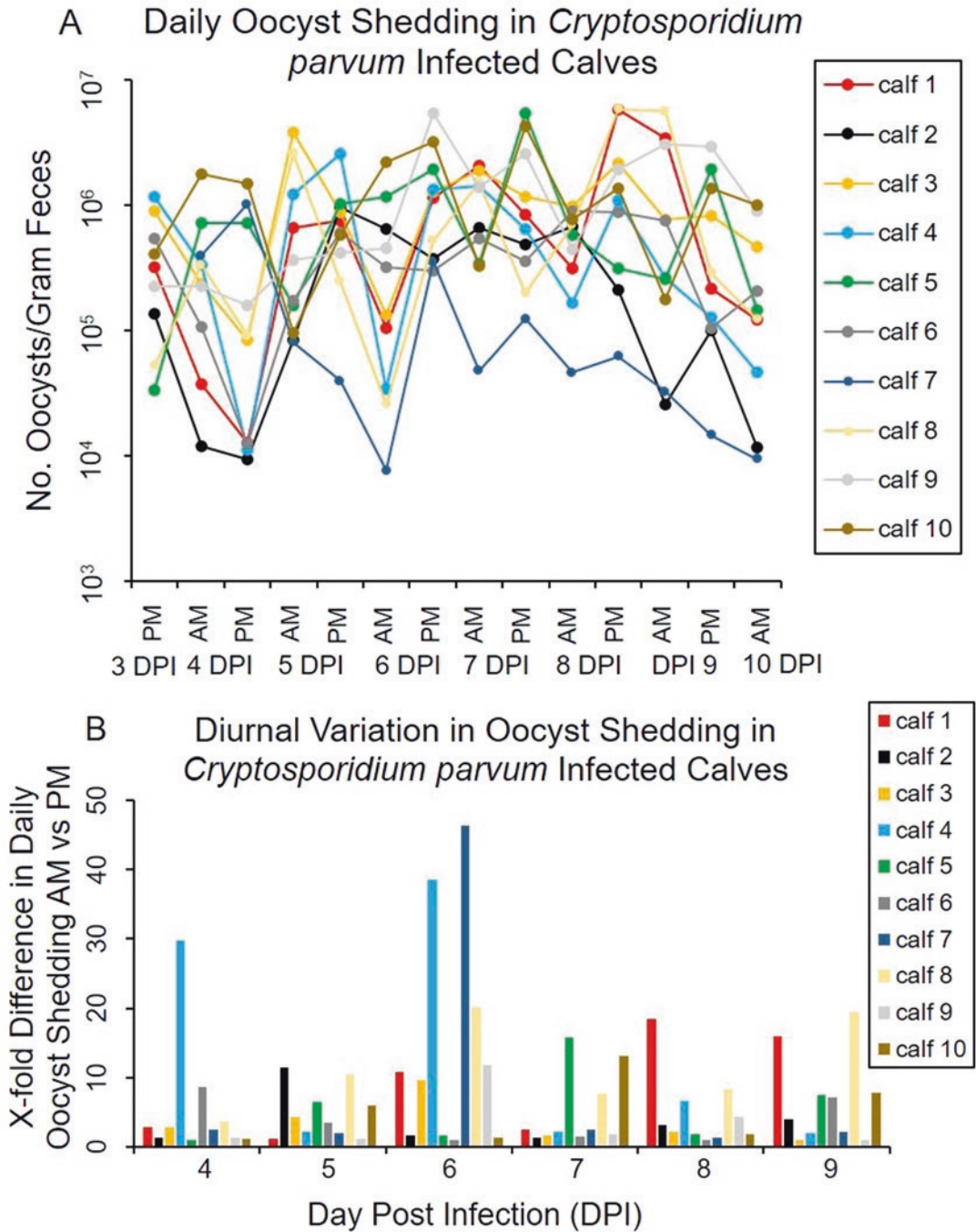
**Fig. 2** Diurnal variation in oocyst shedding in *C. parvum*-infected calves. (**a**) Total AM and total PM feces excreted by each calf were collected separately each day for oocyst quantitation. (**b**) AM and PM oocyst counts per gram of feces from individual calves varied by 10-fold or more on some days, up to 46-fold in one calf. Reproduced in part (data for calves 1–5) from [11] by permission of Oxford University Press. Additional calf data demonstrating diurnal variation in oocyst shedding in identically infected calves used for *C. parvum* oocyst propagation (calves 6–10) are newly reported here

produced highly variable amounts of mucus in feces. We observed microscopically that large numbers (wall to wall sheets) of oocysts were entrapped in mucus in such cases, while more watery fecal components were often only sparsely populated with oocysts. Whether the observed oocyst count variations in AM vs PM collections represent true diurnal variation or skewing of oocyst counts by entrapment of oocysts in fecal mucus produced by some calves is unclear. Nevertheless, based on these observations, we have since used uniformly blended <u>total</u> daily fecal collections (AM and PM combined) to obtain a representative sample for accurate oocyst quantitation. An industrial grade blender is used to ensure that oocysts trapped in clumps of mucus are released and that daily fecal collections from each calf are thoroughly blended to homogeneity before taking a representative sample for oocyst quantitation (# oocysts/mL) and back calculation using total fecal volume (mL) to determine the total number of oocysts shed per day.

2. Peracetic acid is highly toxic and explosive. Follow all precautions stated in the MSDS.

3. Use % excystation to adjust the number of oocysts in the inoculum to deliver $5 \times 10^7$ viable oocysts/calf. For example, if percent excystation is 94%, adjust the oocyst inoculum dose/calf from $5 \times 10^7$ to $5.3 \times 10^7$.

4. Occasionally, the desired number of age-matched calves for a cohort cannot be obtained (such as six calves total to provide three calves each for the treatment and control groups). If only 3–5 age-matched calves are obtained, the cohort can proceed, as long as one calf is a control (vehicle alone).

5. Dairy facilities vary in their willingness to allow laboratory personnel to manually assist delivery of calves on-site because of biosecurity and/or liability concerns for potential personal injury. In such cases, accept only calves for which dairy personnel have manually assisted delivery onto the plastic sheet barrier, then placed into a disinfected wheelbarrow for transport to the designated disinfected concrete area where laboratory personnel can determine acceptability and process acceptable calves further. If there is any inadvertent minimal soiling of otherwise acceptable calves, calves are thoroughly cleaned with 7% iodine scrub and towel dried.

6. Calves have room to rise/lie down and move/maneuver within the stall and may be able to turn completely around and reverse their position in the stall. This is most likely to occur if smaller calves <32 kg are used, during the first few days acclimating to stalls, prior to infection and onset of diarrhea. If calves turn themselves around in the stall such that their

posterior is at the front of the stall, reposition them properly. However, in such cases, most of the feces excreted during the previous 12-h period will have ended up in the urine collection bin. If the feces are normal or loose but not diarrheic, urine tends to float on top of the feces. In such cases, carefully pour or aspirate off urine to allow fecal collection. If the feces are diarrheic, there is mixing with urine and the collection is essentially lost for evaluation and noted in the record as such. However, we have rarely (~1 in 50 calves) observed calves turning around in the stall when larger calves ≥38 kg are used, and this is the primary reason calves <38 kg are excluded. Needless to say but stated to ensure absolute clarity, <u>never</u> use the headlock between 12-h periods to prevent calves from turning around in the stall. This would be grossly inhumane and constitute animal abuse, prevent the calf from laying down, and result in death from asphyxiation if the calf is unable to stand. Also never use the front head gate bars to headlock a calf for more than ~5 min, if at all. The headlock is intended strictly to facilitate brief restraint for blood draws, etc.

7. Some calves suckle so intensely that a vacuum may form in the bottle during nursing, eventually precluding additional ingestion. Only use nipple bottles that incorporate a ~1 mm diameter air release valve port at the nipple base to allow air to enter the bottle as the calf is nursing, thus precluding vacuum formation. If necessary, massage or press next to the valve port to release any vacuum. After feeding is completed verify that the valve is not plugged due to inadequate cleaning and is working properly.

8. Colostrum replacer powder does not go into solution easily. To thoroughly reconstitute and solubilize colostrum replacer, gradually add the specified amount of powder per calf (twice the manufacturer's recommended dose, or 2 packets totaling 940 g powder) to a 6-L bucket containing 2 L of clean tap water at 110–120 °F using a manual whisk. It is critical to whisk the preparation during reconstitution until no visible clumps remain. Following consumption of the full dose of colostrum replacer, add ~250 mL clean warm water to the same nipple bottle, swirl vigorously to rinse residual colostrum replacer, and feed this to the calf to ensure complete dosing.

9. Bovine colostrum contains variable titers of antibody reactive with *C. parvum*, acquired from natural exposure or previous infection. However, no significant <u>neutralizing</u> antibody is present following previous infection as normal colostrum-fed calves are readily infected with *C. parvum*. Nevertheless, while expensive, commercial colostrum replacer is used to eliminate this variable in entirety. A commercial colostrum replacer such as the one we use has been formulated to replace maternal

colostrum and negate lot to lot and inherent variability found in source dairy-acquired colostrum (bovine IgG colostrum replacer, Land O'Lakes, Shoreview, MN). Frozen whole colostrum acquired from most source dairies varies substantially in antibody content, quality, and microbial contamination load, and should be avoided.

10. Calves are born agammaglobulinemic. They require colostral antibodies following birth for passive transfer of immunity against the major enteropathogenic bacteria and viruses responsible for neonatal diarrhea and mortality for which commercially available maternal vaccines exist (there is presently no commercially available maternal vaccine for *Cryptosporidium*). The newborn calf's ability to absorb ingested colostral antibody decreases linearly over the first 24 h of age during the process of gut closure. By 12 h of age, the reduction is substantial and by 24 h of age, the gut is essentially closed to absorption of colostral antibody and other macromolecules. For this reason, ensure that calves suckle the majority of the recommended colostrum replacer dose, preferably within 6 h after birth but no later than 9 h after birth. Feed any colostrum replacer remaining at 9 h after birth by esophageal intubation.

11. Feeding colostrum replacer by esophageal tube should be used as a last resort due to the fact that intubating (unlike suckling) does not stimulate the esophageal (gastric) groove closure reflex and thus delivers replacer into the rumen instead of the abomasum [28].

12. Adjust the width of the stall's front head gate bars to allow only the head to have free access through the bars. This will allow calves to easily access water and/or electrolyte buckets, while also preventing a calf from jumping through the front of the stall and potentially getting trapped or injured. This potential problem is uncommon and typically occurs only if smaller calves <32 kg are used and the head gate bars are improperly positioned. We have never observed this problem using calves ≥38 kg with head gate bars positioned properly. Other than bruising acquired by two small 30–32 kg calves jumping through the front of the stall, we have also never had a calf acquire any injury being housed in elevated stalls. Nevertheless, it is important after assembling newly purchased stalls to file down any sharp or jagged edges on the framing or exposed hardware. Periodically inspect stalls for the same and to ensure that all hardware fasteners are securely tightened to maintain stall stability. Periodically inspect the rubberized flooring for wear and deterioration from use of harsh disinfectants and replace when necessary.

13. Similar to colostrum replacer, milk replacer powder does not go into solution easily. Use warm (110–120 °F) water and a

whisk to thoroughly reconstitute replacer until all clumps are fully dissolved.

14. Feed milk replacer via esophageal tube as a last resort measure for calves which will not consume any of the calculated volume of replacer in a ~25–30-min feeding period. The suckle response resulting in esophageal (gastric) groove closure is voided by tube feeding, delivering milk replacer into the rumen. Additionally, in our experience, calves which are tube fed more than ~3–4 times are reluctant to feed from a nipple bottle subsequently.

15. Failure of passive transfer (FPT) of maternal immunoglobulins is rare if commercial colostrum replacer (at twice the label dose) is suckled within 6-9 h of birth. However, if FPT occurs, but the calf is otherwise healthy at 24 h of age, treat it by intravenous transfusion of commercially available adult bovine plasma (Lake Immunogenics, Ontario, NY) administered under supervision by a veterinarian.

16. We have never experienced a calf not receiving the entire oocyst inoculum using the method described, but it could potentially happen (leakage, expectoration). If this occurs, note in the records the estimated volume of inoculum not received and that data from the calf may not be suitable for use in final analyses.

17. It is potentially possible that some urine may enter the fecal collection bins, voiding accurate fecal volume determination. This possibility is largely eliminated if the plastic diversion sheet is stretched tightly against the bottom at the rear third of the stall and properly secured with reinforced waterproof industrial strength adhesive tape. This ensures that urine flows only in one direction, toward the front of the stall and into the urine collection bin. This is also a secondary reason calves <38 kg are excluded, as smaller calves can move back and forth in the stall to a greater extent and make accurate placement of the plastic diversion sheet difficult, especially for calves <32 kg.

18. Calves infected with *C. parvum* in this model shed extremely large numbers of oocysts, especially the untreated controls. A single experimentally infected newborn calf may shed >$10^{12}$ oocysts in feces during the peak days of diarrhea [11, 13]. Thus, personnel caring for calves are at high risk of zoonotic infection unless appropriate precautions are taken and adequate personal protective equipment is used while in calf rooms, minimally (1) disposable double gloves, (2) mid-calf high plastic or rubber boots over disposable plastic shoe covers, (3) Tyvek coveralls with elastic cinched ankle and wrist cuffs, (4) N95 respirators, (5) disposable hair bonnets, completely covering all hair (long hair must be tied back into a bun), and (6) goggles, safety glasses, or full face shields.

19. An institutional protocol should be in place for evaluation of any calf personnel which develop nausea, vomiting, or diarrhea during or after a trial to determine the etiology and appropriate treatment. We use the University's Occupational Health Division for independent assessment of ill personnel and compliance with HIPPA. If *C. parvum* is identified as the etiologic agent the experimental protocol should be carefully reviewed to determine where the breach leading to zoonotic transmission likely occurred so that it can be addressed. Despite the precautions described here, we have had at least one documented case of zoonotic transmission.

20. Adhering closely to the methods described here for calf procurement, we have never had a calf inadvertently infected with a field isolate of *C. parvum*, and have had only four calves in the last ~100 used inadvertently infected with field acquired enteropathogenic bacteria.

21. We use a standardized daily AM/PM form for each calf that has all health evaluation parameters listed along with the scoring scale for each to record consensus scores; rectal temperature; amount of milk replacer, water and/or oral electrolytes consumed; date and actual AM/PM times (h, min) data were recorded, and calf ear tag #, with a signature box for each of the two attending staff involved (Daily Health Observation Record). Standardized forms are also used for all other information described in methods for which it is stated that records are made (Newborn Calf Record, *Cryptosporidium* Infection Record, Daily Fecal Volume Record, Test-Therapeutic Dosing Record, Concomitant Treatment Record, and Condition(s) Requiring Examination by the Attending Veterinarian Record).

22. The nonsteroidal anti-inflammatory drug flunixin meglumine should be used sparingly, and only when absolutely needed. No more than three doses total during a trial period should be administered to a given experimental calf to avoid nephrotoxicity and gastrointestinal toxicity. Toxicity is of greatest concern when administered to dehydrated calves.

23. Fecal dry matter percentages may optionally be determined on total daily fecal collections as an additional index of diarrhea severity. However, in the model presented here, we found previously that fecal dry matter percentages added little to fecal consistency scores and total daily fecal volumes in determining diarrhea severity and have since discontinued percent dry matter determinations.

24. Calves with diarrhea are prone to cutaneous scalding in the perianal and tail base regions. Use human infant sanitary wipes or warm wet paper towels to gently clean these regions as needed. If indicated, apply generic human infant diaper rash

ointment liberally after cleaning the region. In such cases, this should be done prior to feeding or taking rectal temperature, both of which stimulate defecation.

25. When calves are reluctant but able to stand for evaluation and feeding, rapidly and repeatedly stroke the hair coat from the hip region toward the shoulders. This is usually effective in getting the calf to stand. Alternatively, grasp the hip region and gently rock the calf back and forth laterally to stimulate initiation of the standing motion.

26. The drugs used for sedation and euthanasia are controlled (scheduled) substances. A DEA license is required to obtain these. If the investigator does not have a DEA license, obtain the drugs through the attending veterinarian and comply with all record keeping and other requirements.

## Acknowledgments

## References

1. Tzipori S, Widmer G (2008) A hundred-year retrospective on cryptosporidiosis. Trends Parasitol 24:184–189. https://doi.org/10.1016/j.pt.2008.01.002

2. Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, Panchalingam S, Wu Y, Sow SO, Sur D, Breiman RF, Faruque AS, Zaidi AK, Saha D, Alonso PL, Tamboura B, Sanogo D, Onwuchekwa U, Manna B, Ramamurthy T, Kanungo S, Ochieng JB, Omore R, Oundo JO, Hossain A, Das SK, Ahmed S, Qureshi S, Quadri F, Adegbola RA, Antonio M, Hossain MJ, Akinsola A, Mandomando I, Nhampossa T, Acacio S, Biswas K, O'Reilly CE, Mintz ED, Berkeley LY, Muhsen K, Sommerfelt H, Robins-Browne RM, Levine MM (2013) Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. Lancet 382:209–222. https://doi.org/10.1016/S0140-6736(13)60844-2

3. Wyatt CR, Riggs MW, Fayer R (2010) Cryptosporidiosis in neonatal calves. Vet Clin North Am Food Anim Pract 26:89–103

4. Stockdale HD, Spencer JA, Blagburn BL (2008) Prophylaxis and chemotherapy. In: Fayer R, Xiao L (eds) *Cryptosporidium* and cryptosporidiosis, 2nd edn. CRC Press, Boca Raton, FL

5. Cabada MM, White AC (2010) Treatment of cryptosporidiosis: do we know what we think we know? Curr Opin Infect Dis 23:494–499

6. Amadi B, Mwiya M, Sianongo S, Payne L, Watuka A, Katubulushi M, Kelly P (2009) High dose prolonged treatment with nitazoxanide is not effective for cryptosporidiosis in HIV positive Zambian children: a randomised controlled trial. BMC Infect Dis 9:195. https://doi.org/10.1186/1471-2334-9-195

7. Amadi B, Mwiya M, Musuku J, Watuka A, Sianongo S, Ayoub A, Kelly P (2002) Effect of nitazoxanide on morbidity and mortality in Zambian children with cryptosporidiosis: a randomised controlled trial. Lancet 360:1375–1380. https://doi.org/10.1016/S0140-6736(02)11401-2

8. Santin M, Trout JM (2008) Livestock. In: Fayer R, Xiao L (eds) *Cryptosporidium* and cryptosporidiosis, 2nd edn. CRC Press, Boca Raton, FL

9. Heinrichs AJ (2005) Rumen development in the dairy calf. Adv Dairy Technol 17:179–188

10. Cirle A, Guerrant RL (2008) Clinical disease and pathology. In: Fayer R, Xiao L (eds) *Cryptosporidium* and cryptosporidiosis, 2nd edn. CRC Press, Boca Raton, FL

11. Schaefer DA, Betzer DP, Smith KD, Millman ZG, Michalski HC, Menchaca SE, Zambriski JA, Ojo KK, Hulverson MA, Arnold SL, Rivas KL, Vidadala RS, Huang W, Barrett LK, Maly DJ, Fan E, Van Voorhis WC, Riggs MW (2016) Novel bumped kinase inhibitors are safe and effective therapeutics in the calf clinical model for cryptosporidiosis. J Infect Dis 15(214):1856–1864. https://doi.org/10.1093/infdis/jiw488

12. Imboden M, Schaefer DA, Bremel RD, Homan EJ, Riggs MW (2012) Antibody fusions reduce onset of experimental *Cryptosporidium parvum* infection in calves. Vet Parasitol 188(1–2):41–47. https://doi.org/10.1016/j.vetpar.2012.02.014

13. Perryman LE, Kapil SJ, Jones ML, Hunt EL (1999) Protection of calves against cryptosporidiosis with immune bovine colostrum induced by a *Cryptosporidium parvum* recombinant protein. Vaccine 17:2142–2149

14. Ollivett TL, Nydam DV, Bowman DD, Zambriski JA, Bellosa ML, Linden TC, Divers TJ (2009) Effect of nitazoxanide on cryptosporidiosis in experimentally infected neonatal dairy calves. J Dairy Sci 92:1643–1648. https://doi.org/10.3168/jds.2008-1474

15. Schnyder ML, Kohler L, Hemphill A, Deplazes P (2009) Prophylactic and therapeutic efficacy of nitazoxanide against *Cryptosporidium parvum* in experimentally challenged neonatal calves. Vet Parasitol 160:149–154. https://doi.org/10.1016/j.vetpar.2008.10.094

16. Fayer R, Ellis W (1993) Paromomycin is effective as prophylaxis for cryptosporidiosis in dairy calves. J Parasitol 79:771–774

17. Hulverson MA, Choi R, Arnold SLM, Schaefer DA, Hemphill A, McCloskey MC, Betzer DP, Muller J, Vidadala RSR, Whitman GR, Rivas KL, Barrett LK, Hackman RC, Love MS, McNamara CW, Shaughnessy TK, Kondratiuk A, Kurnick M, Banfor PN, Lynch JJ, Freiberg GM, Kempf DJ, Maly DJ, Riggs MW, Ojo KK, Van Voorhis WC (2017) Advances in bumped kinase inhibitors for human and animal therapy for cryptosporidiosis. Int J Parasitol 47:753–763. https://doi.org/10.1016/j.ijpara.2017.08.006

18. Okhuysen PC, Chappell CL, Crabb J, Valdez LM, Douglass ET, DuPont HL (1998) Prophylactic effect of bovine anti-*Cryptosporidium* hyperimmune colostrum immunoglobulin in healthy volunteers challenged with *Cryptosporidium parvum*. Clin Infect Dis 26:1324–1329

19. Fayer R (2008) The general biology of *Cryptosporidium*. In: Fayer R, Xiao L (eds) *Cryptosporidium* and cryptosporidiosis, 2nd edn. CRC Press, Boca Raton, FL

20. Riggs MW (1987) Dissertation, Washington State University, Pullman, WA

21. Schaefer DA, Auerbach-Dixon BA, Riggs MW (2000) Characterization and formulation of multiple epitope-specific neutralizing monoclonal antibodies for passive immunization against cryptosporidiosis. Infect Immun 68:2608–2616. https://doi.org/10.1128/IAI.68.5.2608-2616.2000

22. Perryman LE, Jasmer DP, Riggs MW, Bohnet SG, McGuire TC, Arrowood MJ (1996) A cloned gene of *Cryptosporidium parvum* encodes neutralization-sensitive epitopes. Mol Biochem Parasitol 80:137–147

23. Heine J, Pohlenz JF, Moon HW, Woode GN (1984) Enteric lesions and diarrhea in gnotobiotic calves monoinfected with *Cryptosporidium species*. J Infect Dis 150:768–775

24. Abrahamsen MS, Templeton TJ, Enomoto S, Abrahante JE, Zhu G, Lancto CA, Deng M, Liu C, Widmer G, Tzipori S, Buck GA, Xu P, Bankier AT, Dear PH, Konfortov BA, Spriggs HF, Iyer L, Anantharaman V, Aravind L, Kapur V (2004) Complete genome sequence of the apicomplexan, *Cryptosporidium parvum*. Science 304:441–445

25. Riggs MW, Perryman LE (1987) Infectivity and neutralization of *Cryptosporidium parvum* sporozoites. Infect Immun 55:2081–2087

26. Riggs MW, Stone AL, Yount PA, Langer RC, Arrowood MJ, Bentley DL (1997) Protective monoclonal antibody defines a circumsporozoite-like glycoprotein exoantigen of *Cryptosporidium parvum* sporozoites and merozoites. J Immunol 158:1787–1795

27. Riggs MW, Cama VA, Leary JR, Sterling CR (1994) Bovine antibody against *Cryptosporidium parvum* elicits a circumsporozoite precipitate-like reaction and has immunotherapeutic effect against persistent cryptosporidiosis in SCID mice. Infect Immun 62:1927–1939

28. Jones CM, Heinrichs AJ (2003) Feeding the newborn dairy calf. In: Agricultural Research and Cooperative Extension Publications. Pennsylvania State University, State College, PA. Available at: https://extension.psu.edu/feeding-the-newborn-dairy-calf

29. Morrill KM, Polo J, Lago A, Campbell J, Quigley J, Tyler H (2013) Estimate of serum

immunoglobulin G concentration using refractometry with or without caprylic acid fractionation. J Dairy Sci 96:4535–4541. https://doi.org/10.3168/jds.2012-5843

30. Hernandez D, Nydam DV, Godden SM, Bristol LS, Kryzer A, Ranum J, Schaefer D (2016) Brix refractometry in serum as a measure of failure of passive transfer compared to measured immunoglobulin G and total protein by refractometry in serum from dairy calves. Vet J 211:82–87

31. Riggs MW, Schaefer DA, Kapil SJ, Barley-Maloney L, Perryman LE (2002) Efficacy of monoclonal antibodies against defined antigens for passive immunotherapy of chronic gastrointestinal cryptosporidiosis. Antimicrob Agents Chemother 46:275–282. https://doi.org/10.1128/AAC.46.2.275-282.2002

32. Arnold SLM, Choi R, Hulverson MA, Schaefer DA, Vinayak S, Vidadala RSR, McCloskey MC, Whitman GR, Huang W, Barrett LK, Ojo KK, Fan E, Maly DJ, Riggs MW, Striepen B, Van Voorhis WC (2017) Necessity of bumped kinase inhibitor gastrointestinal exposure in treating *Cryptosporidium* infection. J Infect Dis 216:55–63

33. Constable PD, Walker PG, Morin DE, Foreman JH (1998) Clinical and laboratory assessment of hydration status of neonatal calves with diarrhea. J Am Vet Med Assoc 212:991–996

34. Guy RA, Payment P, Krull UJ, Horgen PA (2003) Real-time PCR for quantification of *Giardia* and *Cryptosporidium* in environmental water samples and sewage. Appl Environ Microbiol 69:5178–5185. https://doi.org/10.1128/AEM.69.9.5178-5185.2003

# Chapter 16

# Cell Culture Infectivity to Assess Chlorine Disinfection of *Cryptosporidium* Oocysts in Water

**Jennifer L. Murphy and Michael J. Arrowood**

## Abstract

This chapter provides a detailed protocol to assess disinfection efficacy of chlorine against *Cryptosporidium* oocysts including the core chlorine disinfection assay, the in vitro cell culture infectivity assay, and microscopy analysis and data interpretation.

**Key words** Cell culture infectivity, Chlorine, Disinfection, In vitro culture, *Cryptosporidium*, Oocysts

## 1   Introduction

*Cryptosporidium* is a leading cause of waterborne disease in both developing [1, 2] and industrialized [3–5] countries. Due to a robust outer oocyst wall, *Cryptosporidium* oocysts are extremely tolerant to many chemical disinfectants, including chlorine, one of the most widely used water treatment chemicals in the world. Laboratory experiments have assessed the efficacy of a number of chlorine-based disinfectants, including sodium hypochlorite [6–9], stabilized chlorine [10, 11], monochloramine [7, 12], mixed oxidants [9, 13], and chlorine dioxide [7, 14–18]. A variety of methods were used to assess inactivation of *Cryptosporidium* in these disinfection studies, including animal infectivity [7, 9, 15, 18], in vitro excystation [12, 17], cell culture-quantitative polymerase chain reaction [13], and in vitro cell culture infectivity [8, 10, 11, 14, 16, 19]. This chapter provides a detailed protocol we have used to assess efficacy of chlorine against *Cryptosporidium* oocysts including the core chlorine disinfection assay, the in vitro cell culture infectivity assay, and microscopy analysis and data interpretation. These methods can be used as a basis for the design of studies to assess efficacy of new or existing disinfectants, either alone or in combination.

## 2  Materials

1. MDCK (NBL-2) (ATCC CCL-34) Madin Darby canine kidney cells (*see* **Note 1**).

2. Water bath (37 °C).

3. 5% $CO_2$ incubators (37 °C) (one for routine cell passaging, one for infectivity assay).

4. DMEM growth medium: DMEM base (high glucose), 10% heat-inactivated fetal bovine serum, 1× sodium pyruvate, 1× L-glutamine, 1× MEM non-essential amino acids, 0.01 M HEPES buffer, 1× penicillin-streptomycin antibiotic mix, ~0.1% sodium bicarbonate.

5. UltraCULTURE™ synthetic culture medium (Lonza Bioscience, Walkersville, MD).

6. Vacuum aspirator flasks (2 or 4 L) with vacuum flask traps (typically 1 L or 500 mL) outfitted with disposable glass Pasteur pipets connected via silicone tubing.

7. Hydrophobic in-line filter for vacuum aspirator to protect vacuum system from contamination.

8. Micropipettes and tips, including aerosol-barrier tips.

9. Automatic pipet aid (line or battery powered).

10. 1, 5, 10, 50, and 100 mL disposable pipets.

11. 50 mL polypropylene conical tubes.

12. Phosphate-buffered saline (PBS): 0.01 M, pH 7.2.

13. PBS (0.01 M, pH 7.2) supplemented with 0.1% (w/v) bovine serum albumin (BSA).

14. PBS (0.01 M pH 7.2), 0.1% bovine serum albumin (BSA), supplemented with sodium thiosulfate at 50 mg/L per 1 mg/L free chlorine neutralization.

15. Trypsin 1×: ~0.25% w/v trypsin, ~0.5 mM EDTA in PBS for dissociating cells during routine culture.

16. Versene: ~0.5 mM EDTA in PBS (monolayer rinse before trypsinization).

17. T75 (75 cm²) vented or non-vented tissue culture flasks.

18. SensoPlate™ 24-well flat-bottomed microplates with lids (Greiner Bio-One North America, Inc.) with confluent MDCK cell monolayers (*see* Subheading 3.1).

19. Digital colorimeter or spectrophotometer for chlorine determination.

20. DPD 1 reagent (*N*,*N*-diethyl-*p*-phenylenediamine: tablets or pillows) for chlorine determination.

21. Timer.

22. pH meter.

23. Environmental chamber (or recirculating water bath) with multi-position magnetic stir plate.

24. Sodium hypochlorite solution: ~5.65% w/v (laboratory grade).

25. Chlorine demand-free (CDF) water (*see* **Note 2**).

26. Chlorine demand-free (CDF) glassware and magnetic stir bars (*see* **Note 3**).

27. *Cryptosporidium parvum* oocyst stock suspension (*see* **Note 4**).

28. Potassium dichromate: 2.5% w/v aqueous solution.

29. Hemocytometer or Neubauer chamber and coverslips.

30. Vortex mixer.

31. Sodium thiosulfate solution: 10% w/v in distilled water.

32. Refrigerated tabletop centrifuge (should reach $3200 \times g$ or more).

33. Refrigerated microcentrifuge (should reach $16,000 \times g$ or more).

34. Flame (ethanol or butane).

35. 1.5 mL microcentrifuge tubes, low-binding (*see* **Note 5**).

36. Refrigerator (4 °C).

37. Class II biosafety cabinet (BSC).

38. DMEM or UltraCULTURE base medium, plus 0.75% synthetic sodium taurocholate (to promote excystation).

39. Bouin's fixative solution.

40. 70% ethyl alcohol (v/v, prepared from 200 proof anhydrous non-denatured ethanol and deionized water).

41. *Cryptosporidium*-specific monoclonal antibody C3C3 (*see* **Note 6**).

42. 4′,6-diamidino-2-phenylindole (DAPI): 1000× frozen stock solution: 1 mg/mL in sterile distilled water, stored at −20 °C; working solution: dilute 1000× stock into PBS (0.01 M, pH 7.2) to yield 1.0–0.5 μg/mL (i.e., 1/1000 to 1/2000 dilution).

43. Platform shakers (rocking, rotary, and/or orbital wave motion).

44. Goat anti-mouse IgG (H + L) secondary antibody, conjugated with fluorescein isothiocyanate (FITC) or cyanine (Cy3), or similar fluorochromes (e.g., Alexa 555) supplemented with 2 mM sodium azide.

45. Aluminum foil.

46. Coverslip mounting medium (PVA/DABCO) with fluorescence anti-quenching agent (*see* **Note 7** for recipe and preparation instructions).

47. 15 mm #1 round glass coverslips.

48. Suction pen (vacuum sucking pen with silicone tips for manipulating coverslips).

49. Inverted microscope for routine cell culture applications.

50. Inverted epifluorescence microscope with digital camera (*see* **Note 8**).

51. Zeiss AxioVert 200 M microscope (or similar) (Carl Zeiss, Inc., Thornwood, NY).

52. Zeiss HRm digital camera (or similar) (Carl Zeiss).

53. Zeiss AxioVision software (or similar software for capturing images) (Carl Zeiss).

54. ImageJ software (for image analysis and parasite quantification [20]).

55. Microsoft Excel (for data analysis).

## 3  Methods (*See* Note 9)

*3.1  MDCK Cell Maintenance (Twice Weekly Subculture)*

1. Warm all reagents in a 37 °C water bath (or incubator) prior to splitting cells.

2. Aspirate media from T75 culture flask containing confluent MDCK cells (typically ~3.5 days old).

3. Rinse the cell monolayer with 10 mL PBS. Aspirate rinsate.

4. Flood cell monolayer with 7 mL Versene, cap flask, gently rock the flask to get complete coverage of the monolayer, and incubate at 37 °C for 10 min. Aspirate Versene.

5. Add 3 mL trypsin with EDTA. Gently rock the flask to get complete coverage of the monolayer.

6. Incubate the flask in a 5% $CO_2$ incubator at 37 °C for up to 30 min, or until monolayer visibly dissociates from the flask. It might be necessary to dislodge some cells with the tip of pipet (triturate) or cell scraper.

7. Add 7 mL DMEM growth medium to cells.

8. Use a pipet to triturate cells ~10 times (or more to disperse cells into suspension).

9. Remove 9 mL of the cell/media suspension in the T75 flask. This will serve as the backup flask.

10. Transfer 1 mL of the cell-media suspension to a new T75 flask, discard remainder.

11. Add 15 mL of fresh DMEM growth medium to each flask.

12. Tighten (if vented flask) or loosely close (if non-vented flask) cap.

13. Label flasks with passage number and date.

14. Incubate flasks in a 5% $CO_2$ incubator at 37 °C for ~3.5 days.

*3.2 Microplate Preparation*

1. During routine cell passaging, transfer sufficient volume of triturated cells (*see* Subheading 3.1, **step 8**) to a flask containing fresh DMEM growth medium (~1:100 dilution of cells) to prepare the necessary number of culture microplates (*see* **Note 10**). Swirl to mix.

2. Transfer 1.5 mL of this diluted cell suspension to each of 24 wells in a microplate. Place lid on microplate.

3. Incubate microplate in a 5% $CO_2$ incubator at 37 °C for ~3.5 days (until confluent).

4. At ~3.5 days, aspirate media from wells.

5. Add 1.5 mL of fresh DMEM growth medium to each well.

6. Incubate microplate in a 5% $CO_2$ incubator at 37 °C for 1 additional day prior to performing infectivity assay.

*3.3 Titer C. parvum Oocyst Stock*

1. Calculate the total number of oocysts necessary for the experimental run (all treatment and control flasks).

2. Wash out storage medium using sterile PBS (0.01 M, pH 7.2) and centrifugation (~16,000 × $g$ for 3 min).

3. Repeat **step 2** twice more, especially if oocysts previously stored in potassium dichromate.

4. Resuspend oocysts in 1 mL PBS (0.01 M, pH 7.2) by vortexing for 30 s.

5. Oocyst stock concentrations are verified using a hemocytometer (or other cell-counting tool).

*3.4 Disinfection Assay*

1. Set environmental chamber (or prepare recirculating water bath) to desired experimental temperature.

2. Label three CDF glass flasks each containing a CDF magnetic stir bar as experimental replicates for each chlorine concentration being tested (*see* **Note 11**). Label one additional CDF glass flask containing a CDF magnetic stir bar as a control (no chlorine) flask.

3. Prepare stock volume of experimental water at the desired chlorine concentration (*see* **Note 12**).

4. Use a CDF graduated cylinder to add sterile CDF water to a large CDF beaker containing a CDF stir bar. Immediately return foil cover to beaker and keep covered at all times.

5. Add the appropriate volume of 5.65% sodium hypochlorite to CDF water to achieve target chlorine concentration. Stir gently on magnetic stir plate.

6. Measure free chlorine concentration using standardized colorimeter (*see* **Note 13**). Record.

7. Measure pH using calibrated pH meter (*see* **Note 14**). Record.

8. Use a CDF graduated cylinder to transfer an equal volume of chlorinated water into each experimental flask and the control flask (*see* **Note 15**). Immediately return foil cover to flasks and keep covered at all times.

9. Place all flasks on magnetic stir plate in environmental chamber (or in recirculating water bath).

10. Measure free chlorine concentration and pH for each flask to ensure target experimental parameters are met (Time 0). Record.

11. Vortex oocyst stock for 30 s.

12. Add predetermined volume of oocyst stock to the first experimental flask for a final concentration of ~$10^5$ oocysts/mL. Start timer.

13. Repeat **step 12** for the two other experimental flasks.

14. Repeat **step 12** for the control flask. After 1 min, add sodium thiosulfate solution to the control flask at 50 mg/L per 1 mg/L free chlorine (*see* **Note 16)**.

15. At set time points, measure free chlorine and other water parameters (e.g., pH) as required.

16. At set time points, transfer 4, 40, or 120 mL (in triplicate 40 mL volumes) from each flask into sterile 50 mL screw-cap tubes containing PBS-BSA and 10% sodium thiosulfate (*see* **Note 17**). Gently swirl and invert to mix, neutralizing chlorine.

*3.5 Sample Concentration*

1. Immediately after collection, centrifuge 50 mL tube(s) at ~3290 × *g* for 10 min at 4 °C.

2. Carefully aspirate supernatant down to just above the pellet (~1.5 mL should remain in the tube). Be careful not to disturb the pellet (*see* **Note 18**).

3. Resuspend the pellet by gently triturating the remaining material at least five times with a micropipettor equipped with a new aerosol-barrier tip.

4. Transfer the resuspended pellet solution to a 1.5 mL microcentrifuge tube.

5. Centrifuge the microcentrifuge tube at ~16,000 × *g* for 3 min.

6. Carefully aspirate supernatant down to the 0.1 mL demarcation on the microcentrifuge tube. Be careful not to disturb the pellet.

7. Rinse the 50 mL tube with 1 mL PBS and transfer rinsate to the corresponding 1.5 mL microcentrifuge tube containing the pellet.

8. Centrifuge the microcentrifuge tube at ~16,000 × $g$ for 3 min.

9. Carefully aspirate supernatant down to the 0.1 mL demarcation on the microcentrifuge tube. Be careful not to disturb the pellet.

10. Resuspend the pellet up to 1 mL with 0.9 mL DMEM media containing 0.75% synthetic sodium taurocholate. Gently triturate suspension as you slowly add the DMEM to resuspend the pellet. Incubation in DMEM/taurocholate facilitates excystation.

11. Incubate tubes at ambient temperature for 10 min.

*3.6   Cell Culture Infectivity Assay*

1. During oocyst incubation in DMEM base medium or UltraCULTURE with 0.75% synthetic sodium taurocholate, aspirate media from MDCK monolayers in culture plate wells and replace with 1 mL fresh, room temperature DMEM growth medium or UltraCULTURE.

2. Following incubation, invert the microcentrifuge tube containing the resuspended oocyst pellet three times.

3. Inoculate 150 µL of the resuspended pellet onto MDCK monolayers in duplicate microplate wells (*see* **Note 19**).

4. Inoculate 150 µL of the appropriate dilution of the *C. parvum* oocyst stock (Subheading 3.3, **step 5**) that will result in an inoculum of ~$10^4$ oocysts onto MDCK monolayers in duplicate microplate wells (positive oocyst control).

5. Inoculate 150 µL of DMEM media containing 0.75% synthetic sodium taurocholate onto MDCK monolayers in duplicate microplate wells (reagent blank/no parasites).

6. Incubate the microplate for 48–60 h at 37 °C under 5% $CO_2$. Ensure that control and treatment wells are incubated for the same time period.

7. After incubation, aspirate media from microplate wells (media will have changed color from red to yellow/orange).

8. Add and remove (aspirate) 1 mL sterile PBS to wash remaining media from wells.

9. Add 0.5 mL Bouin's fixative solution to each well. Place microplate on a platform shaker and gently rock for 30 min at ambient temperature (*see* **Note 20**).

10. After incubation, use a micropipettor to transfer Bouin's fixative solution into appropriate waste container for disposal as hazardous chemical waste.

11. Add 0.5 mL 70% ethanol to each well. Place microplate on platform shaker and gently rock for 10 min at ambient temperature. After incubation, transfer solution into hazardous waste container (as in **step 8**).

12. Repeat **step 9** four additional times. At this point, waste can be aspirated into regular aspiration flasks and does not need to go into a specialized waste container.

13. Add and remove (aspirate) 1 mL sterile 0.01 M PBS pH 7.2 to wash remaining 70% ethanol from wells.

14. Add 1 mL PBS containing 0.1% BSA to each well and incubate at ambient temperature for at least 30 min ("blocking" step). At this point slides can be stored overnight at 4 °C, if necessary.

15. Remove (aspirate) PBS containing 0.1% BSA from each well.

16. Add 350 µL working DAPI solution (prepared in PBS) and incubate for 10 min at room temperature on a platform shaker (with light-protective cover).

17. Remove (aspirate) DAPI solution from each well.

18. Add 0.25 mL appropriately diluted C3C3 monoclonal antibody (or alternative reagent) to each well. Place microplate on platform shaker and gently rock for 60 min at ambient temperature (*see* **Note 21**).

19. After incubation, remove C3C3 monoclonal antibody from each well.

20. Add 1 mL sterile PBS to each well to wash unbound C3C3 antibody from wells. Place microplate on platform shaker and gently rock for 10 min at ambient temperature. After incubation, remove PBS from each well by aspiration.

21. Repeat **step 18** two additional times.

22. [Skip to **step 24** if using fluorochrome-labeled primary reagent to label developing parasites in host cells]. Add 0.25 mL FITC-goat anti-mouse IgG (H + L) antibody (or alternative reagent, as desired) to each well. Place microplate on platform shaker (with light-protective cover) and gently rock for 60 min at ambient temperature.

23. After incubation, remove FITC-goat anti-mouse IgG (H + L) antibody from each well.

24. Add 1 mL sterile PBS to each well to wash unbound FITC-goat anti-mouse IgG (H + L) antibody from wells. Place microplate on platform shaker (with light-protective cover)

and gently rock for 10 min at ambient temperature. After incubation, remove PBS from each well by aspiration.

25. Repeat **step 22** two additional times.

26. Add 2–3 drops of room temperature PVA/DABCO to each well.

27. Use suction pen tool to place 15 mm coverslip in well (*see* **Note 22**).

28. Aspirate excess PVA-DABCO from each well (*see* **Note 23**).

29. Cover microplate with foil and transfer to microscope for image capture. Alternatively, allow microplate to sit overnight at 4 °C before microscopic examination.

*3.7 Microscopy and Data Analysis*

1. If previously stored at 4 °C, remove culture plates from refrigerator and allow to warm to ambient temperature (make sure there is no condensation on plate bottom).

2. Set up inverted epifluorescent microscope for use (*see* **Note 24**).

3. Place 24 well plate on microscope stage.

4. Run software image capture protocol/experiment to capture up to 64 images per culture well at 100× or 200× magnification (depending on the desired imaging coverage and analysis configuration). Capture images of parasite developing stages, and if desired, host cell nuclei (DAPI staining). The latter will facilitate characterizing parasite to host cell ratios and is also useful in assessing whether host cell monolayers vary significantly between wells (i.e. reveal differences in host cell growth or perhaps show signs of toxicity, bacterial or fungal infection).

5. Because most native image capture files are very large, it may be useful (for portability and to conserve storage space) to convert the files to the Jpeg format. We find no differences in parasite quantification between Jpeg images and uncompressed native image file formats. The ImageJ macro language has convenient tools for file format conversion.

6. Using relevant criteria (*see* **Note 25**), use ImageJ to count parasites and host cell nuclei for each frame (digital image) within a microscopical field (*see* **Note 26** and Electronic Supplementary File ImageJ example macro: "CountParasiteFolder_Universal_8-bit.txt"). Note that derivative images are generated for each original image (Fig. 1, panels a and c) with the identified parasites (or host cell nuclei) indicated as numbered, schematic "ring" objects (Fig. 1, panels b and d). The ImageJ-generated data file (MS Excel *.xls format) tabulates the relevant data for each countable object (i.e. Area, X, Y, Perim., BX, BY, Width, Height, Major, Minor, Angle, Circ., Feret, FeretX, FeretY, FeretAngle, MinFeret,
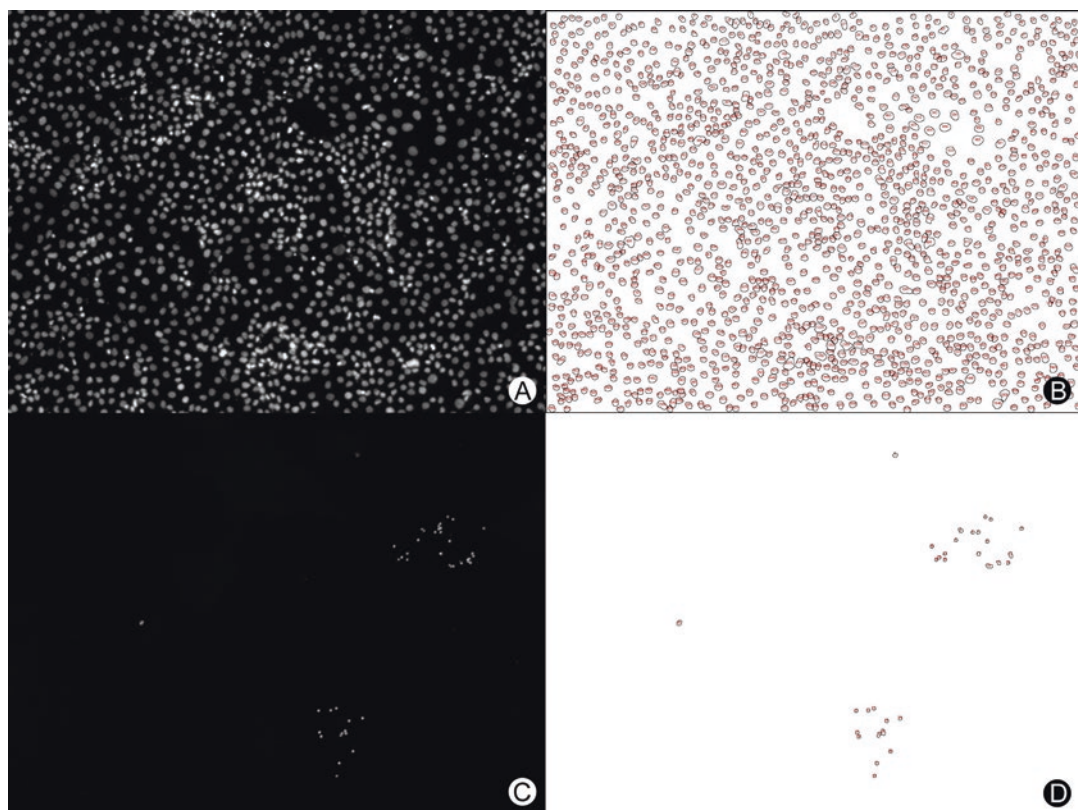
**Fig. 1** Representative images captured from an MDCK cell culture of chlorine-treated *C. parvum* oocysts and corresponding images showing ImageJ-quantified parasites or host cell nuclei (after DAPI staining). Panel **a** illustrates MDCK cell nuclei stained with DAPI and panel **b** shows the ImageJ-identified nuclei (note nucleus perimeter/outlines and numbers corresponding to each nucleus in red font). Panel **c** illustrates reduced numbers of *C. parvum* life cycle stages labeled with specific monoclonal antibody (C3C3) while panel **d** shows the corresponding ImageJ-identified parasites. Microscopical images captured at a total magnification of 100×. See Subheading 4 for details on ImageJ criteria and macro used to identify parasites under these assay conditions

AR, Round, Solidity, XStart, YStart). The most useful data (for quantification purposes) include the area (volume in pixels), perimeter, and circularity of each object. The ImageJ macro "CountParasiteFolder_Universal_8-bit.txt" generates a summary data file (Well ID + "Summary") that consolidates the results for all of the images in each folder analyzed (corresponding to each culture well) (*see* **Note 27**). These tables are imported into Microsoft Excel to calculate average number and size of developing parasite stages (or host cell nuclei) per culture well. The macro language included in MS Excel can automate the importing of data files and calculating descriptive statistics.

7. Parasite quantification is used to calculate the $\log_{10}$ inactivation of oocysts in each condition over time as compared with the oocyst concentration in the control flasks. For a basic

disinfection analysis, calculate $\log_{10}$ inactivation or percent reduction of parasites:

$$\log\text{reduction} = \log_{10}\left(\frac{A}{B}\right)$$

Alternatively, calculate percent reduction using this formula:

$$\text{Percent reduction} = \frac{(A - B) * 100}{A}$$

where

$A$ = parasite stage count in control wells.

$B$ = parasite stage count in treatment wells.

Adjust calculations based on any dilutions incorporated into well inocula values. For example, if a ten-fold higher oocyst inocula was added to a culture well, the parasite count for that well would need to be decremented by a factor of 10 before calculating log or percent reduction values (*see* **Note 28**). Consider more elaborate analyses if accounting for disinfectant decay, etc. [14, 21, 22].

# 4    Notes

1. We routinely use MDCK cells for immunofluorescence-based assays of *C. parvum* growth primarily because they are robust, support cryptosporidial growth comparable to or exceeding HCT-8 cells, and MDCK cells grow "flat" in culture chambers. The flat monolayer topology facilitates image capture of developing parasites, as they are located in a narrow focal plane. HCT-8 cells, however, grow much more three-dimensionally ("hills and valleys"), complicating the autofocus of parasites during image capture-based microscopic analyses (this is more pronounced at microscope magnifications >100×). While recent advancements in cell culture environments facilitate extended cultivation of *C. parvum*, the conditions described in this chapter are well suited to evaluating and enumerating the survival of disinfectant-treated oocysts.

2. Prepare a 0.01 M phosphate buffer solution (pH 7.5) in reverse osmosis-treated water using dibasic sodium phosphate (anhydrous) and monobasic sodium phosphate (monohydrate). If different pH is desired, quantities of mono and dibasic sodium phosphate can be adjusted empirically. Add sufficient 5.65% sodium hypochlorite solution to achieve ≥5 mg/L free chlorine. Cover tightly with aluminum foil. After 2 days, ensure that the solution contains at least 2 mg/L free chlorine; discard if not. Place uncovered container under an ultraviolet lamp to remove all remaining free chlorine.

Ensure that there is no detectable free chlorine in the solution, cover tightly with aluminum foil, and autoclave at 121 °C at 15 psi for 15 min. CDF water can be used up to 2 days after preparation, otherwise prepare fresh CDF water.

3. Add sufficient 5.65% sodium hypochlorite solution to deionized water in a large tank to achieve ≥10 mg/L free chlorine. Submerge glass beakers and Erlenmeyer flasks each containing magnetic stir bars and glass graduated cylinders. After ≥3 h, rinse thoroughly with CDF water, cover tightly with aluminum foil, and autoclave at 121 °C at 15 psi for 15 min.

4. *C. parvum* oocysts are available from commercial vendors or may be produced and isolated from experimentally infected animals, from naturally infected animals (e.g., calves), or from clinical stool samples collected from patients with cryptosporidiosis (see chapter in this book on oocyst purification). If oocysts are derived from human samples, genotyping may be warranted to determine the species of *Cryptosporidium* present. In general, *C. parvum* is more readily cultured in vitro than *C. hominis* when using MDCK cells. For disinfection studies, oocysts are typically processed and stored in saline or PBS supplemented with antibiotics. Such oocysts may harbor contaminating bacteria that can overgrow control wells in cell culture studies. Consider using a short-term bleach exposure of control oocysts (1/10 dilution of commercial bleach for 5 min) to "sanitize" and provide a reference "zero" for comparison to experimentally treated oocysts. Oocysts stored in 2.5% aqueous potassium dichromate are likewise suitable for disinfection studies, although some investigators prefer antibiotic-stored oocysts. One advantage of using potassium dichromate-stored oocysts is that they are "sanitized" already and can be used directly in cultures after washing out the dichromate via centrifugation. While "freshly passaged" oocysts (≤4 months old) are preferred for disinfection studies, we have found that as long as the oocysts used are from the same lot, results are consistent using oocysts up to 6 months old. Slight differences might be observed between antibiotic-stored and potassium dichromate-stored oocysts, these differences are typically less than observed in lot-to-lot variation.

5. Microcentrifuge tubes should be "low binding" or "low retention" to minimize losses of oocysts during centrifugation and other processing steps. Conventional polypropylene microcentrifuge tubes are not recommended. If tubes are labeled "RNase, DNase, and/or DNA free," they can typically be considered suitable for use without the need to sterilize by autoclave (just be sure to handle aseptically).

6. C3C3 monoclonal antibody can be used in a direct or indirect fluorescence assay format [19, 23]. If using the fluorochrome-

labeled antibody, no secondary antibody step is necessary. If using the unlabeled C3C3 antibody, use an appropriate secondary antibody (e.g., fluorescein-conjugated goat anti-mouse IgG reagent). Alternatively, *Vicia villosa* lectin has been successfully used to label and identify developing stages of *C. parvum* in cell cultures [24].

7. Antiquenching (anti-fading) long-term coverslip mounting medium for immunofluorescence assays. DABCO (1,4-diazabicyclo-[2.2.2]octane) is recommended as a fluorescence antiquenching agent due to its great solubility and chemical stability [25]. DABCO protects fluorochrome dyes against quenching by scavenging free radicals liberated by the excitation of the fluorochrome. The higher pH of this recipe typically enhances fluorescence intensity versus neutral pH recipes. Slides prepared with PVA/DABCO can be stored at 4 or −20 °C for years with little loss of fluorescence. Preparation: Mix 2.4 g polyvinyl alcohol (PVA, average molecular weight 30,000–70,000) gradually into 6 g glycerol. Mix carefully to avoid creating air bubbles. Add 6 mL of $H_2O$, mix and allow to stand at room temperature for several hours. Add 12 mL Tris buffer (0.2 M, pH 8.5), mix and heat to ~50 °C for 10 min with occasional mixing. When the PVA has dissolved, clarify by centrifugation at ~3000 × *g* for 15 min. Decant supernatant (discard pellet, if any) and add DABCO to a final concentration of 2.5% (w/v). The mounting medium is viscous, so use a pipet to mix in the DABCO. Avoid introducing bubbles while mixing. When dissolved, decant aliquots of the "working" PVA/DABCO into "snap cap" polypropylene tubes (~4 mL/tube) and store at −20 °C (long-term storage) or at 4 °C (short-term storage: i.e., several months).

8. Ideally, the inverted epifluorescence microscope will have objectives suitable for fluorescence imaging at 10× and 20×. Epifluorescence light source (e.g., mercury vapor lamp or LED source) should produce wavelengths needed for nuclear stain observation (DAPI) as well as for fluorescein, cyanine, or other fluorochromes used in the parasite and host cell labeling portion of the assay. Typical fluorescence filter sets will have the following or similar features: For DAPI observation, excitation filter ~340–380 nm; dichroic mirror ~400 nm; barrier filter ~420 nm. For fluorescein or Alexa Fluor 488 observation, excitation filter ~450–490 nm; dichroic mirror ~510 nm; barrier filter ~515–550 nm. For cyanine dye (e.g., Cy3) or TRITC or Alexa Fluor 555 observation, excitation filter ~530–560 nm; dichroic mirror ~565 nm; barrier filter ~580–660 nm. Digital camera should be suitable for automatic capture of fluorescent images. Users will need to calculate the frame size scale at the magnification of choice and determine

how many frames/fields cover the area of the culture well. While we use 24-well plates, other investigators have used 96-, 384-, and 1536-well formats successfully.

9. Unless otherwise indicated, all steps are performed in an approved Class II Biosafety Cabinet.

10. Each volume of water sampled from experimental and control flasks (*see* **Note 17**) will be individually concentrated and inoculated onto cell monolayers in duplicate microplate wells. This number of wells, plus additional wells needed for infectivity assay controls (*see* Subheading 3.6, **steps 4** and **5**) will dictate the required number of culture microplates needed per experiment.

11. At least 3 flasks per chlorine concentration should be used. All experimental flasks should be of equal volume. Use flasks that will be no more than 2/3 full at maximum volume during the experiment.

12. If experiment is conducted in triplicate, prepare at least five times the volume needed for each replicate. Experimental water should be prepared on the day of the experiment. A slightly higher concentration should be prepared to account for chlorine off-gassing when pouring chlorinated water into flasks. Preliminary experiments to determine dose volume and to assess chlorine decay during pouring steps should be conducted to determine target chlorine concentration in stock water.

13. If target chlorine concentrations are near the detection limit of the colorimeter, use CDF water to prepare dilutions for measurement within the working range of the colorimeter.

14. Preliminary experiments to assess pH changes due to chlorine addition should be conducted to ensure that target pH is achieved after addition of sodium hypochlorite.

15. After measuring free chlorine, proceed immediately to the following step because chlorine will begin to decay.

16. Sodium thiosulfate is added to stop hypochlorite oxidation and sanitization of control oocysts. Measure free chlorine in control flask right after sodium thiosulfate neutralization to verify it is zero. Note that this hypochlorite step assures that the control oocysts (stored in PBS/antibiotics) are free of residual (viable) bacterial contaminants, preventing potential bacterial infection of positive control wells. Also, the short hypochlorite exposure provides a slight enhancement of the excystation of the control oocysts, providing a better reference for the experimentally treated oocysts. The latter is why we recommend using the bleach treatment step for potassium dichromate stored oocysts, too. If the bleach step is omitted,

early time points in the chlorine-treatment of oocysts can actually generate surprising infection results where treated oocysts yield higher infection levels compared to control oocysts.

17. Volume assayed is dependent on efficacy of disinfectant on oocyst inactivation. Smaller sample volumes are needed when a high number of infectious oocysts is expected (e.g., short contact time). Larger sample volumes are needed when a low number of infectious oocysts (survivors) is expected (e.g., long contact time) to increase the detection limit of the assay. Preliminary "range-finding" experiments may be necessary to help determine optimal sample volumes required at assay time points.

18. The pellet will likely not be visible to the naked eye. When aspirating, keep suction low and keep the Pasteur pipet tip just below the surface of the supernatant.

19. Oocysts suspended in DMEM/taurocholate (or UltraCULTURE/taurocholate) can be directly inoculated into culture wells. The residual sodium taurocholate actually enhances cryptosporidial growth (keep final sodium taurocholate concentration at ≤0.1%).

20. Platform shaker should provide gentle rocking, or orbital wave (variable tilt) motion to ensure that the monolayer is continuously covered with reagent, but not agitated so aggressively that the monolayer detaches.

21. Alternatives to the use of unlabeled monoclonal antibody include using a fluorochrome-labeled version (e.g. C3C3-Cy3, 1/500 dilution, 250 μL/chamber cell) or use of fluorochrome-labeled lectin at an appropriate dilution. Red fluorescent dyes are typically preferred as host cell monolayers typically exhibit lower background fluorescence using "red" filter sets than when observed using filter sets for fluorescein or similar dyes.

22. Use suction pen tool with silicone tip to pick up 15 mm coverslip. Tilt 24 well plate at an angle that causes PVA/DABCO mounting media to pool to the lower side of the well. Carefully place the edge of the glass coverslip toward the base of the well to contact mounting medium. Carefully release the cover slip (release suction) to allow coverslip to drop into place. Avoid introducing air bubbles under the coverslip. It may be useful to "tap" the coverslip with the tip of the aspirating Pasteur pipet to dislodge any bubbles trapped beneath the coverslip before aspirating excess mounting medium.

23. PVA/DABCO mounting medium will "gel" (immobilizing the coverslip), protect the labeled monolayer from drying, and protect fluorochromes from quenching (fading) under microscope lamp illumination.

24. Power up microscope and computer. Load microscopy software that controls the microscope and captures images using the digital camera. Load image capture protocol or experiment in software that will capture images. For the described protocol, we employed Zeiss AxioVision software (Carl Zeiss, Thornwood, NY), a Zeiss HRm digital camera and a Zeiss AxioVert 200M microscope to capture 64 images in an $8 \times 8$ "checkerboard" grid at 100× total magnification (10× objective) which is equivalent to approximately 22% of the well (monolayer) surface area. Ideally, the entire well should be captured for optimal sensitivity, but the subset of 64 images has been successful for us and covers a significant portion of the monolayer. Each microscope system will require optimization and it is beyond the scope of this chapter to describe all alternatives.

25. Criteria for identifying and counting parasites and host cells include the following. Developing parasite life cycle stages can be selectively identified by parasite-specific monoclonal antibody reactivity (or by general labeling by lectin reactivity). Immediately after sporozoite invasion, early trophozoites are approximately 1–2 μm in diameter (normally round in morphology). While some sporozoites do not successfully invade host cells, they typically "round up" anyway even if they remain free in the culture medium or adhere to host cells (though do not actually invade, grow, or proliferate). If fluorescing objects smaller than ~2 μm are excluded (non-viable sporozoites/trophozoites or debris), and objects much larger than ~6 μm are excluded (some parasite clumps and large debris), the quantification can be confidently limited to developing stages (late trophozoites, meronts, gamonts, and zygotes, depending on the time of analysis post inoculation). Also keep in mind that there is little, if any, "recycling" of the asexual stages in MDCK or HCT-8 cells under conventional cell culture conditions. This means the life cycle development progresses essentially directly through the life cycle, going from sporozoite to Type I meront, on to Type II meront and then directly to the gamont stages. This effectively limits the amplification potential to the numbers predicted by a direct path through the life cycle. Consequently, the developing stage numbers (which "peak" between 48 and 72 h post inoculation) are predictive of the number of surviving oocysts after disinfection treatment.

26. Pre-process images using the ImageJ built-in commands "Make Binary", "Fill Holes", and "Watershed". The ImageJ command "Analyze Particles" is used to count parasites with the criteria described below (realizing the values indicated may need to be customized for the microscopy hardware,

magnification, fluorescent labeling reagents, etc.). Based on our configuration (*see* Subheading 3) where imaging is performed at 100× total magnification, the following settings can be used as defaults: Size min: 30, Size max: 250 (the size range of 30–250 is a volumetric range of pixels that developing parasites occupy at this magnification); Circularity min: 0.7, Circularity max: 1 (the circularity range of 0.7–1 indicates the parasites are essentially round to slightly oval); Brightness/contrast min: 15, Brightness/contrast max: 255; Threshold min: 25, Threshold max: 255. Depending on the host cells used in the culture assays, determine appropriate conditions and values to identify DAPI-stained host cell nuclei (if calculating these data for determining host cell monolayer characteristics and to calculate parasite: host cell ratios). ImageJ has robust macro options for automating the image analysis process. See example in Electronic Supplementary File ImageJ macro: "CountParasiteFolder_Universal_8-bit.txt." Note, the "Size min" and "Size max" can be adjusted to conform to the characteristics of the images captured by the microscope, digital camera, and fluorescence labeling result in use by respective investigators. The size parameters used above actually capture slightly larger objects than might be expected from uniformly dispersed parasites (i.e., closely adjacent parasites that cannot be split using the "Watershed" command may be counted as a single parasite); consequently, the total counts may be somewhat conservatively estimated depending on the parasite density in the image and the quality of the fluorescence labeling). Manually scanning image sets (raw images and the processed images) is warranted to determine if excessive over- or under-counts have occurred because of heavy parasite density in the images (tightly packed clusters of parasites can be undercounted because they may not be resolvable into individual parasites and may be excluded because the estimated size exceeds the maximum for inclusion in the overall count). This result can also be gleaned by examining the average object (parasite) size in the data tables generated for the images.

27. Several options are available for the analysis of the parasite and host cell nuclei data. Data for each well can be simply summed from the total number of frames captured (64) and used as a data point. Alternatively, the average number of parasites per frame can be calculated from the 64 frames and used as a data point. If an estimate of the total number of parasites per well is preferred, the estimate can be calculated using the following information (we are using the data from our microscope configuration in this example): total magnification (100×), well area (1.9 cm²), digital camera frame dimension (1388 × 1040

pixels), total number of frames per well at 100× magnification (293), subset of frames actually captured per well (64, representing ~21.8% of total area). The following calculation can be made: total number of parasites per well = (average number of parasites in the 64 frames captured) × 293. Alternatively, parasites per mm² can be derived using a variation on the formula described by Augustine [26] where stages/mm² = ((mean # stages per frame)/($K$ × PC)) where PC is the average percent confluence for each field and the constant $K$ represents the area in mm² for each video frame digitized at 100× (in our configuration $K$ = ~0.648).

28. After range-finding experiments, it may be useful to adjust the treated inoculum oocyst numbers introduced to test wells/monolayers. For example, if it is expected that a treatment will produce a 2–3 log reduction in oocyst viability (infectivity), the inoculum can be increased by 1–2 logs (or more) compared to the control. In this way, the numbers of developing parasites will occupy the optimal range for the assay. Too few survivors and the parasite counts will be at the lower limit of detection, while too many survivors will lead to monolayer injury, or parasite overcrowding which can interfere with the identification of individual life cycle stages (reducing the sensitivity of the assay). We find that viable oocyst inocula should be in the following range for the 24-well plates described in this assay: ~$1 \times 10^2$ to $1 \times 10^5$ oocysts, but preferable close to $1 \times 10^4$ for the best data.

## Acknowledgments

*Disclosures*: Use of trade names is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Department of Health and Human Services. The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

## References

1. Snelling WJ, Lin Q, Moore JE, Millar BC, Tosini F, Pozio E, Dooley JS, Lowery CJ (2007) Proteomics analysis and protein expression during sporozoite excystation of *Cryptosporidium parvum* (Coccidia, Apicomplexa). Mol Cell Proteomics 6(2):346–355. https://doi.org/10.1074/mcp.M600372-MCP200

2. Sow SO, Muhsen K, Nasrin D, Blackwelder WC, Wu Y, Farag TH, Panchalingam S, Sur D, Zaidi AK, Faruque AS, Saha D, Adegbola R,

Alonso PL, Breiman RF, Bassat Q, Tamboura B, Sanogo D, Onwuchekwa U, Manna B, Ramamurthy T, Kanungo S, Ahmed S, Qureshi S, Quadri F, Hossain A, Das SK, Antonio M, Hossain MJ, Mandomando I, Nhampossa T, Acacio S, Omore R, Oundo JO, Ochieng JB, Mintz ED, O'Reilly CE, Berkeley LY, Livio S, Tennant SM, Sommerfelt H, Nataro JP, Ziv-Baran T, Robins-Browne RM, Mishcherkin V, Zhang J, Liu J, Houpt ER, Kotloff KL, Levine MM (2016) The burden of *Cryptosporidium* diarrheal disease among children <24 months of age in moderate/high mortality regions of Sub-Saharan Africa and South Asia, utilizing data from the Global Enteric Multicenter Study (GEMS). PLoS Negl Trop Dis 10(5):e0004729. https://doi.org/10.1371/journal.pntd.0004729

3. MacKenzie WR, Hoxie NJ, Proctor ME, Gradus MS, Blair KA, Peterson DE, Kazmierczak JJ, Addiss DG, Fox KR, Rose JB et al (1994) A massive outbreak in Milwaukee of *Cryptosporidium* infection transmitted through the public water supply. N Engl J Med 331(3):161–167

4. Shirley DA, Moonah SN, Kotloff KL (2012) Burden of disease from cryptosporidiosis. Curr Opin Infect Dis 25(5):555–563. https://doi.org/10.1097/QCO.0b013e328357e569

5. Hlavsa MC, Cikesh BL, Roberts VA, Kahler AM, Vigar M, Hilborn ED, Wade TJ, Roellig DM, Murphy JL, Xiao L, Yates KM, Kunz JM, Arduino MJ, Reddy SC, Fullerton KE, Cooley LA, Beach MJ, Hill VR, Yoder JS (2018) Outbreaks associated with treated recreational water—United States, 2000-2014. MMWR 67(19):547–551. https://doi.org/10.15585/mmwr.mm6719a3

6. Carpenter C, Fayer R, Trout J, Beach MJ (1999) Chlorine disinfection of recreational water for *Cryptosporidium parvum*. Emerg Infect Dis 5(4):579–584

7. Korich DG, Mead JR, Madore MS, Sinclair NA, Sterling CR (1990) Effects of ozone, chlorine dioxide, chlorine, monochloramine, on *Cryptosporidium parvum* oocyst viability. Appl Environ Microbiol 56:1423–1428

8. Shields JM, Hill VR, Arrowood MJ, Beach MJ (2008) Inactivation of *Cryptosporidium parvum* under chlorinated recreational water conditions. J Water Health 6(4):513–520

9. Venczel LV, Arrowood M, Hurd M, Sobsey MD (1997) Inactivation of *Cryptosporidium parvum* oocysts and *Clostridium perfringens* spores by a mixed-oxidant disinfectant and by free chlorine. Appl Environ Microbiol 63:1598–1601

10. Murphy JL, Arrowood MJ, Lu X, Hlavsa MC, Beach MJ, Hill VR (2015) Effect of cyanuric acid on the inactivation of *Cryptosporidium parvum* under hyperchlorination conditions. Environ Sci Technol 49(12):7348–7355. https://doi.org/10.1021/acs.est.5b00962

11. Shields JM, Arrowood MJ, Hill VR, Beach MJ (2009) The effect of cyanuric acid on the disinfection rate of *Cryptosporidium parvum* in 20-ppm free chlorine. J Water Health 7(1):109–114

12. Driedger AM, Rennecker JL, Mariñas BJ (2001) Inactivation of *Cryptosporidium parvum* oocysts with ozone and monochloramine at low temperatures. Water Res 35(1):41–48

13. Keegan AR, Fanok S, Monis PT, Saint CP (2003) Cell culture-Taqman PCR assay for evaluation of *Cryptosporidium parvum* disinfection. Appl Environ Microbiol 69(5):2505–2511

14. Murphy JL, Haas CN, Arrowood MJ, Hlavsa MC, Beach MJ, Hill VR (2014) Efficacy of chlorine dioxide tablets on inactivation of *Cryptosporidium* oocysts. Environ Sci Technol 48(10):5849–5856. https://doi.org/10.1021/es500644d

15. Peeters JE, Mazas EA, Masschelein WJ, Martinez de Maturana IV, Debacker E (1989) Effects of disinfection of drinking water with ozone or chlorine dioxide on survival of *Cryptosporidium parvum* oocysts. Appl Environ Microbiol 55:1519–1522

16. Chauret CP, Radziminski CZ, Lepuil M, Creason R, Andrews RC (2001) Chlorine dioxide inactivation of *Cryptosporidium parvum* oocysts and bacterial spore indicators. Appl Environ Microbiol 67(7):2993–3001. https://doi.org/10.1128/AEM.67.7.2993-3001.2001

17. Ruffell KM, Rennecker JL, Mariñas BJ (2000) Inactivation of *Cryptosporidium parvum* oocysts with chlorine dioxide. Water Res 34(3):868–876

18. Finch GR, Li H (1999) Inactivation of *Cryptosporidium* at 1°C using ozone or chlorine dioxide. Ozone Sci Eng 21(5):477–486. https://doi.org/10.1080/01919512.1999.10382886

19. Arrowood MJ (2002) In vitro cultivation of *Cryptosporidium* species. Clin Microbiol Rev 15(3):390–400

20. Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9(7):671–675

21. Haas CN, Joffe J (1994) Disinfection under dynamic conditions: modification of Hom's

model for decay. Environ Sci Technol 28(7):1367–1369. https://doi.org/10.1021/es00056a028

22. Haas CN, Kaymak B (2003) Effect of initial microbial density on inactivation of *Giardia muris* by ozone. Water Res 37(12):2980–2988. https://doi.org/10.1016/S0043-1354(03)00112-X

23. Arrowood MJ, Mead JR, Xie L, You X (1996) In vitro anticryptosporidial activity of dinitroaniline herbicides. FEMS Microbiol Lett 136(3):245–249

24. Gut J, Nelson RG (1999) *Cryptosporidium parvum*: synchronized excystation in vitro and evaluation of sporozoite infectivity with a new lectin-based assay. J Eukaryot Microbiol 46(5):56S–57S

25. Harlow E, Lane D (1988) Cell staining. In: Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, p 418

26. Augustine PC (1980) Effects of polyions, $Ca^{++}$, and enzymes on penetration of cultured cells by *Eimeria meleagridis* sporozoites. J Parasitol 66:498–501

# Chapter 17

# High-Content Screening for *Cryptosporidium* Drug Discovery

## Melissa S. Love and Case W. McNamara

## Abstract

High-content screening (HCS) is a cell-based type of phenotypic screening that combines multiple simultaneous readouts with a high level of throughput. A particular benefit of this form of screening for drug discovery is the ability to perform the interrogation in a biologically relevant system. This approach has greatly advanced the field of drug discovery for cryptosporidiosis, a diarrheal disease caused by protozoan parasites of *Cryptosporidium* spp. These parasites are obligate intracellular parasites and cannot be cultured in vitro without the support of a host cell, limiting the options for potential assay readout. Here we describe an established 384- or 1536-well format high-content imaging (HCI) assay of *Cryptosporidium*-infected HCT-8 human ileocecal adenocarcinoma cells. This HCS assay is a powerful tool to assess large numbers of compounds to power drug discovery, as well as to phenotypically characterize known *Cryptosporidium*-active compounds.

**Key words** Phenotypic screening, High-throughput screening, High-content imaging, *Cryptosporidium*, Cryptosporidiosis, Anti-cryptosporidial, Drug discovery, Neglected tropical diseases, Infectious diseases

## 1 Introduction

Recent growing interest in developing novel anti-cryptosporidial drugs [1, 2] has encouraged advances in characterizing *Cryptosporidium* infections in vitro [3–5], including screening of diverse compound libraries. Screening efforts have included targeted screens of compounds with known mechanisms of action [6–10], compounds with known activity against related pathogens [11, 12], libraries of known bioactive compounds [13], as well as broader small-molecule compound libraries [14]. The largest screen published to date against *Cryptosporidium* spp. parasites included ~80 K compounds in total, and all published screening efforts to date have led to a diverse set of lead compounds in the pipeline.

Here, we describe a method of high-throughput screening (HTS) for *Cryptosporidium* spp. The compound treatment of *Cryptosporidium*-infected host cells is carried out in a microtiter plate and analyzed via high-content imaging (HCI). Specifically, HCT-8 host cells are seeded in microtiter plates 24 h prior to infection, compounds of interest are transferred into the wells, and then "activated" *Cryptosporidium* oocysts are added to allow for cell infection and subsequent intracellular parasite proliferation over a 48-h period. After 48 h, the cells are fixed and differentially stained with fluorescent markers to allow for enumeration of both HCT-8 cells and parasites. All steps are capable of automation to allow for maximum throughput. This method can be used for *C. parvum* or *C. hominis* parasites and is amenable to 384-well or 1536-well formats [6, 14].

HCI capabilities have also greatly advanced over the past 15 years, with many systems that allow for automated imaging of microtiter plates [15, 16]. An advantage to using HCI as the readout for anti-cryptosporidial HTS is that phenotypic effects on both host cells and parasites can be assessed concomitantly. Host cell effects can be used for determination of cytotoxicity, and therefore, compounds can be quickly triaged as either *Cryptosporidium*-specific or more generally cytotoxic. The assay described herein is designed to be flexible for phenotypic characterization of a wide range of molecules. Although we have capabilities of automation to increase throughput, the 384-well format is easily carried out "by hand" with multichannel pipettors and is amenable to most laboratory setups.

## 2    Materials

### 2.1    Media and Cell Culture Reagents

1. Propagation medium for HCT-8 host cells: RPMI 1640 medium with L-glutamine, 10% heat-inactivated (55 °C for 30 min) fetal bovine serum, 100 IU penicillin, 100 μg/mL streptomycin. Combine all components and sterile filter through a 0.22 μm filter flask. Store at 4 °C.

2. Assay medium: phenol red-free RPMI 1640 with L-glutamine, 2% heat-inactivated horse serum, 100 IU penicillin, 100 μg/mL streptomycin. Combine all components and sterile filter through a 0.22 μm filter flask. Store at 4 °C.

3. Dulbecco's Phosphate Buffered Saline (DPBS).

4. TrypLE™ dissociation reagent (recombinant trypsin, EDTA) (Thermo Fisher Scientific, Inc., Norcross, GA).

5. T75-treated tissue culture flasks.

6. Cell scrapers.

7. 70 μm mesh filters.

8. C-Chip disposable hemocytometer.

**2.2 *Cryptosporidium* Oocysts and Excystation Reagents**

1. *C. parvum* oocysts, Iowa strain, propagated through neonatal Holstein calves, isolated and stored at 4 °C in a solution with 0.01% Tween 20, 100 U of penicillin, 100 μg of gentamicin/mL (*Cryptosporidium* Production Laboratory, School of Animal and Comparative Biomedical Sciences, University of Arizona).

2. *C. parvum* oocysts, Iowa strain, propagated through neonatal calves, isolated and stored at 4 °C in a solution with 0.01% Tween 20, 100 U of penicillin, 100 μg of gentamicin/mL (Bunch Grass Farm, Deary, ID).

3. *C. parvum* oocysts, wild-type field isolate, isolated from dairy calves naturally infected with *Cryptosporidium* and stored at 4 °C in a solution with 0.01% Tween 20, 100 U of penicillin, 100 μg of gentamicin/mL (Zambriski Laboratory, Paul G. Allen School for Global Animal Health, Washington State University).

4. *C. hominis* oocysts, TU502 strain, propagated through neonatal gnotobiotic piglets, isolated and stored at 4 °C in a solution with 0.01% Tween 20, 100 U of penicillin, 100 μg of gentamicin/mL (Tzipori Laboratory, Cummings School of Veterinary Medicine, Tufts University).

5. 10 mM hydrochloric acid (HCl).

6. Excystation buffer: dissolve 10 mg of sodium taurocholate hydrate in 9 mL of DPBS with 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$, sterile filter (*see* **Note 1**), giving a final concentration of 2 mM sodium taurocholate.

7. Microcentrifuge.

8. 37 °C water bath incubator.

**2.3 *Cryptosporidium* Cell Assay**

1. Low-evaporation metal lids or comparable plastic lids.

2. BioTek MultiFlo™ FX Multi-Mode dispenser (BioTek Instruments Inc., Winooski, VT).

3. Cassettes: 1 and 5 μL sizes.

4. Labcyte Echo® 550 acoustic transfer liquid-handling system (Labcyte Inc., San Jose, CA) (*see* **Note 2**).

5. Echo®-compatible 384-well source plates (*see* **Note 3**).

6. Dimethyl sulfoxide (DMSO) as a solvent and neutral control.

7. 2′-Deoxy-5-fluorouridine (FDU) and nitazoxanide (NTZ) as positive controls for *Cryptosporidium* growth inhibition; puromycin (Pur) as a positive control for HCT-8 host cell growth inhibition.

8. Multichannel electronic pipettor.

9. Tabletop centrifuge with microplate adapters.

10. Tissue culture incubator programmable for 37 °C with 5% $CO_2$.

11. Assay plates: 384-well black, µclear bottom, or 1536-well black, µclear bottom (LoBase), microtiter plates (Greiner Bio-One, Monroe, NC).

*2.4 High-Content Imaging*

1. Microplate washer/dispenser (*see* **Note 4**).

2. 20% Paraformaldehyde for cell fixation.

3. Wash buffer A: phosphate buffered saline (PBS) with 0.05% sodium azide.

4. Permeabilization buffer: 0.25% triton X-100 in PBS.

5. Wash buffer B: PBS with 0.1% Tween 20 (PBS-T) and 0.05% sodium azide.

6. Blocking agent: SuperBlock™ T20 (PBS) Blocking Buffer (Thermo Fisher Scientific, Inc.).

7. Visualization buffer: 10% SuperBlock™ T20 (PBS) Blocking Buffer diluted with PBS-T, 1 µg/mL fluorescein-labeled *Vicia villosa* lectin, and 3 µM 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). Protect from light.

8. Adhesive foil seals.

9. CellInsight CX5 high content screening platform (Thermo Fisher Scientific, Inc.).

## 3   Methods

HTS workflow is typically as follows: first is a single-point primary screen, followed by reconfirmation at a single concentration in triplicate, and then dose–response reconfirmation in either duplicate or triplicate. Secondary or complementary assays may be employed for follow-up characterization, such as to assess the compound stage of action, mode of action, cidality, or rapidity of killing.

This cell-based screening assay takes place with multiple steps over multiple days. On day 1, host cell seeding occurs. On day 2, compounds of interest and controls are transferred onto the cells in a "wet" dispense with an acoustic liquid handler. Compound preparation depends on the step in the overall screening workflow. After compound transfer, *Cryptosporidium* oocysts are subjected to an excystation protocol and then dispensed onto the cells and left to infect and replicate within cells while remaining in the presence of compound for 48 h. On day 4, the infected cells are fixed and then labeled with fluorescent markers for imaging. This can be broken up into multiple steps if needed, and stopping points are indicated in Subheading 4. The final steps are imaging the

microplates to obtain cell and parasite counts, and then data analysis to determine inhibition and finding either "hits" or the half-maximal effective concentration ($EC_{50}$) of a confirmed hit compound.

Our compound preparation is geared toward the use of an acoustic liquid handler, and therefore all compound dilutions are made in compatible source plates. DMSO is the preferred solvent, although aqueous transfers are also possible. For primary screening of a compound library, compounds should be prepared in a single concentration, usually 2 or 10 mM stock. These same stocks can be used throughout the reconfirmation stages, though a resupply from the vendor or resynthesis of compounds (especially small molecules) is recommended as a final step to ensure the quality and purity of the active molecule. For dose–response reconfirmation, serial dilutions can be carried out ahead of time on the source plate, or all points can come from a single source well by programming the acoustic liquid handler and employing an intermediate plate as necessary.

Cell densities and any automation should be optimized within each laboratory setup. Statistical analyses and reproducibility using negative and positive controls are essential to ensure assay quality. Factors that should be examined include the signal-to-noise ratio (S/N), signal-to-background ratio (S/B), standard deviation (SD), coefficient of variation (CV), and the $Z'$-factor (Eq. 1) [17]. A $Z'$-factor of >0.5 is considered to be a high-quality assay, though $Z'$-factors of 0.2–0.4 may be acceptable depending on the separation ban between the samples and controls. Automation of liquid-handling steps can greatly improve throughput for this assay, though is not critical.

$$S/N = \frac{\text{mean signal} - \text{mean background}}{\text{standard deviation of background}}$$

$$S/B = \frac{\text{mean signal}}{\text{mean background}}$$

$$SD = \sqrt{\frac{\Sigma(x-\mu)^2}{n}} \tag{1}$$

$$CV = \frac{SD}{\mu}$$

$$Z' \;\; \text{factor} = 1 - \frac{3(SD_p + SD_n)}{|\mu_p - \mu_n|}$$

Statistical parameters for high-throughput screening. Signal-to-noise ratio (S/N), signal-to-background ratio (S/B), standard deviation (SD), coefficient of variation (CV), and the $Z'$-factor, where $x$ = each value in the data set, $\mu$ = mean, $n$ = number of

values in the data set, and ($SD_p$, $\mu_p$, $SD_n$, $\mu_n$) are the standard deviations and means of both the positive (p) and negative (n) controls [17]

*3.1 Host Cell Seeding*

1. HCT-8 cells should be passaged twice per week with a 2:5 split in T75 flasks (*see* **Note 5**).

2. Harvest cells 3–4 days after a passage (*see* **Note 6**) by first rinsing with 6 mL DPBS, and then adding 3 mL TrypLE™.

3. Cells will not fully release with TrypLE™ alone. Use a cell scraper (can be used across multiple flasks) to release the cells from the bottom of the flask.

4. Add 7 mL of assay medium to collect the cells and transfer to a 50 mL conical tube.

5. Pellet cells in a benchtop centrifuge at $300 \times g$ for 5 min.

6. Aspirate the supernatant and resuspend cells in 10 mL assay medium.

7. Strain the cell suspension through a 70 μm mesh filter into a fresh 50 mL conical tube. Wash the filter once with an additional 5 mL of assay medium.

8. Dilute the cell suspension 1:10 in 100 μL suspension and count on a C-Chip hemocytometer.

9. Dilute the cell suspension to $6 \times 10^5$ cells/mL and prepare enough suspension for the number of assay plates required, plus an additional 30% for priming and loss.

    *For 384-well plates*: 25 μL/well or 9.6 mL/plate; 12.5 mL with dead volume. This is equivalent to 15,000 cells/well.

    *For 1536-well plates*: 5 μL/well, or 7.68 mL/plate; 10 mL with dead volume. This is equivalent to 3,000 cells/well.

10. Prepare the MultiFlo™ dispenser and 5 μL cassette for dispense. Assemble the cassette and prime the line with 70% ethanol to disinfect, followed by sterile water, assay medium, and finally the cell suspension. If the volume and setup allow, a sterile stir bar may be added and the cell suspension is placed on a stir plate to prevent cells from settling and causing uneven quantities dispensed into the assay plates.

11. Dispense cells into assay plates at the above-indicated volumes for each microplate type.

12. Cover the plates with the low-evaporation metal lids, and place in the 37 °C incubator for 24 h.

*3.2 Compound Preparation and Transfer*

1. Prepare a 10 mM stock of compounds of interest and add to a 384-well Echo® Qualified microplate. This will serve as the source plate.

2. If testing in dose–response, an 8-point 1:3 serial dilution can be prepared on the source plate at this time.
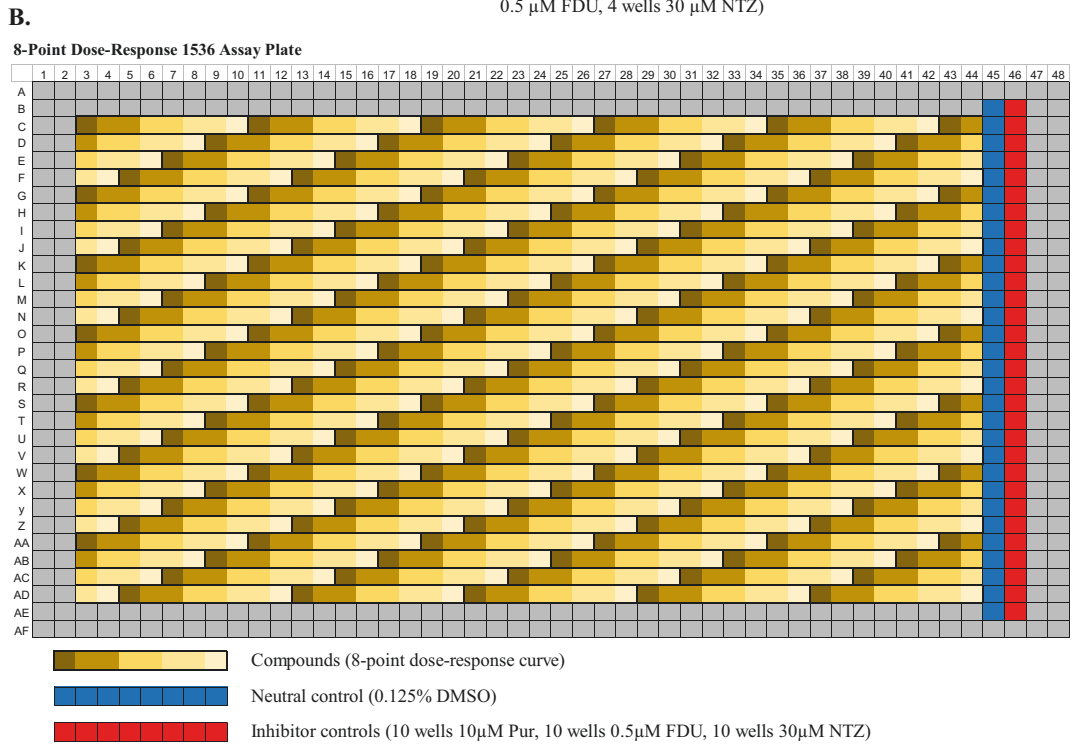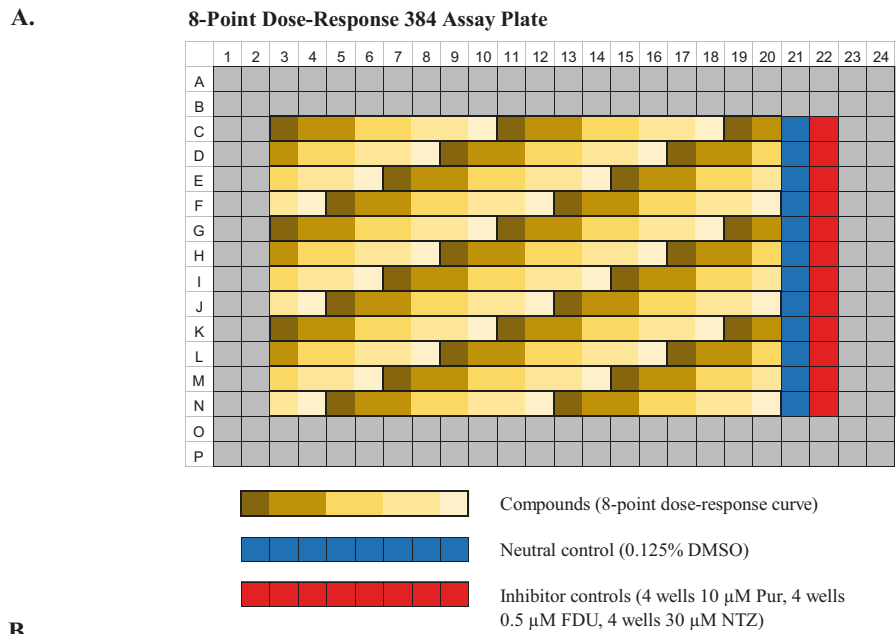
3. 24 h after host cell seeding, remove plates from the 37 °C incubator (*see* **Note 7**).

4. Using the Echo®, transfer compounds of interest into the cell-seeded assay plates in either single-point (Fig. 1) or 8-point dose–response format (Fig. 2).

**A.**

**Single Point 384 Assay Plate**



Compounds (1.875 μM FAC)

Neutral control (0.125% DMSO)

Inhibitor controls (4 wells 10 μM Pur, 4 wells 0.5 μM FDU, 4 wells 30 μM NTZ)

**B.**

**Single Point 1536 Assay Plate**



Compounds (1.875 μM FAC)

Neutral control (0.125% DMSO)

Inhibitor controls (10 wells 10 μM Pur, 10 wells 0.5 μM FDU, 10 wells 30 μM NTZ)

**Fig. 1** Plate layouts for (**A**) 384- and (**B**) 1536-well formats in single-point testing. These formats are for both primary and triplicate reconfirmation steps. *FAC* final assay concentration. The values provided are the most often used in our *Cryptosporidium* screening assays

**A.**

**8-Point Dose-Response 384 Assay Plate**



| | Compounds (8-point dose-response curve) |
| | Neutral control (0.125% DMSO) |
| | Inhibitor controls (4 wells 10 μM Pur, 4 wells 0.5 μM FDU, 4 wells 30 μM NTZ) |

**B.**

**8-Point Dose-Response 1536 Assay Plate**



| | Compounds (8-point dose-response curve) |
| | Neutral control (0.125% DMSO) |
| | Inhibitor controls (10 wells 10μM Pur, 10 wells 0.5μM FDU, 10 wells 30μM NTZ) |

**Fig. 2** Plate layouts for (**A**) 384- and (**B**) 1536-well formats in dose–response testing. A 1:3 serial dilution is recommended for the dose–response titration

5. Ensure that controls at the proper concentration, as well as DMSO in an equivalent volume (for negative or neutral control), are added to each assay plate.

6. After compound transfer, place the assay plates back into the 37 °C incubator until the infection step.

*3.3  Cryptosporidium Oocyst Excystation and Infection*

1. Prewarm 1 mL of 10 mM HCl in a microcentrifuge tube to 37 °C in a water bath incubator for ≥10 min.

2. Calculate the volume of oocyst suspension needed to infect the number of plates seeded the day prior:

   *For 384-well plates*: 15 µL/well or 5.76 mL/plate; 7.5 mL with dead volume.

   *For 1536-well plates*: 3 µL/well or 4.61 mL/plate; 6 mL with dead volume.

3. Calculate the quantity of oocysts needed (*see* **Note 8**).

4. Remove the number of oocysts required from the stock and transfer to the microcentrifuge tube containing prewarmed 10 mM HCl. If the volume of oocyst stock is >200 µL, separate into equal volumes across 2–3 microcentrifuge tubes and centrifuge at $16,000 \times g$ for 2 min. Remove the supernatant, and resuspend with the 10 mM HCl.

5. Incubate at 37 °C in a water bath incubator for 10 min.

6. Centrifuge at $16,000 \times g$ for 2 min. Carefully remove the supernatant.

7. Resuspend in enough excystation buffer to achieve 0.05 mM sodium taurocholate in the final dispense volume.

8. Incubate 40–60 min in the excystation buffer at room temperature.

9. Dilute excysted oocysts with assay medium to an optimized density (*see* **Note 9**).

10. Remove 10 µL of oocyst suspension and count on a C-Chip disposable hemocytometer. Both unexcysted oocysts and sporozoites should be examined to determine the excystation ratio, though this is more qualitative than quantitative. For 100% excystation, there should be four sporozoites to each empty oocyst shell. If excystation has failed, there will be no visible sporozoites. If excystation has initiated in the 40–60 min incubation, the dispensing can commence as oocysts will continue to excyst in the assay plates.

11. Prepare the MultiFlo™ dispenser and 1 µL cassette for dispense: assemble the cassette and prime the line with 70% ethanol to disinfect, followed by sterile water, assay medium, and finally, the oocyst suspension. If the volume and set-up allow, a sterile stir bar may be added and the oocyst suspension is placed

on a stir plate to prevent oocysts and sporozoites from settling and causing uneven quantities dispensed into the assay plates.

12. Dispense excysted oocysts into assay plates at the indicated volumes in **step 2** for each microplate type.

13. Centrifuge plates in a tabletop centrifuge with microplate adapters at $150 \times g$ for 3 min with soft deceleration.

14. Replace the low-evaporation metal lids onto each plate, and place in the 37 °C incubator for 48 h.

*3.4  Post-Assay Microplate Processing and Cell Staining*

1. After 48 h, remove the assay plates from the incubator.

2. Using the microplate bottle valve washer, add 2 μL (for 1536-well plates) or 10 μL (for 384-well plates) of 20% paraformaldehyde to each well to fix the cells (final well volume 10 μL in 1536-well plates; 50 μL in 384-well plates; final concentration 4% paraformaldehyde).

3. Incubate at room temperature for 15 min.

4. Aspirate the assay media:paraformaldehyde solution.

5. For all remaining steps, use the following volumes:

    8 μL/well for 1536-well plates.

    40 μL/well for 384-well plates

6. Wash 2× with wash buffer A (*see* **Note 10**).

7. Dispense permeabilization buffer.

8. Incubate at room temperature for 5–10 min. If possible, keep incubation ≤20 min.

9. Wash 2× with wash buffer B.

10. Dispense blocking agent.

11. Incubate at room temperature for 1 h (*see* **Note 11**).

12. Aspirate the blocking agent.

13. Dispense the visualization buffer.

14. Incubate at room temperature for 1 h.

15. Wash 2× with wash buffer B.

16. Seal each plate with adhesive foil seals and keep plate bottoms protected from light.

*3.5  High-Content Imaging and Data Processing*

1. Image plates with a cellomics instrument coupled with High Content Screening analysis software. We use the CellInsight CX5 system with HCS Studio 2.0, Spot Detector module. The images are obtained with a 10× objective using two channels: excitation/emission (nm) 384/440 for DAPI (host nuclei) and 485/521 for FITC (*Cryptosporidium* parasites).

    *For 384-well plates*, up to nine microscopic fields ($802{,}511.39 \ \mu m^2$/field) can be captured.

*For 1536-well plates*, only one microscopic field per well can be captured.

2. DAPI-stained host cell nuclei is assessed through Selected Object Count, and FITC-labeled *Cryptosporidium* intracellular parasites are assessed through Spot Count, which is the total count of FITC spots within the allowed "cell area" as determined by the optimized protocol (Fig. 3).

3. Host cell and *Cryptosporidium* counts are loaded into Genedata analysis software (Version 13.0 Standard) (Genedata, Basel, Switzerland).

4. *Cryptosporidium* spot counts and HCT-8 selected object counts are normalized to neutral controls minus inhibitor controls. We used 0.5 μM FDU as the normalizing inhibitor control for *Cryptosporidium* proliferation and 10 μM Pur as the HCT-8 cytotoxicity control.

5. Correct for any obvious effects due to liquid-handling errors in specific wells or groups of wells, e.g., if there is a tip that failed
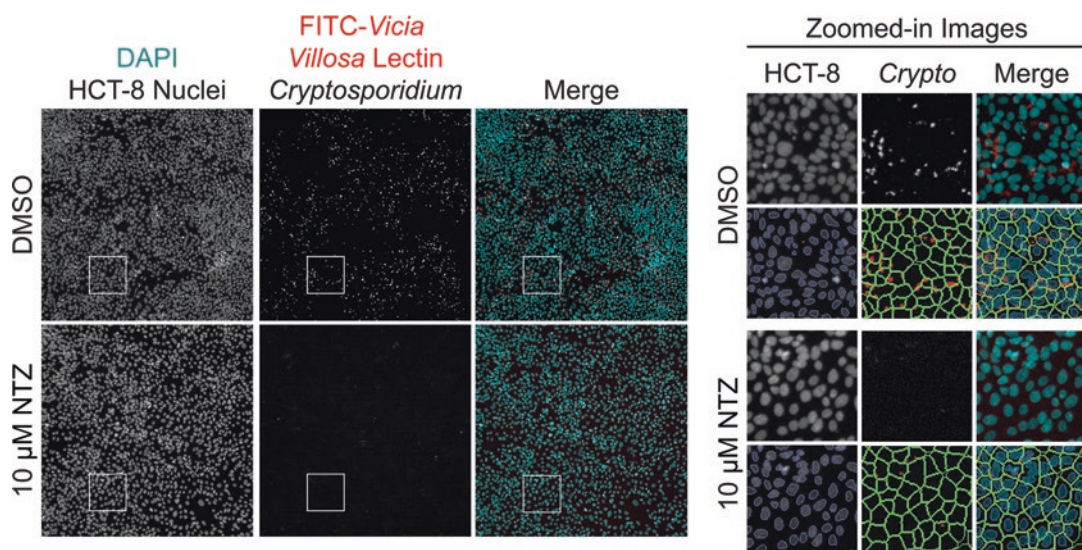


**Fig. 3** Screening images and software analysis. Representative images and software analysis from 1536-well High Content Imaging on the Thermo Fisher Scientific CellInsight CX5 HCS Platform. (Left) Images of two wells mock-treated with either dimethyl sulfoxide (DMSO) or 10 μM nitazoxanide. A merged image of the two fluorescent channels is artificially colored to show contrast: DAPI in cyan and FITC in red. Well area for each full-sized image is 802,511.39 μm². Zoomed-in images from the inset white squares in the left panel (area is 24,318.53 μm²) are shown. The image overlays are applied by the imaging software to assess the assay metrics: HCT-8 cell count and *Cryptosporidium* spot count. First, the host cell nuclei are identified and counted based on DAPI signal; cell debris and other particles are rejected based on a size filter (orange). Next, a region of interest, or "cell area" is drawn around each host cell nuclei to encompass where *Cryptosporidium* parasites may be located. Finally, the software identifies and counts "spots" within the "cell area" based on signal from the FITC-conjugated *Vicia villosa* lectin (Reprinted from ref. 14 used under the Creative Commons BY 4.0 license)

to dispense evenly, those wells affected may need to be excluded.

6. Select hits based on an appropriate activity cutoff (*see* **Note 12**).

7. If testing in dose–response, fit curves to determine the $EC_{50}$ value of each compound. We used the Smart Fit option in the Genedata software.

8. Values for both host cells and *Cryptosporodium* parasites can be determined (*see* **Note 13**).

# 4   Notes

1. The excystation buffer should be prepared fresh before each assay.

2. There are several acoustic transfer liquid handlers on the market, but we are equipped with the Labcyte Echo® 555 system. This type of liquid handler is not essential for 384-well, but necessary for 1536-well as pipetting "by hand" is difficult in this format.

3. The two types of Echo® qualified microplates include a polypropylene plate with a 15–65 μL working volume, and a cyclic olefin copolymer plate with a 3–12 μL working volume.

4. We use a "bottle-valve" type of dispenser/washer fitted with a 32-head aspirator for 1536-well formats. Our dispenser was custom made, but any 384- or 1536-well compatible washer will do. If an automated instrument is not available, "flicking" plates is also an option. This involves holding a microplate upside-down and flicking in a downward motion to expel the liquid out of the wells. For an infectious agent such as *Cryptosporidium*, care must be taken to contain the expelled liquid into a trap. Flicking works for both 1536- and 384-well formats.

5. We find that HCT-8 cells grow best in a 75 cm² flask (T75) with 20 mL of propagation medium and gentle splitting. This cell line maintains a consistent phenotype when left in high confluency and a 2:5 split ratio twice per week. We find that we have consistent infections from passages 30 to 45 (P30–P45), and therefore, we keep frozen stocks of cells around P28 to quickly reach an "aged" passage number from thaw. Also of note is that the propagation medium contains fetal bovine serum, whereas the assay medium contains horse serum. The recommended serum for this cell line from the source (ATCC) is horse serum, but we found that the cells did not grow consistently, though we use horse serum for the *Cryptosporidium* infection as these conditions gave better results.

6. Prior to setting up an assay, be sure to propagate the proper amount of cells needed for seeding the quantity of microplates intended. A single T75 flask contains roughly $4 \times 10^7$ HCT-8 cells when at 90–100% confluency.

7. As this is a "wet" transfer, as opposed to transferring compounds into an empty assay plate, this is a time-sensitive protocol. Compound transfer should take ≤2 h, and plates should be kept at 37 °C whenever possible.

8. The oocyst density is calculated from the stock and not from the number of excysted sporozoites. This is because the excystation rate is variable across strains, species, and even assays. A quick check to ensure that excystation has initiated is necessary, but oocysts will continue to excyst within the assay plates. For the four lines that we have routinely used, these are the optimized densities:

    For University of Arizona *C. parvum* (Iowa) oocysts, use 3,125 oocysts/well (MOI = 4).

    For Bunch Grass Farm *C. parvum* (Iowa) oocysts, use 1,500 oocysts/well (MOI = 2).

    For wild-type *C. parvum* (field isolate) oocysts, use 1,500 oocysts/well (MOI = 2).

    For *C. hominis* (TU502) oocysts, use 3,125 oocysts/well (MOI = 4).

9. The optimized densities are based on the starting number of oocysts as in **Note 8**. The densities for each commonly used *Cryptosporidium* line in assay medium are as follows:

    For University of Arizona *C. parvum* (Iowa) oocysts, $1.04 \times 10^6$ oocysts/mL.

    For Bunch Grass Farm *C. parvum* (Iowa) oocysts, $5.0 \times 10^5$ oocysts/mL.

    For wild-type *C. parvum* (field isolate) oocysts, $5.0 \times 10^5$ oocysts/mL.

    For *C. hominis* (TU502) oocysts, $1.04 \times 10^6$ oocysts/mL.

10. This is the first stopping point. Plates can be kept at 4 °C until processing can continue.

11. This is the second stopping point. Plates can be kept at 4 °C until processing can continue.

12. We have found that due to high variability (1–3× across samples and negative controls), a high hit cutoff of 70% inhibition improved our assay reproducibility and reconfirmation rates.

13. We incorporated a cytotoxicity cutoff into our workflow for hit selection. Compounds that inhibited *Cryptosporidium* propagation by ≥70% but inhibited HCT-8 growth ≥40% were excluded from our hit lists. These cutoffs were selected for our assay specifically, and can be changed depending on the goals of the screen.

## References

1. Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, Panchalingam S, Wu Y, Sow SO, Sur D, Breiman RF, Faruque AS, Zaidi AK, Saha D, Alonso PL, Tamboura B, Sanogo D, Onwuchekwa U, Manna B, Ramamurthy T, Kanungo S, Ochieng JB, Omore R, Oundo JO, Hossain A, Das SK, Ahmed S, Qureshi S, Quadri F, Adegbola RA, Antonio M, Hossain MJ, Akinsola A, Mandomando I, Nhampossa T, Acacio S, Biswas K, O'Reilly CE, Mintz ED, Berkeley LY, Muhsen K, Sommerfelt H, Robins-Browne RM, Levine MM (2013) Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. Lancet 382 (9888):209–222. https://doi.org/10.1016/S0140-6736(13)60844-2

2. Platts-Mills JA, Babji S, Bodhidatta L, Gratz J, Haque R, Havt A, McCormick BJ, McGrath M, Olortegui MP, Samie A, Shakoor S, Mondal D, Lima IF, Hariraju D, Rayamajhi BB, Qureshi S, Kabir F, Yori PP, Mufamadi B, Amour C, Carreon JD, Richard SA, Lang D, Bessong P, Mduma E, Ahmed T, Lima AA, Mason CJ, Zaidi AK, Bhutta ZA, Kosek M, Guerrant RL, Gottlieb M, Miller M, Kang G, Houpt ER (2015) Pathogen-specific burdens of community diarrhoea in developing countries: a multisite birth cohort study (MAL-ED). Lancet Glob Health 3(9):e564–e575. https://doi.org/10.1016/s2214-109x(15)00151-5

3. Arrowood MJ (2002) In vitro cultivation of *Cryptosporidium* species. Clin Microbiol Rev 15(3):390–400

4. Hijjawi NS, Meloni BP, Morgan UM, Thompson RC (2001) Complete development and long-term maintenance of *Cryptosporidium parvum* human and cattle genotypes in cell culture. Int J Parasitol 31(10):1048–1055

5. Upton SJ, Tilley M, Nesterenko MV, Brillhart DB (1994) A simple and reliable method of producing in vitro infections of *Cryptosporidium parvum* (Apicomplexa). FEMS Microbiol Lett 118(1–2):45–49

6. Bessoff K, Sateriale A, Lee KK, Huston CD (2013) Drug repurposing screen reveals FDA-approved inhibitors of human HMG-CoA reductase and isoprenoid synthesis that block *Cryptosporidium parvum* growth. Antimicrob Agents Chemother 57(4):1804–1814. https://doi.org/10.1128/aac.02460-12

7. Murphy RC, Ojo KK, Larson ET, Castellanos-Gonzalez A, Perera BG, Keyloun KR, Kim JE, Bhandari JG, Muller NR, Verlinde CL, White AC Jr, Merritt EA, Van Voorhis WC, Maly DJ (2010) Discovery of potent and selective inhibitors of calcium-dependent protein kinase 1 (CDPK1) from *C. parvum* and *T. gondii*. ACS Med Chem Lett 1(7):331–335. https://doi.org/10.1021/ml100096t

8. Sharling L, Liu X, Gollapalli DR, Maurya SK, Hedstrom L, Striepen B (2010) A screening pipeline for antiparasitic agents targeting *Cryptosporidium* inosine monophosphate dehydrogenase. PLoS Negl Trop Dis 4(8):e794. https://doi.org/10.1371/journal.pntd.0000794

9. Umejiego NN, Gollapalli D, Sharling L, Volftsun A, Lu J, Benjamin NN, Stroupe AH, Riera TV, Striepen B, Hedstrom L (2008) Targeting a prokaryotic protein in a eukaryotic pathogen: identification of lead compounds against cryptosporidiosis. Chem Biol 15(1):70–77. https://doi.org/10.1016/j.chembiol.2007.12.010

10. Gorla SK, McNair NN, Yang G, Gao S, Hu M, Jala VR, Haribabu B, Striepen B, Cuny GD, Mead JR, Hedstrom L (2014) Validation of IMP dehydrogenase inhibitors in a mouse model of cryptosporidiosis. Antimicrob Agents Chemother 58(3):1603–1614. https://doi.org/10.1128/aac.02075-13

11. Bessoff K, Spangenberg T, Foderaro JE, Jumani RS, Ward GE, Huston CD (2014) Identification of *Cryptosporidium parvum* active chemical series by repurposing the open access malaria box. Antimicrob Agents Chemother 58(5):2731–2739. https://doi.org/10.1128/aac.02641-13

12. Manjunatha UH, Vinayak S, Zambriski JA, Chao AT, Sy T, Noble CG, Bonamy GMC, Kondreddi RR, Zou B, Gedeck P, Brooks CF, Herbert GT, Sateriale A, Tandel J, Noh S, Lakshminarayana SB, Lim SH, Goodman LB, Bodenreider C, Feng G, Zhang L, Blasco F, Wagner J, Leong FJ, Striepen B, Diagana TT (2017) A *Cryptosporidium* PI(4)K inhibitor is a drug candidate for cryptosporidiosis. Nature 546(7658):376–380. https://doi.org/10.1038/nature22337

13. Chen JJ, Rateb ME, Love MS, Xu Z, Yang D, Zhu X, Huang Y, Zhao LX, Jiang Y, Duan Y, McNamara CW, Shen B (2018) Herbicidins from *Streptomyces* sp. CB01388 showing anti-*Cryptosporidium* activity. J Nat Prod 81(4):791–797. https://doi.org/10.1021/acs.jnatprod.7b00850

14. Love MS, Beasley FC, Jumani RS, Wright TM, Chatterjee AK, Huston CD, Schultz PG,

McNamara CW (2017) A high-throughput phenotypic screen identifies clofazimine as a potential treatment for cryptosporidiosis. PLoS Negl Trop Dis 11(2):e0005373. https://doi.org/10.1371/journal.pntd.0005373

15. Giuliano KA, Haskins JR, Taylor DL (2003) Advances in high content screening for drug discovery. Assay Drug Dev Technol 1(4): 565–577. https://doi.org/10.1089/154065803322302826

16. Ang ML, Pethe K (2016) Contribution of high-content imaging technologies to the development of anti-infective drugs. Cytometry A 89(8):755–760. https://doi.org/10.1002/cyto.a.22885

17. Zhang JH, Chung TD, Oldenburg KR (1999) A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J Biomol Screen 4(2):67–73. https://doi.org/10.1177/108705719900400206

# Chapter 18

# High-Throughput Screening of Drugs Against the Growth of *Cryptosporidium parvum* In Vitro by qRT-PCR

## Haili Zhang and Guan Zhu

## Abstract

An effective method to quantify the parasite loads is the key to the evaluation of anti-cryptosporidial drug efficacy in vitro. However, high-throughput screening (HTS) of drugs against *Cryptosporidium parvum* in vitro was impractical by the labor-intensive traditional assays. Here we describe a simplified quantitative RT-PCR assay suitable for HTS of compounds and for evaluating drug efficacy against the growth of *C. parvum* in vitro.

Key words *Cryptosporidium parvum*, In vitro cell culture, Real-time quantitative RT-PCR (qRT-PCR), Drug screening

## 1 Introduction

High-throughput screening (HTS) of compounds in vitro is important in the process of drug discovery against infectious diseases, but it was previously impractical for the intracellular parasite *Cryptosporidium parvum* due to the lack of suitable assays. Recently, two phenotypic screening assays have been developed. The first assay was based on high-content imaging analysis ($Z' = 0.21$–$0.47$), in which cells were grown in 384-well, black-walled, clear-bottom microwell plates, and selected microscopic fields of immunofluorescence-labeled intracellular parasites were captured for batch image analysis with ImageJ [1]. Our laboratory has developed a second assay based on quantitative RT-PCR (qRT-PCR) detection of the parasite 18S rRNA [2]. In this assay, host cells were grown in regular 96-well cell culture plates for inoculation of *C. parvum* and drug treatment, and HTS was achieved by directly using cell lysates as the templates in qRT-PCR in 384-well plates.

In comparison to qPCR, qRT-PCR has several apparent advantages in assessing the parasite loads in vitro: (1) First, the standard curves between cycle threshold ($C_T$) values and the logarithm of parasite loads produced by qRT-PCR are much more linear than

those produced by qPCR; (2) the levels of RNA molecules are a better indication of cell viability than those of DNA, and they are also degraded more quickly than DNA after the death of cells; and (3) total RNA can be more easily extracted, making it possible to directly use cell lysates prepared by appropriate lysis buffer in high-throughput format. In comparison to the high-content imaging analysis, which faces the challenge of distinguishing live parasites from those died but yet undegraded and separating mature meronts (containing multiple unreleased merozoites), just released merozoites, or early-stage meronts (representing a fraction of a mature meront), and broken parasitophorous vacuole membranes (that may also be fluorescent-labeled after merozoites are released), qRT-PCR avoids any sampling and counting errors by using cell lysates extracted from whole wells.

In the qRT-PCR assay, we also detect the levels of host cell 18S rRNA for normalization. Together with the use of multi-channel pipettors and robotic pipetting, the qRT-PCR-base assay is able to achieve excellence in assay uniformity (i.e., <5% coefficient of variation [CV] and satisfactory intra-plate, inter-plate, and inter-day uniformity), signal to-noise ratios (SW = 7.4–44.1 and $Z'$ = 0.73–0.87), and linear dynamic range (i.e., >150-fold linear range in detecting the parasite loads). The procedure of the qRT-PCR assay for drug screening is highlighted in Fig. 1.
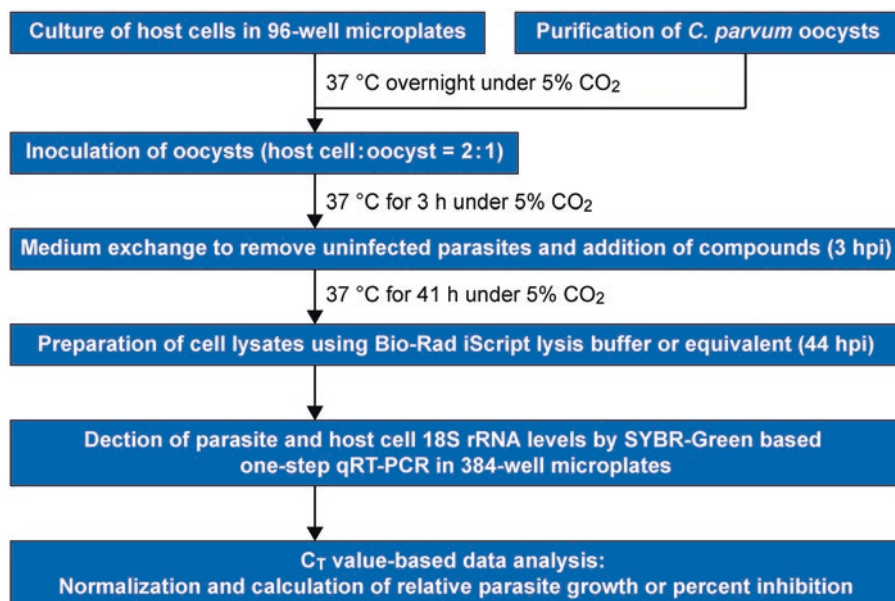


**Fig. 1** Illustration of the procedure for high-throughput screening of drugs or evaluating drug efficacy against *Cryptosporidium parvum* in vitro by qRT-PCR detection of the relative levels of the parasite 18S rRNA (Cp18S) normalized with levels of the host cell 18S rRNA (Hs18S)

In addition to drug screening, the qRT-PCR assay may also be used for detecting the parasite loads in other studies (e.g., the growth of *C. parvum* under various conditions) and adapted to the drug screening against other intracellular pathogens with specified primers. When more sensitive detection and better linear dynamic range are needed, such as to evaluate lower level of parasite loads in the early stage of development, RNA samples may be prepared by traditional column-based purification procedure to achieve up to $10^4$ folds of linear dynamic range [3].

## 2  Materials

### 2.1  Common Reagents and Kits

1. Ultrapure water.
2. Phosphate-buffered saline (PBS; pH 7.4).
3. Taurodeoxycholic acid (TDC).
4. Trypsin.
5. RPMI 1640 culture medium.
6. Fetal bovine serum (FBS).
7. 10× Trypsin/EDTA cell detachment solution (0.5% trypsin and 0.2% EDTA) and Detachin cell detachment solution (Genlantis, Inc., San Diego, CA).
8. Percoll solution.
9. iScript RT-qPCR sample preparation reagent (Bio-Rad Laboratories, Inc., Hercules, CA).
10. qScript one-step SYBR green qRT-PCR kit (Quanta Biosciences, Beverly, MA) or iTaq Universal SYBR® Green One-Step Kit (Bio-Rad).

### 2.2  Common Equipment and Supplies

1. 96-well cell culture plates.
2. 384-well real-time PCR plates.
3. NanoDrop ND-1000 spectrophotometer or equivalent is used for quantitation of total RNA (NanoDrop Technologies, Inc., Wilmington, DE).
4. Bio-Rad CFX384 Touch Real-Time PCR Detection System (Bio-Rad).
5. Multi-tube vortexer.
6. Automated pipetting system (epMotion 5070, Eppendorf North America, Hauppauge, NY).
7. Class II biosafety cabinet.
8. $CO_2$ incubator.
9. Inverted microscope.
10. Adhesive sealing films.
11. PCR plate sealing film (Bio-Rad).

| **2.3** | **Host Cells** | An adherent ileocecal colorectal adenocarcinoma cell line (HCT-8; ATCC # CCL-244) is used to host the growth of *C. parvum* in vitro. This cell line supports superior development of *C. parvum* in comparison with 10 other cell lines [4, 5]. However, other cell lines may be used for various purposes (e.g., Caco-2 cells). HCT-8 cells are maintained in RPMI 1640 medium supplied with 10% fetal bovine serum (FBS) under 5% $CO_2$ atmosphere and subcultured by 1:4 to 1:8 dilution of cells with the exchange of fresh medium every 2–3 days. |

| **2.4** | **Parasite** | Several strains of *C. parvum* are propagated in, and may be acquired from, commercial sources or research laboratories, e.g., Bunch Grass Farm (Deary, ID) and Waterborne, Inc. (New Orleans, LA). We typically purchase partially purified fresh *C. parvum* oocysts from Bunch Grass Farm (Deary, ID) and Waterborne, Inc. (New Orleans, LA) and store them at 4 °C in phosphate buffered saline (PBS) containing penicillin (100 units/mL) and streptomycin (100 μg/mL). It is noticed that the original strain propagated in Brunch Grass Farm was the Iowa-1 strain (subtype IIaA15G2R1 based on the *gp60* gene), but has been replaced by a local isolate with a subtype IIaA17G2R1. Oocysts are further purified by a Percoll-based gradient centrifugation protocol (see details in Subheading 3.4). Oocysts used in the in vitro drug efficacy assay should be less than 3 months old with an in vitro excystation rate at >80% (see details in Subheading 3.5). |

| **2.5** | **RNA Isolation Reagents** | For HTS or most drug testing assays, several lysis buffers are commercially available to prepare cell lysates, including Bio-Rad iScript RT-qPCR sample preparation reagent (termed as Bio-Rad iScript lysis buffer in this protocol) and Ambion Cell-to-cDNA kit (Ambion, Inc., Foster City, CA). The cell lysates can be diluted and directly used for synthesizing cDNA from RNA. When a larger linear dynamic range is needed, such as in the case samples containing a much lower level of parasite loads during the invasion and early stage development, total RNA can be isolated using traditional resin column-based isolation kits, such as Qiagen RNeasy MiniKit (Qiagen Inc., Germantown, MD). |

| **2.6** | **qRT-PCR Primers** | The protocol uses the relative levels of *C. parvum* 18S rRNA (Cp18S; Reference sequence in Genbank: NC_006986.1) normalized with those of host cell 18S rRNA (Hs18S; Reference sequence in Genbank: NR_003286) as the indicator of the relative parasite loads [2]. We prefer using 18S rRNA because 18S and 28S rRNA make up ~80% of a total RNA sample and 18S rRNA is less likely to fluctuate under conditions affecting the expression of housekeeping genes. The primers listed in Table 1 are selected from several pairs of primers in our earlier optimization procedure of the qRT-PCR protocol. |

**Table 1**
**Primers used in determining the relative loads of *C. parvum* in vitro by qRT-PCR**

| Target | Primer name | Primer sequence (5′–3′) | Final conc. | Product length |
|---|---|---|---|---|
| Parasite 18S rRNA (Cp18S) | Cp18S-1011F | TTGTTCCTTACTCCTTCAGCAC | 250 nM | 175 nt |
|  | Cp18S-1185R | TCCTTCCTATGTCTGGACCTG | 250 nM |  |
| Host cell 18S rRNA (Hs18S) | Hs18S-1F | GGCGCCCCTCGATGCTCTTA | 700 nM | 189 nt |
|  | Hs18S-1R | CCCCCGGCCGTCCCTCTTA | 700 nM |  |

**2.7  qRT-PCR Kit**

Several commercial sources of one-step RT-qPCR kits are available. We had previously used the iScript One-step RT-PCR kit with SYBR (Bio-Rad), and are currently using a more cost-effective qScript one-step SYBR green qRT-PCR kit (Quanta BioSciences, Inc., Beverly, MA).

## 3  Methods

All procedures are carried out at room temperature unless otherwise specified. Strictly follow biosafety level 2 (BSL2) practices when handling live *C. parvum* parasites.

**3.1  Preparation of Cell Culture Medium**

1. Add 100 mL of RPMI 1640 medium 10× stock solution (or dissolve powdered RPMI 1640 medium for 1.0 L) in ~500 mL ultrapure water into a sterilized 1.0 L conical glass flask containing a magnetic stir bar.

2. Add 2.0 g of sodium bicarbonate, 1.0 g of glucose, 100 mL of heat-inactivated fetal bovine serum (FBS), and 10 mL of antibiotics mixture, containing 10,000 unit/mL of penicillin and 10,000 μg/mL of streptomycin, to the medium.

3. Gently mix the solution well on a magnetic stir plate.

4. Add ultrapure water to make a final volume of 1000 mL and mix gently.

5. Sterilize the culture medium by filtration with a 0.2 μm filter unit.

6. Store the medium at 4 °C.

**3.2  Recovery of Cryopreserved Host Cells**

1. Thaw one vial of cryopreserved cells in a 37 °C water bath with gentle agitation.

2. Remove the vial from the water bath as soon as cells are thawed (usually within ~2 min), and decontaminate the surface of the vial by spraying with 70% ethanol.

3. Move the vial to a biosafety cabinet and carry out all remaining procedures under strict aseptic conditions.

4. Transfer thawed cells to a centrifuge tube containing 9.0 mL culture medium, and centrifuge for 5 min at $125 \times g$.

5. Resuspend cell pellet with a few mL of cell culture medium and dispense cell suspension into a new culture flask.

6. Incubate cells at 37 °C in a cell culture incubator supplied with 5% $CO_2$ until the cell monolayer grows to confluence. Exchange medium every 2 days.

**3.3 Subculture of HCT-8 Cells**

HCT-8 cells can be used in routine cultivation of *C. parvum* in various experiments, including drug efficacy assay, because this cell line supports superior development of *C. parvum* in comparison with 10 other cell lines [4, 5]. However, other cell lines may be used for various purposes.

All cell lines need to be routinely monitored for potential contaminations, particularly for mycoplasma contamination by fluorescent staining (e.g., DAPI or Hoechst 33258), ELISA, PCR, immunostaining, autoradiography, or microbiological assays. We routinely use DAPI-staining, or commercially available PCR detection primers for more sensitive and/or specific detection of mycoplasma contamination.

1. Remove old culture medium from the flask containing cultured HCT-8 cells with ~90–100% confluence.

2. Briefly rinse the cell monolayer one time with 10 mL sterile PBS (PH 7.4) to remove all traces of serum.

3. Add 2.0 mL of Detachin cell detachment solution (Genlantis) to a flask, and rock the flask back and forth to cover the entire cell monolayer with Detachin.

4. Incubate the flask at 37 °C for 5–10 min and observe cells under an inverted microscope until the cell layer is dispersed.

5. To facilitate dispersal, gently tap the flask three times on a sterilized absorbance pad.

6. Add 8.0 mL of culture medium to neutralize the Detachin. There is no need to wash the Detachin solution away or add any enzyme inhibitors in cells.

7. Inoculate 10–20% of the detached cells into a new 75 cm² cell culture flask containing 10 mL fresh culture medium (i.e., 1:5 to 1:10 subcultivation ratio).

8. Incubate cells until the cell monolayer reaches to ~100% confluence (typically in 2 days) with medium exchange every 2 days.

9. Continue subculture of cells or prepare frozen cells in complete cell culture medium containing 5% DMSO using a standard cryopreservation protocol.

**3.4 Purification of** *C. parvum* **Oocysts**

The oocysts of *C. parvum* purchased from Bunch Grass Farm are partially purified by a sucrose-based gradient centrifugation procedure, which can be further purified by a Percoll-based gradient centrifugation and surface cleaned with 10% bleach [6, 7]. All solutions are sterilized by either autoclave or filtration with 0.2 μm filter units.

1. Resuspend a desired number of oocysts in PBS in a 1.5 mL microcentrifuge tube.

2. Centrifuge the oocyst suspension at $2000 \times g$ for 10 min, remove supernatant, and vortex to loosen the oocyst pellet.

3. Prepare 1.0 mL of 57% isotonic Percoll solution (Percoll/$H_2O$/1.5 M NaCl = 5.7:3.3:1).

4. Resuspend oocysts in 1.0 mL of 57% Percoll solution in 1.5 mL microcentrifuge tube and mix well by pipetting.

5. Centrifuge the suspension at $2000 \times g$ for 30 min at 4 °C. Dead bacteria and other debris will form a sticky pellet at the bottom while oocysts remain in the supernatant.

6. Transfer supernatant with oocysts to a 15 mL tube, and dilute the suspension with >4× volume of $dH_2O$.

7. Wash oocysts with $dH_2O$ for three times by centrifugation at $2000 \times g$ for 10 min at 4 °C.

8. Resuspend oocysts in 0.9 mL of ultrapure water.

9. Add 0.1 mL commercial bleach (~5% hypochlorite) into the suspension, mix well, and incubate the suspension on ice for 5 min. Do not prepare bleach in PBS, which may reduce the viability of oocysts.

10. Centrifuge the bleached oocyst suspension at $10,000 \times g$ for 2 min at 4 °C and remove the supernatant.

11. Wash oocysts twice with sterilized ultrapure water by centrifugation at $10,000 \times g$ for 2 min at 4 °C.

12. Resuspend oocysts in ~1.2 mL of PBS in a new microcentrifuge tube.

13. Continue washing oocysts 4–5 times with PBS by centrifugation.

14. Examine the purified oocysts under a microscope for purity.

15. Count the oocysts in a hemocytometer.

16. Store the cleaned oocysts in PBS with 20 units/mL of penicillin and 20 μg/mL of streptomycin at 4 °C.

**3.5 Evaluation of Oocyst Viability by In Vitro Excystation**

Before in vitro infection assay, the viability of oocysts may be examined by an in vitro excystation assay in a medium containing taurodeoxycholic acid (TDC) and trypsin. The oocysts of *C. parvum* may also undergo excystation in PBS or culture medium when incubated at 37 °C, but at lower excystation rates.

1. Prepare 1.0 mL of excystation medium in a 1.5 mL microcentrifuge tube by dissolving 0.5% taurodeoxycholic acid (TDC) and 0.25% trypsin in PBS.

2. Add ~$10^4$–$10^5$ oocysts into the excystation medium and mix.

3. Incubate the suspension at 37 °C for 45 min with occasional shake of the microcentrifuge tube.

4. Add 0.1 mL formalin to fix the parasite.

5. Centrifuge the suspension (5000 × $g$ for 5 min), remove supernatant, and resuspend the pellet in 0.1 mL PBS.

6. Prepare three microscopic slides with ~10 μL suspension/slide.

7. Count free sporozoites and intact oocysts under a microscope in three or more randomly selected microscopic fields until a total number of >300 sporozoites or oocysts are counted.

8. Calculate the in vitro excystation rate in each slide by the following equation:

$$\text{Excystation rate} \,(\%) = \frac{\text{No.of sporozoites}}{(\text{No.of sporozoites}) + ((\text{No.of intact oocysts}) \times 4)} \times 100 \,(\%) \qquad (1)$$

9. It is preferred using oocysts less than 3 months old with a higher than 80% excystation rate.

*3.6 Standard 44-h Infection Assay for Evaluating Drug Efficacy In Vitro*

The 44-h infection time point is preferred for general in vitro drug assay because no or a much smaller number of parasite merozoites or gametes are released into the medium at this time point, and it allows the subsequent preparation of cell lysates and qRT-PCR experiments to start in the morning of the day [2]. The time points may be adjusted according to the purpose of the assay.

1. Grow a sufficient number of host cells by subcultivation in 75 cm² cell culture flasks (i.e., ~3 × $10^6$ host cells for one 96-well culture plate). One 75 cm² flask may provide $10^7$ cells when cell monolayer reaches to ~100% confluence. However, do not overgrow host cells.

2. Detach cells, prepare cell suspensions in culture medium, count host cells with a hemocytometer, and resuspend cells in complete culture medium at a concentration of $1.25 \times 10^5$ cells/mL.

3. Seed host cells into 96-well culture plates by adding 200 μL host cell suspension into each well by an 8-channel pipettor (i.e., $2.5 \times 10^4$ host cells/well). Cell suspensions can be placed in a multichannel pipetting reagent reservoir. Mix cell suspension well before each pipetting step.

4. Incubate microplates containing host cells at 37 °C under 5% $CO_2$ atmosphere overnight until the cell monolayers reach to ~90% confluence.

5. Prepare cleaned *C. parvum* oocysts in complete culture medium at a concentration of $2 \times 10^5$ oocysts/mL.

6. Remove culture medium from 96-well plates containing host cell monolayers.

7. Add 100 μL of oocyst suspension per well (i.e., $2 \times 10^4$ oocysts; 2:1 host cell to oocyst ratio) using an 8-channel pipettor.

8. Incubate the cell culture plates containing *C. parvum* oocysts and host cell monolayers for 3 h at 37 °C to allow excystation of sporozoites from oocysts and invasion of sporozoites into host cells.

9. At 3 h post-infection time (hpi), remove supernatants containing oocyst walls and oocysts/sporozoites failed to undergo excystation or invasion.

10. Add 200 μL of culture medium containing drugs at desired concentrations. Remember to include appropriate negative control (diluent) and positive control (e.g., paromomycin or nitazoxanide at ~$IC_{70}$ concentrations), as well as two to three biological replicates for each experimental condition. It is recommended to include five to six biological replicates for the negative control (containing diluent only) because it sets up the baseline for all other experimental groups. Because compound libraries are typically stored in dimethyl sulfoxide (DMSO), it is recommended to include 0.5% DMSO in all wells, including treatment groups and controls.

11. Incubate the plates at 37 °C under 5% $CO_2$ atmosphere for an additional 41 h (i.e., 44 h total infection time).

12. Proceed to the preparation of cell lysates as described below.

### 3.7 Preparation of Cell Lysates Containing Total RNA from Host Cells and Parasites

The volumes described here are based on 96-well plates, which can be proportionally reduced or increased based on the volumes of culture vessels. In addition to this protocol, total RNA may also be isolated using a regular column resin-based RNA isolation kit (e.g., Qiagen's RNeasy RNA isolation kit) when a better sensitivity and higher linear dynamic range of the assay are needed.

1. Remove plates containing *C. parvum*-infected HCT-8 cells from incubator.

2. Centrifuge the plates for 10 min at $1000 \times g$ in a plate centrifuge.

3. Medium was removed using an 8-channel multichannel pipette, followed by two gentle rinses with PBS (200 μL/well).

4. Remove PBS, place plates on ice, and add 200 μL of ice-cold Bio-Rad iScript RT-qPCR sample preparation reagent into each well.

5. Seal plates with adhesive sealing films and incubate microplates at room temperature for 2 min.

6. Secure the plates in a multi-tube vortexer (VX-2500, VWR International, LLC, Suwanee, GA), and vortex for 20 min at the speed set at 7.

7. Remove plates from the vortexer, transfer 50 μL of cell lysate to 96-well microplates, and incubate the plates at 75 °C for 15 min on a PCR thermal cycler.

8. Centrifuge the plates at 2000 × *g* for 15 min to precipitate cell debris.

9. The plates can be placed on ice for short-term storage or stored at −80 °C until use.

*3.8   RNA Quality and Quantity*

The RNA concentration and quality can be determined using the NanoDrop 1000 spectrophotometer, in which 1.0 μL of RNA sample is sufficient to produce accurate and reproducible results. The lysis buffer is used as control for baseline. Sample concentrations can be estimated according to the absorbance at 260 nm. The ratio of absorbance at 260 and 280 nm (260/280) is used to assess the purity of RNA, in which a ratio of ~2.0 is generally acceptable. The ratio of absorbance at 260 and 230 nm (260/230) is a secondary measure of contamination by organic compounds, which is typically in the range of 1.8–2.2 [8].

*3.9   qRT-PCR Assay*

The current protocol has been optimized in our laboratory for reactions in 384-well plates. Different laboratories may consider optimizing the protocol under their own conditions, such as when different cell lines, parasite strains, reagents, and/or kits are used. These include optimal primer concentrations and the dilution factors of the cell lysates for Cp18S and Hs18S rRNA. Because cell lysates are directly used in qRT-PCR, they need to be appropriately diluted to reduce the inhibitory effect of the lysis buffer, but maintain sufficient sensitivity of the assay.

The reaction master mixes for both Cp18S and Hs18S genes are designed for 10 μL reaction volume in 384-well plates, which can be scaled up for reactions performed in 96-well plates. The pipetting of reaction cocktails and RNA templates into 384-well plates may be assisted using an automated pipetting machine (e.g., epMotion 5070 automated pipetting system). qRT-PCR reactions should include two technical replicates for each biological sample.

1. Thaw all components on ice.

2. Prepare the reaction cocktails for Cp18S and Hs18S as described in Tables 2 and 3. Scale up the volume of the cocktails based on the total numbers of reactions.

3. Mix the cocktails gently and centrifuge them to collect the contents to the bottom of the tube.

4. Load 7.0 μL of reaction master mix into each well of 384-well plates for real-time PCR system using an automated pipetting system.

5. Prepare RNA templates in 96-well plates or microcentrifuge tubes by diluting the supernatants of individual cell lysates 100× for detecting Cp18S rRNA (~3.0 ng total RNA) and 2000× for Hs18S rRNA (~0.3 ng total RNA).

6. Load 3.0 μL of RNA template to each reaction using an automated pipetting system.

7. Seal plates with PCR plate sealing films (Bio-Rad) and transfer them to a real-time PCR system (e.g., Bio-Rad CFX384 Touch Real-Time PCR Detection System).

8. Perform real-time qRT-PCR reactions according to the thermal cycling protocol described in Table 4.

9. After qRT-PCR reactions are completed, examine the amplification and melting curves to assess the quality and specificity of the reactions. A typical sigmoidal amplification curve plotted with relative fluorescence unit (RFU) against the logarithm of thermal cycles and a single peak in a melting curve plotted with the change of RFU (−dRFU/dT) against the temperature indicate specific amplification of an RNA template (Fig. 2).

**Table 2**
**Reaction cocktail for Cp18S gene in 10 μL reaction volume**

| Component | Volume/reaction | Final concentration |
|---|---|---|
| 2× One-Step SYBR Green Master mix | 5.0 μL | 1× |
| Cp18S-1011F primer (10 μM) | 0.25 μL | 0.25 μM |
| Cp18S-1185R primer (10 μM) | 0.25 μL | 0.25 μM |
| qScript One-Step RTase | 0.2 μL | 1× |
| Nuclease-free water | 1.3 μL | – |
| Total cocktail volume | 7.0 μL | – |

**Table 3**
**Reaction cocktail for Hs18S gene in 10 μL reaction volume**

| Component | Volume/reaction | Final concentration |
|---|---|---|
| 2× One-step SYBR Green Master mix | 5.0 μL | 1× |
| Hs18S-1F primer 10 (μM) | 0.7 μL | 0.7 μM |
| Hs18S-1R primer 10 (μM) | 0.7 μL | 0.7 μM |
| qScript one-step RT | 0.2 μL | 1× |
| Nuclease-free water | 0.4 μL | – |
| Total cocktail volume | 7.0 μL | – |

**Table 4**
**qRT-PCR thermal cycling protocol**

| Reaction step | Temperature | Duration |
|---|---|---|
| cDNA synthesis | 50 °C | 10 min |
| RTase inactivation | 95 °C | 5 min |
| Two-step thermal cycles (40×) | 95 °C | 10 s |
| | 58 °C | 30 s (data collection) |
| Melting curve | 65–95 °C with increment by 0.5 °C | 0.5 s/step (data collection) |
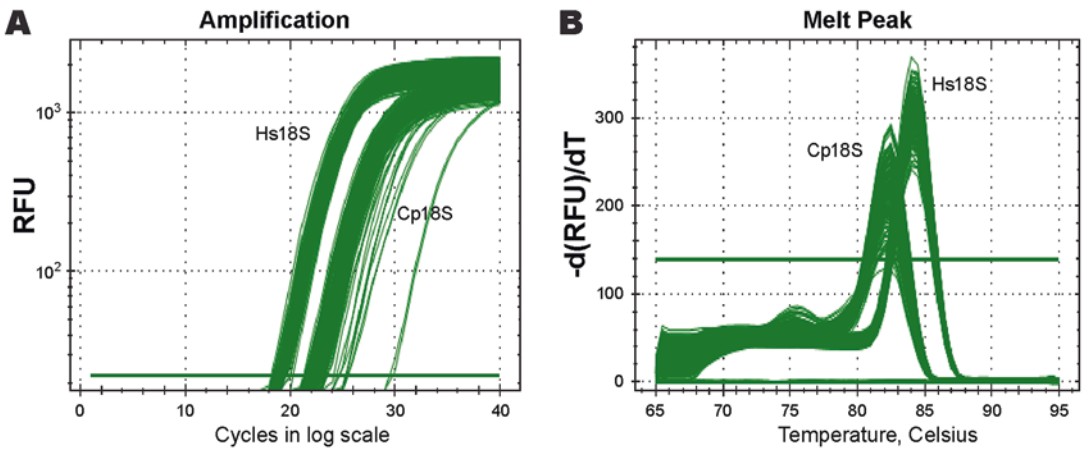


**Fig. 2** Specific detection of Cp18S and Hs18S rRNA transcripts as demonstrated by amplification curve (**a**) and melting curve (**b**) analyses of a representative plates. Adapted from "Quantitative RT-PCR assay for high-throughput screening (HTS) of drugs against the growth of *Cryptosporidium parvum* in vitro," by Haili Zhang and Guan Zhu, 2015, Frontier in Microbiology, 6:991. doi: https://doi.org/10.3389/fmicb.2015.00991. Copyright 2015 by Zhang and Zhu [2]

***3.10 Generation of Standard Curves***

A standard curve is useful in determining the linear dynamic range and amplification efficiency in the qRT-PCR assay. There are two approaches to generate standard curves. The first approach uses serially diluted RNA templates, which is indicative of the amplification efficiency in the qRT-PCR step. The second approach uses RNA samples isolated from cells infected with various numbers of parasites, which could correct systematic errors from infection. Using the second approach, we have previously shown that this qRT-PCR protocol could give a >150-fold linear dynamic range (Fig. 3). The procedure is briefly described as follows.

1. Seed and grow host cell monolayers in a 96-well cell culture plate until they reach ~90% confluence as described in Subheading 3.6.
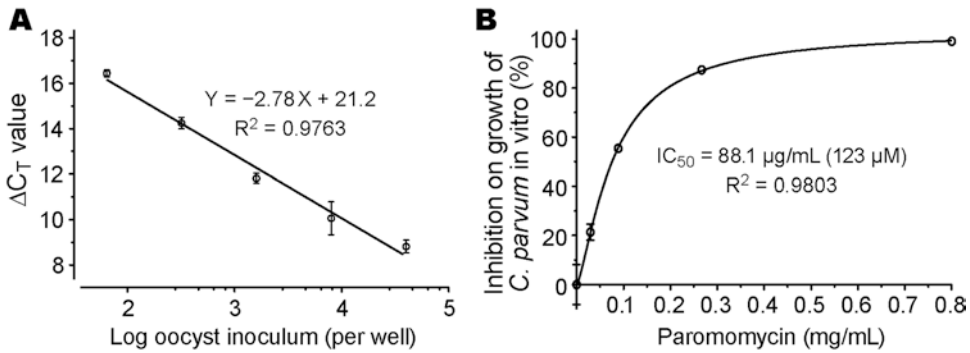
**Fig. 3** Evaluation of the qRT-PCR-based high-throughput screening assay. (**a**) Standard curves showing the relationship between the number of inoculated oocysts and $\Delta C_{T[Cp18S-Hs18S]}$ values. (**b**) Dose–response curve on the in vitro anti-cryptosporidial activity of paromomycin. Bars represent standard errors of the mean (SEM) derived from at least two biological replicates. Adapted from "Quantitative RT-PCR assay for high-throughput screening (HTS) of drugs against the growth of *Cryptosporidium parvum* in vitro," by Haili Zhang and Guan Zhu, 2015, Frontier in Microbiology, 6:991. doi: https://doi.org/10.3389/fmicb.2015.00991. Copyright 2015 by Zhang and Zhu [2]

2. Prepare fivefold serial dilutions of *C. parvum* oocysts in 300 μL culture medium for three replicates of 100 μL suspensions, each containing 40,000, 8000, 1600, 320, and 64 oocysts per 100 μL.

3. Remove culture medium from the cell culture plate and add 100 μL of oocyst suspensions into individual wells.

4. Place the plate into the 37 °C incubator to allow the parasite oocysts to undergo excystation and invasion into host cells for 3 h at 37 °C.

5. Replace the culture medium at 3 hpi with 200 μL/well of fresh medium to remove free parasites.

6. Incubate the infected cell monolayers at 37 °C with 5% $CO_2$ for additional 41 h.

7. Prepare cell lysates and perform qRT-PCR as described in Subheadings 3.7 and 3.9.

8. Plot the means of $\Delta C_T$ values from technical and biological replicates against the logarithm of the numbers of inoculated oocysts (Fig. 3a).

9. Conduct linear regression to obtain the standard curve, slope value, and $R^2$ value for determining the detection efficacy and data coefficient (Fig. 3a).

*3.11 Calculation of Parasite Loads or Drug Efficacy*

The relative parasite loads or inhibition of the parasite growth are calculated based on the cycle threshold ($C_T$) values of Cp18S and Hs18S transcripts [2, 3].

1. Compute the means of $C_{T[Cp18S]}$ and $C_{T[Hs18S]}$ values from technical replicates for each biological replicate.

2. Normalize the level of Cp18S with Hs18S in each sample by computing the $\Delta C_T$ between $C_{T[Cp18S]}$ and $C_{T[Hs18S]}$:

$$\Delta C_T = C_{T[Cp18S]} - \Delta C_{T[Hs18S]} \qquad (2)$$

3. Calculate the mean of $\Delta C_T$ values derived from all replicates of the negative control group in each plate or for each set of experimental condition:

$$\overline{\Delta C}_{T[ctl]} = \frac{1}{n}\sum_{i=1}^{n}\Delta C_{T[ctl]i} = \frac{\left(\Delta C_{T[ctl]1} + \Delta C_{T[ctl]2} + \cdots + \Delta C_{T[ctl]n}\right)}{n} \qquad (3)$$

4. Determine the relative level between individual sample and the mean of negative control by computing the $\Delta\Delta C_T$ values:

$$\Delta\Delta C_{T[sample]} = \Delta C_{T[sample]} - \overline{\Delta C}_{T[ctl]} \qquad (4)$$

Individual $\Delta\Delta C_T$ values of all biological replicates from the negative control group should also be calculated for later determining the standard error of the mean (SEM) or standard deviation of the mean (SD).

5. Calculate the normalized relative parasite loads or percent inhibition by one of the following two approaches.

*Approach 1* considers the efficiency of detection derived from the slope value of the standard curve (i.e., parameter $A$ in the following equation). The deduction of the equations below has been previously described in more detail [3, 2]:

$$A = 1 / Slope \qquad (5)$$

$$\text{Relative parasite load}\,(\%) = 10^{A\cdot\Delta\Delta C_T} \times 100 \qquad (6)$$

$$\text{Inhibition}\,(\%) = \left(1 - 10^{A\cdot\Delta\Delta C_T}\right) \times 100 \qquad (7)$$

*Approach 2* assumes perfect or near-perfect efficiency of detection using the empirical formulae:

$$\text{Relative parasite load}\,(\%) = 2^{-\Delta\Delta C_T} \times 100 \qquad (8)$$

$$\text{Inhibition}\,(\%) = \left(1 - 2^{-\Delta\Delta C_T}\right) \times 100 \qquad (9)$$

For comparison using the standard curve in Fig. 3a (the slope = −2.78), when $\Delta\Delta C_T$ value is 1.0, the percent inhibitions calculated using Eqs. 7 and 9 will be 56.3% and 50.0%, respectively. Therefore, under the same experimental conditions here, the empirical Eq. 9 would slightly underestimate the drug efficacy, thus providing more conservative estimation of drug efficacy.

**3.12 Validation of the Assay Using Paromomycin**

Before applying the assay to evaluating the anti-*C. parvum* efficacy in vitro, it is necessary to first validate the assay by testing the dose–response of a known anti-cryptosporidial compound, such as paromomycin.

1. Dissolve paromomycin in PBS and prepare twofold serial dilutions of paromomycin ranging from 800 to 50 µg/mL in cell culture medium, each containing a final concentration of 0.5% DMSO.

2. Prepare host cell monolayers in 96-well plates and infect host cells with cleaned fresh *C. parvum* oocysts for 3 h at 37 °C as described in Subheading 3.6.

3. At 3 hpi, remove the medium supernatants, add paromomycin at various concentrations and the diluent control into the plate (200 µL/well), and incubate the plate for 41 h at 37 °C with 5% $CO_2$.

4. At 44 hpi, prepare cell lysates and perform qRT-PCR as described in Subheadings 3.7 and 3.9.

5. Calculate the percentage inhibitions or relative parasite loads as described in Subheading 3.11.

6. Plot dose–response curve on percent inhibition or relative parasite loads against the drug concentrations. The anti-cryptosporidial $EC_{50}$ value of paromomycin would be around 90 µg/mL (Fig. 3b) [2].

**3.13 Validation of Assay for High-Throughput Screening (HTS)**

When the assay is applied to HTS screening, it is necessary to evaluate the intra-plate, inter-plate, and inter-day uniformity of the assay using approaches described in the chapter "HTS Assay Validation" at http://www.ncbi.nlm.nih.gov/books/NBK53196/ [9]. This method is recommended by the National Center for Advancing Translational Sciences (NCATS), National Institutes of Health (NIH) (see "Pre-Clinical Research Toolbox" at http://www.ncats.nih.gov/expertise/preclinical).

Briefly, inter-day tests can be performed by three independent experiments. Each experiment used three 96-well plates to assess inter-plate variations, and each plate contains three experimental groups, which include uninfected HCT-8 cells (blank "Min" background signal), cells infected with parasites but containing only 0.5% DMSO diluent (untreated, "Max" signal), and cells that are infected with parasites and treated with 120 µg/mL (168 µM) of PRM (drug-treated "Mid" signal). These groups (32 wells each) can be arranged in an interleaved-signal format according to the Assay Guidance Manual.

After typical infection for a total of 44 h as described in Subheading 3.6, qRT-PCR detections are performed with two technical replicates for each biological sample as described in Subheadings 3.7 and 3.9. Based on the qRT-PCR data, mean

(AVG), standard deviation (SD), and coefficient of variation (CV) were calculated for each group in each plate. Signal window (SW) and $Z'$ factor for each plate were calculated based on the Assay Guidance Manual as follows [9, 2]:

$$SW = \frac{\left(AVG_{max} - 3SD_{max} / \sqrt{n}\right) - \left(AVG_{min} + 3SD_{min} / \sqrt{n}\right)}{SD_{max} / \sqrt{n}} \tag{9}$$

$$Z' = \frac{\left(AVG_{max} - 3SD_{max} / \sqrt{n}\right) - \left(AVG_{min} + 3SD_{min} / \sqrt{n}\right)}{AVG_{max} - AVG_{min}} \tag{10}$$

The Assay Guidance Manual recommends the following acceptable thresholds: SW $\geq$ 2, $Z' \geq$ 0.4, and $\leq$20% for the CV values. However, for better quality control, we recommend to use 5% or 10% as the threshold for the $C_T$ values in detecting Cp18S and Hs18S transcripts and the $\Delta C_T$ values between Cp18S and Hs18S.

## Acknowledgments

## References

1. Bessoff K, Sateriale A, Lee KK, Huston CD (2013) Drug repurposing screen reveals FDA-approved inhibitors of human HMG-CoA reductase and isoprenoid synthesis that block *Cryptosporidium parvum* growth. Antimicrob Agents Chemother 57(4):1804–1814. https://doi.org/10.1128/AAC.02460-12

2. Zhang H, Zhu G (2015) Quantitative RT-PCR assay for high-throughput screening (HTS) of drugs against the growth of *Cryptosporidium parvum* in vitro. Front Microbiol 6:991. https://doi.org/10.3389/fmicb.2015.00991

3. Cai X, Woods KM, Upton SJ, Zhu G (2005) Application of quantitative real-time reverse transcription-PCR in assessing drug efficacy against the intracellular pathogen *Cryptosporidium parvum* in vitro. Antimicrob Agents Chemother 49(11):4437–4442. https://doi.org/10.1128/AAC.49.11.4437-4442.2005

4. Upton SJ, Tilley M, Brillhart DB (1994) Comparative development of *Cryptosporidium parvum* (Apicomplexa) in 11 continuous host cell lines. FEMS Microbiol Lett 118(3):233–236

5. Upton SJ, Tilley M, Brillhart DB (1995) Effects of select medium supplements on in vitro development of *Cryptosporidium parvum* in HCT-8 cells. J Clin Microbiol 33(2):371–375

6. Arrowood MJ, Sterling CR (1987) Isolation of *Cryptosporidium* oocysts and sporozoites using discontinuous sucrose and isopycnic Percoll gradients. J Parasitol 73(2):314–319

7. Suresh P, Rehg JE (1996) Comparative evaluation of several techniques for purification of *Cryptosporidium parvum* oocysts from rat feces. J Clin Microbiol 34(1):38–40

8. Glasel JA (1995) Validity of nucleic acid purities monitored by 260nm/280nm absorbance ratios. BioTechniques 18(1):62–63

9. Iversen PW, Benoit Beck B, Yun-Fei Chen Y, Dere W, Devanarayan V, Eastwood BJ, Farmen MW, Iturria SJ, Montrose C, Moore RA, Weidner JR, Sittampalam GS (2012) HTS Assay Validation. Eli Lilly & Company and the National Center for Advancing Translational Sciences, Bethesda, MD. http://www.ncbi.nlm.nih.gov/books/NBK53196/

# Chapter 19

# In Vitro Culture of *Cryptosporidium parvum* Using Hollow Fiber Bioreactor: Applications for Simultaneous Pharmacokinetic and Pharmacodynamic Evaluation of Test Compounds

## Nigel Yarlett, Mary Morada, Mohini Gobin, Wesley Van Voorhis, and Samuel Arnold

## Abstract

Hollow fiber technology is a powerful tool for the culture of difficult-to-grow cells. *Cryptosporidium parvum* has a multistage sexual and asexual life cycle that has proved difficult to culture by conventional in vitro culture methods. Here, we describe a method utilizing a hollow fiber bioreactor for the continuous in vitro growth of *C. parvum* that produces sexual and asexual stages. The method enables the evaluation of potential therapeutic compounds under conditions that mirror the dynamic conditions found in the gut facilitating preliminary pharmacokinetic and pharmacodynamic data to be obtained.

**Key words** Hollow fiber bioreactor, *Cryptosporidium parvum*, In vitro culture, Pharmacokinetic, Pharmacodynamics

## 1 Introduction

Despite the significant health risks and global distribution of cryptosporidiosis, there is no consistently effective therapy for the most-at-risk populations, that is, malnourished infants and immunocompromised individuals. Recent advances in manipulating the parasite genome [1] and chemotherapeutic profiling [2–4] are an encouraging sign that this bleak situation will change. Recent advances in continuous culture of *Cryptosporidium* has focused on developing a 3D culture system using adult murine colon cells [5] or novel bioengineered human intestinal cells [6] which has produced novel insights into parasite invasion and significantly extended the length of culture time compared to the 2D culture method. However, these techniques are limited in terms of parasite numbers obtained. The 3D models overcome the major obstacle associated with conventional 2D culture methods where host cells

receive nutrients and oxygen from the apical surface (except for those systems that use porous membrane inserts like the Costar Transwell system); this is contrary to the in vivo situation where the enterocytes receive nutrients and oxygen from the basal surface and the apical surface faces the lumen of the gut. However, current intestinal implant models fail to provide the low oxygen environment present inside the gut lumen, which restricts the long-term growth of the parasite. The use of hollow fiber technology allows the creation of the biphasic environment present in the gut and overcomes many problems associated with the long-term culture of *C. parvum* [7, 8]. This protocol describes the establishment of hollow fiber bioreactors that can be used to simulate in vivo conditions by providing oxygen and nutrients to the basal surface of host intestinal cells that are attached to the outside of the hollow fibers (Fig. 1). The environment inside the reactor is adjusted to mimic the lumen of the gut hence the apical surface of the intestinal cells is established in a low redox, high-nutrient environment that favors high growth rates and long-term maintenance of *C. parvum*. The use of this method provides $10^8$–$10^9$ oocysts which can be used for molecular and biochemical studies. It also has the advantage of avoiding the use of harsh chemicals such as potassium dichromate, which is used as a long-term storage medium at 4 °C and has the advantage of sanitizing the oocysts; and chlorine is currently used as both a sanitizer and to enhance excystation of oocysts obtained from animal sources [9].

## 2    Materials

All solutions are prepared using distilled, deionized water. The intracapillary space (ICS) medium, vitamin mix, and lipid mix is stored for a maximum of 6 months at −18 °C. The extracapillary space (ECS) medium and thiol mix is prepared fresh for each batch. The thiol mix is prepared using sterile distilled water that is boiled and cooled under a jet of nitrogen gas that is filtered using a 0.4 μm pore size filter.

*2.1  Intracapillary Space Medium*

To 1 L of Minimum Essential Medium (MEM) with l-glutamine and phenol red, without HEPES add 0.058 g heparin, 0.29 g l-glutamine, 23.8 g 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethane-sulfonic acid (HEPES) pH 7.8 with 5 M NaOH, 4.5 g d-glucose, 0.035 g ascorbic acid, 0.04 g p-aminobenzoic acid, 0.02 g Ca pantothenate, 0.001 g folic acid, and 100 mL horse serum. The base ICS medium is filter-sterilized using sterile disposable 0.2 μm bottle top filters with a polyethersulfone (PES) membrane.
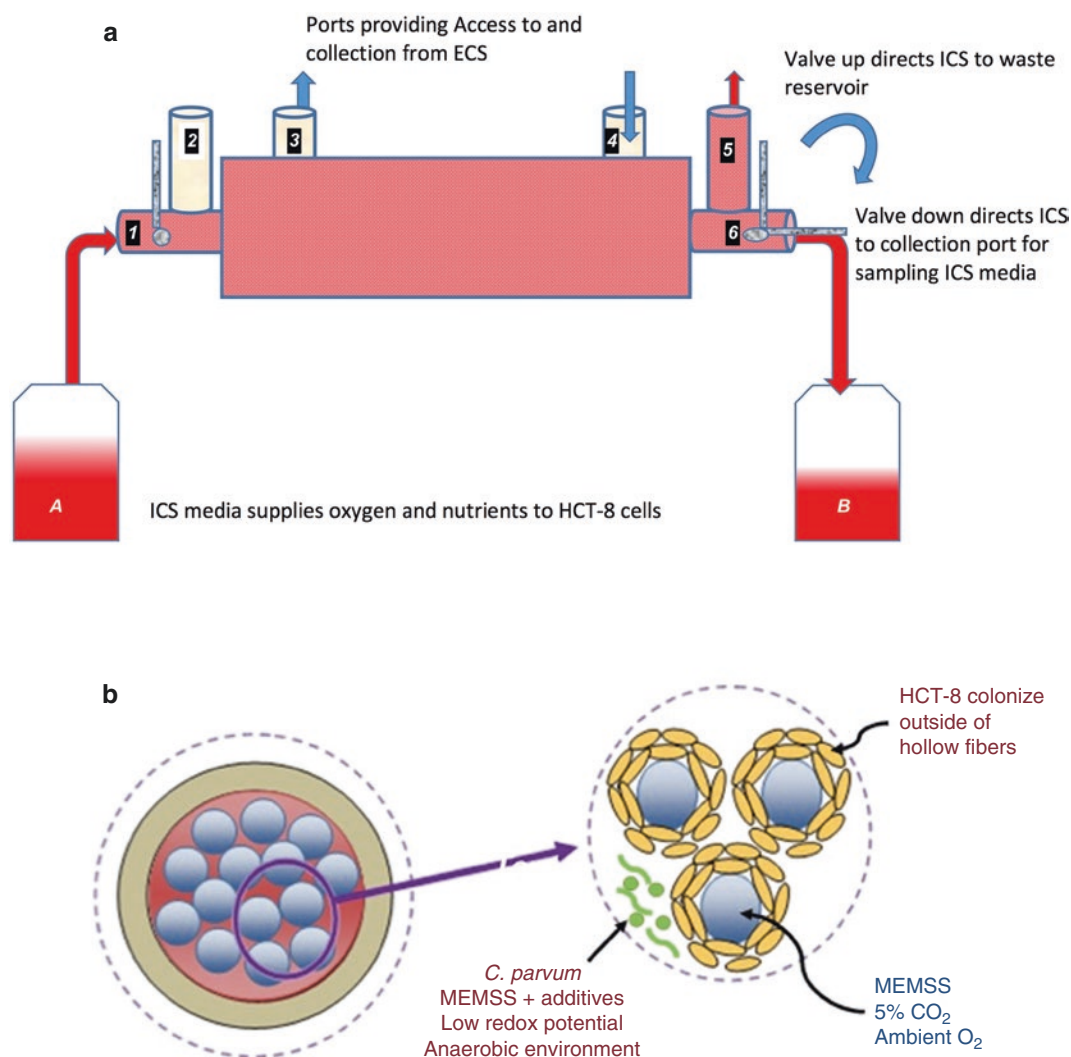
**Fig. 1** Hollow fiber bioreactor (HFB) modified for pK/pD analysis of test compounds. (**a**) MEM containing 10% horse serum is placed in the container on the left and pumped through port [1] into the hollow fibers providing oxygen and nutrients for the HCT-8 cells. Test compound is added to the ECS via port [3] and mixed by alternating depressing of syringes attached to ports [3, 4]. Test compound that is transported through the HCT-8 cells and excreted into the ICS is collected by diverting the waste from port [6] to port [5]. (**b**) Section through the HFB showing ICS (blue circles) and ECS (red). Host HCT-8 cells (yellow) attach and grow around the hollow fibers, while parasite stages (green) develop in the ECS

| | |
|---|---|
| **2.2 Extracapillary Space Medium** | To 10 mL of Minimum Essential Medium (MEM) with l-glutamine and phenol red, without HEPES, add 0.15 g Na taurodeoxycholate, 0.005 g Na thioglycolate, and 0.055 g mannitol. The thiol mixture is prepared by mixing 0.2 g of each of reduced glutathione, taurine, betaine, and cysteine in a glass vial containing 10 mL of distilled water, that has been boiled and cooled under a 0.4 μm filtered stream of nitrogen gas. The thiol mix is stored under a nitrogen |

atmosphere in 2-mL borosilicate glass vials (screw caps with septa). The thiol mix (0.15 mL) is aseptically added to 9 mL of the ECS medium in a sterile hood.

A lipid mix is prepared by adding 0.067 g of oleic acid and 0.18 g of cholesterol (water-soluble) to 10 mL of sterile distilled water. The lipid mix (0.2 mL) is added to 9.15 mL of the base mix to produce the ECS working medium, and brought to a pH of 7.8. The medium is sterilized using a 0.22-μm syringe driven filter with PES membrane.

### 2.3 Host Cells— HCT-8 Cell Line

Human adenocarcinoma intestinal epithelial cells are grown to confluence in MEM with l-glutamine and phenol red, without HEPES using 75 cm² "tissue culture" treated cell culture flasks in a 5% $CO_2$ incubator at 37 °C. HCT-8 cells are collected by replacing the growth medium with 2 mL of trypsin– EDTA (0.25%), phenol red solution, and incubating at 37 °C for 15 min. The trypsin is removed by centrifugation at $4000 \times g$ for 5 min, and the HCT-8 pellet gently resuspended in MEM to a density of $10^6$ cells per mL of ICS mix.

### 2.4 C. parvum

*C. parvum* oocysts (Bunch Grass Farm, Deary, ID) sterilized by immersion in 10% diluted commercial bleach (~5% hypochlorite) at 4 °C for 5 min are washed with a tenfold volume of distilled water six times and enumerated using a hemocytometer. Oocysts are resuspended to $10^5$ per mL in the ECS medium.

### 2.5 Test Compound

Bump kinase inhibitor BKI-1294 (Fig. 2) was provided by the Center for Emerging and Reemerging Infectious Diseases (CERID), Division of Allergy and Infectious Diseases, Department of Medicine, University of Washington (Seattle, WA, USA). The compound is stored as a 20 mM stock solution in dimethyl sulfoxide at −20 °C and diluted to a working solution of 0.512 mM by adding 102 μL of the stock solution to 2 mL of dimethylsulfoxide (DMSO) and brought to a final volume of 4 mL in sterile distilled water. Details of the extraction and liquid chromatography mass spectrometry (LC MS/MS) analysis of BKI-1294 in samples collected from the bioreactor are provided by Hulverson et al. [10].

### 2.6 Analysis of C. parvum Growth

*C. parvum* growth is determined by analysis of the amount of *C. parvum* 18S-rRNA present compared to the HCT-8 18S-rRNA as previously described [11]. Briefly, this was performed using 0.5 mL samples collected from the ECS after centrifugation at $14,000 \times g$ for 5 min. Gently resuspend the pellet in 100 μL of iScript buffer and subject to six freeze/thaw cycles comprising 1 min in liquid nitrogen followed by 1.5 min at 70 °C. Centrifuge the sample at $16,162 \times g$ for 5 min and remove the lysate. Add 1 volume of 70% ethanol, and transfer the sample to an RNeasy Mini spin column
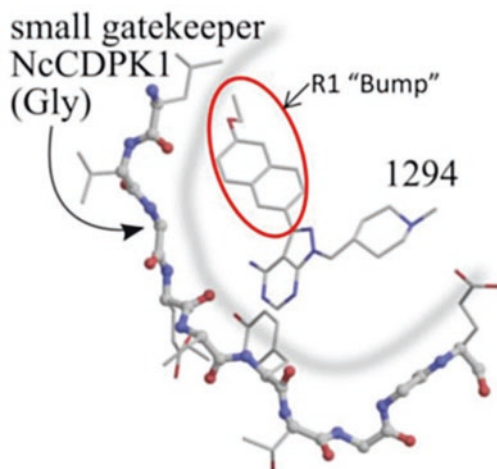
**Fig. 2** Structure and mechanism of Bumped Kinase inhibitor 1294. BKI-1294 was designed specifically as a *Toxoplasma gondii* cyclin dependent protein kinase 1 (CDPK1) inhibitor. A key structural difference between parasite and human CDPKs occurs at the gatekeeper residue in the adenosine triphosphate (ATP)-binding pocket which contains a small glycine residue at this position, compared to human kinases that have larger amino acid residues in this position. A pyrazolopyrimidine scaffold, comprising a 6-alkoxy-2-naphthyl group at the C-3 position binds in the ATP-binding pocket. This bulky C-3 substituent is sterically hindered by the larger gatekeeper residues found in human kinases. A 4-piperidinylmethylene group at the N-1 position fully occupies the ribose-binding pocket in many parasites and forces the bulky C-3 group into a position that cannot be accommodated by human kinases

(Qiagen, Inc., Germantown, MD) placed in a 2 mL collection tube and centrifuge for 15 s at $8000 \times g$. Discard the flow-through and follow the manufacturer's instructions (iScriptTM, RT-qPCR sample preparation kit, Bio-Rad Laboratories, Hercules, CA). *C. parvum* is enumerated by quantitating the *Cp*18S-rRNA using the following specific forward and reverse primers - *Cp*18S-995F: 5′-TAGAGATTGGAGGT TCCT-3′ and *Cp*18S-1206R: 5′-CTCCACCAACTAAGAACGCC-3′, and comparing it to the human *Hs*18S-rRNA using the following primer sets - *Hs*18S-F1373: 5′-CCGATAAC GAACGAGACACTCTGG-3′ and *Hs*18S-R1561: 5′-TAGGGTAGGCACACGCTGAGC C-3′. The amount of 18S-rRNA is determined in 5 ng samples by RT-qPCR as described by the manufacturer using a Luna Universal Probe One-Step® RT-qPCR kit (New England BioLabs, Ipswich, MA) and a Quant Studio 6 Flex 44 instrument (Applied Biosystems Inc., Beverly, MA). The cell number is calculated from the $C_T$ means using a set of standards prepared from $10^4$, $10^5$, and $10^6$ *C. parvum* oocysts and compared to $10^4$, $10^5$, and $10^6$ HCT-8 cells.

**2.7 Microscopic Evaluation of C. parvum Stages**

Dual stain *C. parvum* samples (50 μL) are prepared by the addition of 20 μL each of a fluorescein-labeled mouse monoclonal antibody to the oocyst outer wall protein (Crypt-a-Glo™, Waterborne Inc., New Orleans, LA) and a fluorescent polyclonal antibody to

sporozoites and other motile stages (Sporo-Glo™, Waterborne Inc., New Orleans, LA). Samples are incubated at room temperature in the dark for 30 min and excess antibody is removed by centrifugation at 14,000 × $g$ for 3 min. Samples are gently resuspended with 40 µL of phosphate buffered saline (PBS) and examined microscopically using a fluorescent microscope at 400× magnification. Crypt-a-Glo™ stained oocysts are visualized using an excitation wavelength of 410–485 nm and an emission wavelength of 515 nm; Sporo-Glo™ stained motile stages are visualized using an excitation wavelength of 535–550 nm and an emission wavelength of 580 nm.

# 3    Methods

## 3.1    Preparation of the Bioreactor

1. The culture reservoir bottles are prepared by loosely attaching a 33 mm reservoir cap and tubing (included with the cartridge) into a 125 mL bottle. Loosely wrap the Luer fittings on the end of the reservoir cap tubing with foil and place the entire assembly into an autoclave bag and seal.

2. Autoclave at 121 °C, 15 lb./in² for 20 min (high vacuum setting).

3. Inside a biosafety cabinet, remove the 20 kD MWCO polysulfone fiber cartridge (FiberCell Systems, New Market, MD) from its packaging and check the Luer fittings between the hollow fiber module and the flow path to ensure that they are finger tight.

4. The 20 kD MWCO polysulfone fiber cartridge has additional ports for access to the intracapillary space (ICS) as well as the extracapillary space (ECS) and an ECS volume of 3.2 mL, making it suitable for testing experimental compounds. For production of large parasite numbers, the 20 kD MWCO medium cartridge (FiberCell Systems) with an ECS volume of about 20 mL is recommended. However, the medium cartridge lacks additional ports to access the ICS and the larger ECS volume requires the use of five times more compound, making it unsuitable for pK/pD studies.

5. Remove the foil from one of the Luer fittings on the 33 mL reservoir cap and attach to the inlet flow path on the cartridge. Spray with 70% ethanol and wipe with an alcohol pad. Apply ½ turn counter rotation to the tubing prior to attachment to prevent kinking of the tubing. Attach the other Luer fitting to the outlet flow path. Check fittings to ensure that they are tightly attached.
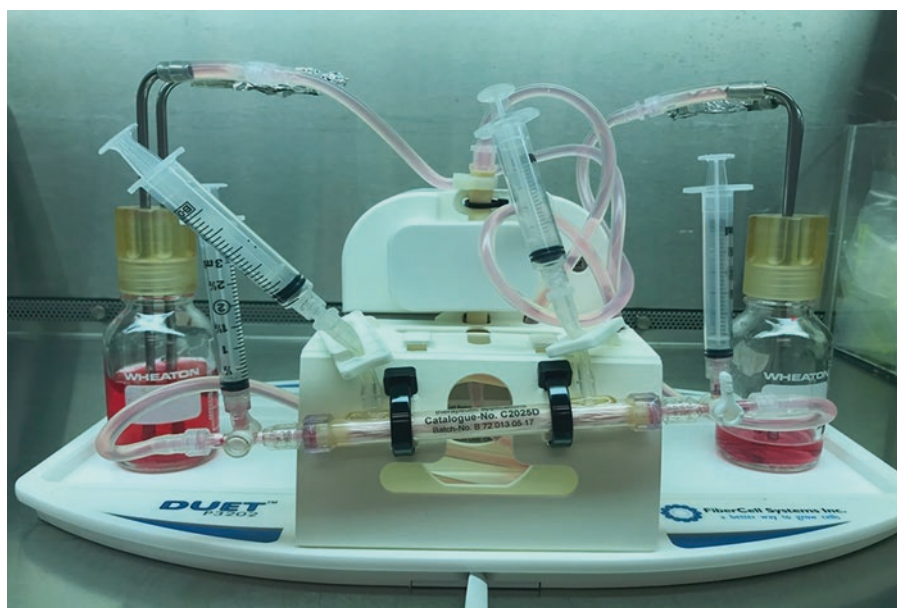
**Fig. 3** Hollow Fiber Bioreactor pK/pD set up

6. Connect the cartridge to the Duet Pump (FiberCell Systems) and ensure that the lines are properly engaged in the pump housing (Fig. 3).

*3.2   Preculture*

1. When working with adherent cells, the cartridge should be washed with two volumes of 125 mL of sterile PBS each for 24 h prior to filling the cartridge with 125 mL of cell culture medium.

2. Ensure that the left and right end port side clamps are open.

3. Aseptically fill 100 mL of sterile PBS into 125 mL bottle attached to cartridge using the 33 mm caps (the flow path and cartridge for medium cartridge holds about 30 mL of media). Prime the pump manually using the thumb and forefinger until all the air is pushed out of the tubing then close the left and right end port side clamps.

4. Attach a sterile syringe containing 30 mL sterile PBS to the left extracapillary space (ECS) port and attach an empty sterile syringe to the right ECS port.

5. Open the clamps on the left and right ECS side ports and push the sterile PBS into the ECS. Tilt cartridge upwards and fill medium taking care to remove all air present in the ECS. If the volume is not sufficient, repeat with more sterile PBS.

6. Close the left and right ECS side ports, spray with 70% alcohol and remove the syringes, spray with 70% alcohol and wipe with

an alcohol pad prior to attaching clean sterile 3 mL syringes to the ports. Open the left- and right-side port clamps.

7. Connect the hollow fiber cartridge to the Duet pump unit in a 37 °C, 5% $CO_2$ incubator. Set a medium flow rate of 5, leave for 24 h.

8. After 24 h, the left and right end port clamps are closed and the PBS reservoir bottle is aseptically changed for a fresh 125 mL bottle containing 100 mL of sterile PBS and returned to the incubator for 24 h.

9. Aseptically fill a sterile 125 mL reservoir bottle with 100 mL of MEM media.

10. Leave the reservoir cap loose by ½ turn. Attach a sterile syringe containing 30 mL of MEM to the left ECS port and an empty 60 mL syringe to the right ECS port.

11. Open the clamps on the left and right ECS side ports and push the sterile MEM into the ECS. Tilt cartridge upwards and fill medium taking care to remove all air present in the ECS. If the volume is not sufficient, repeat with fresh MEM.

12. Close the left and right ECS side ports, spray with 70% alcohol and remove the syringes, spray with 70% alcohol and wipe with an alcohol pad prior to attaching clean sterile 3 mL syringes to the ports.

13. Open the left and right ICS side port clamps (Fig. 1). Connect the hollow fiber module to the Duet pump unit in a 37 °C, 5% $CO_2$ incubator. Set a medium flow rate of 5 and leave overnight.

14. Repeat using MEM plus 10% horse serum.

**3.3 Addition of Host Cells**

1. Close the left and right ICS end port slide clamps of the cartridge.

2. Into a 10 mL sterile syringe take up 5 mL of HCT-8 cells ($5 \times 10^6$) suspended in ICS mix.

3. Spray the left and right ECS side port Luer connections with 70% ethanol and wipe with an alcohol pad.

4. Replace both 3 mL syringes on the ECS ports with the syringe containing the HCT-8 cells on the left ECS port and an empty 10 mL syringe to the right ECS side port.

5. Open the ECS side port clamps.

6. *Gently* flush the cell suspension through the ECS back and forth between the two syringes three to five times, to ensure equal distribution of the cells.

7. When finished, both syringes should contain an equal volume (2.5 mL in each syringe).

8. Loosen the reservoir cap ½ turn, close the left ECS end port slide clamp, open the right-hand ICS end port slide clamp (this

allows excess medium to flow into the reservoir bottle), and empty the syringe on the right hand side into the ECS (be careful not push air into the column).

9. Close the right ECS side port slide clamp, open the left ECS clamp, and empty the syringe on left hand side into the ECS.

10. Close all the side port slide clamps and spray with 70% ethanol and use a sterile alcohol swab before replacing both syringes with new 3 mL sterile syringes.

11. Tighten the reservoir cap. Open the left and right ICS end port slide clamp. Place the cartridge into the Duet pump inside a 37 °C, 5% $CO_2$ incubator, and set flow rate to 5. After 3 days, increase the flow rate to 10.

12. Change the MEM plus 10% horse serum medium every 24 h and the check the glucose concentration using a digital glucose meter (typically used for blood glucose monitoring).

13. The glucose concentration of the MEM plus horse serum medium should be 75 mg/dL. When the glucose concentration falls to approx. 50% (30 mg/dL) of its starting concentration, the bioreactor is inoculated with *C. parvum*, at which time the MEM plus horse serum is changed for the complete ICS medium mix.

### 3.4 Inoculation of (HFB) with Parasites

1. Treat $10^5$ *C. parvum* oocysts with 10% (v/v in distilled water) diluted commercial bleach and wash seven times with 30 mL of sterile distilled water, prior to suspending in 3 mL of MEM containing the ECS supplements described in the Materials section.

2. Close the left and right ICS end port clamps.

3. Spray the left and right ECS ports with 70% alcohol, and remove the left ECS syringe using an alcohol swab.

4. Attach the syringe containing the 3 mL of *C. parvum* oocysts to the left ECS port, and a clean sterile 3 mL syringe to the right ECS port.

5. Open the slide clamps to the left and right ECS ports and slowly push the oocysts into the cartridge, while gently pulling up on the right syringe.

6. Gently mix the contents of the ECS by alternating filling of the right and left syringes approximately five times, *caution should be taken not to introduce air into the cartridge*. With both left and right ECS syringes containing equal volumes (approx. 1.5 mL), close the right ECS port clamp, open the right ICS end port slide clamp, and push down on the syringe attached to left ECS port until the contents have been added to the ECS, *avoid adding air into the cartridge*.

7. Slide the left ECS port clamp to close and open the right ECS slide clamp and push down on the syringe until the contents of the syringe are added, *avoid adding air into the cartridge.*

8. Slide the right ECS port clamp closed (*see* **Note 1**).

9. Open the left ICS end port and spray the left and right ECS ports with 70% alcohol and using an alcohol pad replace the syringes on the left and right ECS ports with clean sterile 10 mL syringes.

10. Replace the reservoir bottle with 125 mL of the complete ICS medium mix, return the unit to the incubator, and set the flow rate to 10.

11. The pH and glucose in the ICS medium are monitored every 48 h, by removing 5 mL directly from the reservoir bottle. When the glucose concentration falls to undetectable levels in 48 h, the reservoir is changed for a 500 mL bottle of the complete ICS medium mix.

12. Samples (0.5 mL) are collected and examined microscopically for parasites and 0.25 mL used for RT-qPCR analysis of parasite numbers as described in Materials, Subheading 2.7.

*3.5 pK/pD Arrangement*

1. After 7–10 days in culture the ICS inlet is connected to a fresh bottle containing 600 mL of media and 0.5 mL of the ICS media removed for use as a starting time point.

2. Connect the ICS outlet to an empty sterile flask to prevent re-circulation of the media.

3. Remove 0.5 mL from the ECS for microscopic examination and enumeration of parasites by spraying with 70% ethanol and attaching a clean 3 mL syringe containing 0.5 mL of ECS media to the left ECS port, and an empty clean sterile 3 mL syringe to the right ECS port.

4. Depress the left syringe to displace 0.5 mL of the ECS to the right hand syringe.

5. Gently alternate pressure on the syringes about five times to mix the contents, and end with the left syringe containing ECS sample and the right syringe empty.

6. Close the left and right ECS ports and remove the left ECS syringe containing the ECS sample and keep for analysis.

*3.6 pK/pD: Addition of Test Compound*

1. Spray the left sampling port with 70% alcohol and attach a fresh sterile 3 mL syringe containing 0.5 mL of 0.512 mM of BKI-1294.

2. Add the test compound as previously described for addition of parasites (Subheading 3.4) but end with 0.25 mL of the ECS medium in both left and right syringes.

3. Close the right ECS port and make sure the left ICS port is closed, with the right ICS port open, depress the syringe on the left ECS port, *do not to introduce an air bubbles.*

4. Close the left ECS port and open the right ECS port and depress the syringe on the right, again ensure that *no air bubbles are introduced to the cartridge.*

5. Close the right ECS port and spray with 70% ethanol before replacing both the left and right ECS ports with new sterile 3 mL syringes.

6. The right and left ICS ports are turned to open and the unit placed back into the incubator at a pump setting of 0.5 mL/min.

*3.7 Collecting Samples for Pharmacokinetic/ Pharmacodynamics Analysis*

1. At 1 h intervals for the first 4 h, collect 0.5 mL samples from the ICS using the sampling port on the right-hand side that samples the ICS medium directly exiting the hollow fibers that contains any test compound taken up and excreted by the host cell epithelial layer (Fig. 1).

2. ICS samples are collected by attaching a sterile 3-mL syringe to the sampling port via the Luer lock fitting (Fig. 1) and the lever turned from the upright position to the right which directs the ICS media to the syringe. The left ICS sampling port should remain open (lever points up) to prevent pulling fluid into the ICS from the ECS.

3. The ICS sampling port is returned to open (lever up) after sampling, and the cartridge returned to the incubator.

*3.8 pK/pD: Days 2 to 4*

After 24 h, sample 0.5 mL from the ECS to quantitate the parasite load as described in Subheading 3.5. Add 0.5 mL of 0.512 mM of the bump kinase inhibitor BKI-1294 as described in Subheading 3.6; and sample 0.5 mL from the ICS at 1 h intervals for 4 hours as described in Subheading 3.7.

*3.9 Analysis of Bump Kinase Inhibitor 1294*

Test compounds can be administered by addition to the ICS, equivalent to serum dosing, or by addition to the ECS, equivalent to oral dosing. Cryptosporidiosis is an intestinal disease and hence delivery via the ECS port was selected. The half-maximal effective concentration ($EC_{50}$) for the bumped kinase inhibitor BKI-1294 was previously determined to be 2.65 μM [9]. Preliminary experiments were designed to determine a dose that would achieve a sustained $EC_{50}$ concentration for the treatment period. The method described permits evaluation of both the concentration of BKI-1294 required to completely inhibit cryptosporidial growth in the culture and the time required to achieve this goal. Based on an $EC_{50}$ of 2.7 μM and gastrointestinal BKI concentrations observed with an efficacious oral dose in mice, a 3- or 4-day dosing regimen of 20 μM, 40 μM, or 80 μM BKI-1294 was evaluated [2].
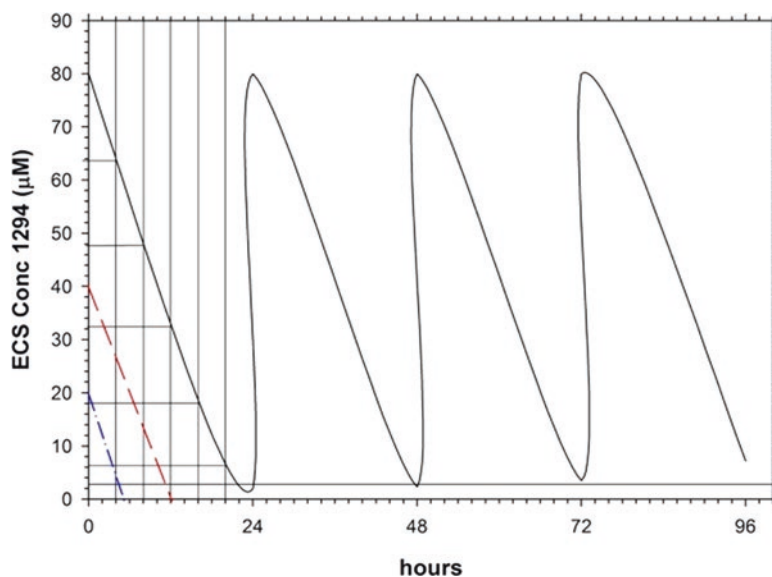
**Fig. 4** Pharmacodynamics of 1294 in the ECS during a 4-day dosing schedule of 80 μM per day. BKI-1294 decreases exponentially in the ECS over 24 h, but remains above the $EC_{50}$ of 2.65 μM for 22 h. Subsequent doses on days 2–4 show that BKI-1294 remains above the ECS for 24 h. Predicted doses of 40 μM (----) and 20 μM (---) will fall below the $EC_{50}$ in 12 and 6 h, respectively

The BKI-1294 concentration in the ECS following a 20 μM and 40 μM daily dose revealed that exposure to the $EC_{50}$ concentration was ≤4 h and 11 h, respectively (Fig. 4), and parasite growth although reduced by 2 and 3 logs was not completely inhibited and recovered regaining the starting numbers after 4 days posttreatment for the 20 μM dose (Fig. 5), and 8 days posttreatment for the 40 μM dose (not shown).

The BKI-1294 concentration in the ECS following an 80 μM daily dose revealed an ECS exposure equal to or greater than the $EC_{50}$ for the first 22 h; subsequent doses of 80 μM every 24 h provides an $EC_{50}$ exposure for ≥24 h (Fig. 4).

The pharmacokinetics of BKI-1294 following an 80 μM per day for 4 days experiment reveal that the concentration of BKI-1294 in the ICS, equivalent to absorption and excretion of the

**Fig. 5** (continued) of $8 \times 10^6$ parasites by day 4. (**b**) Three-day dosing with 80 μM BKI-1294. BKI-1294 was dosed on days 1–3, and parasite numbers were evaluated by qRT-PCR daily out to 37 days. Parasites had an initial 4-log reduction in growth compared to controls, and continued to slightly decrease up to 5 days past the last dose. However, parasite numbers gradually recovered and by day 23, regained control numbers. (**c**) Four-day dosing with 80 μM BKI-1294. BKI-1294 was dosed on days 1–3, and parasite numbers were evaluated by quantitative polymerase chain reaction (qRT-PCR) daily out to 30 days. Parasites had a 6-log reduction in growth compared to controls by day 5, and continued to decrease reaching undetectable numbers by 5 days past the last dose. Parasite numbers did not recover out to 26 days post the final dose
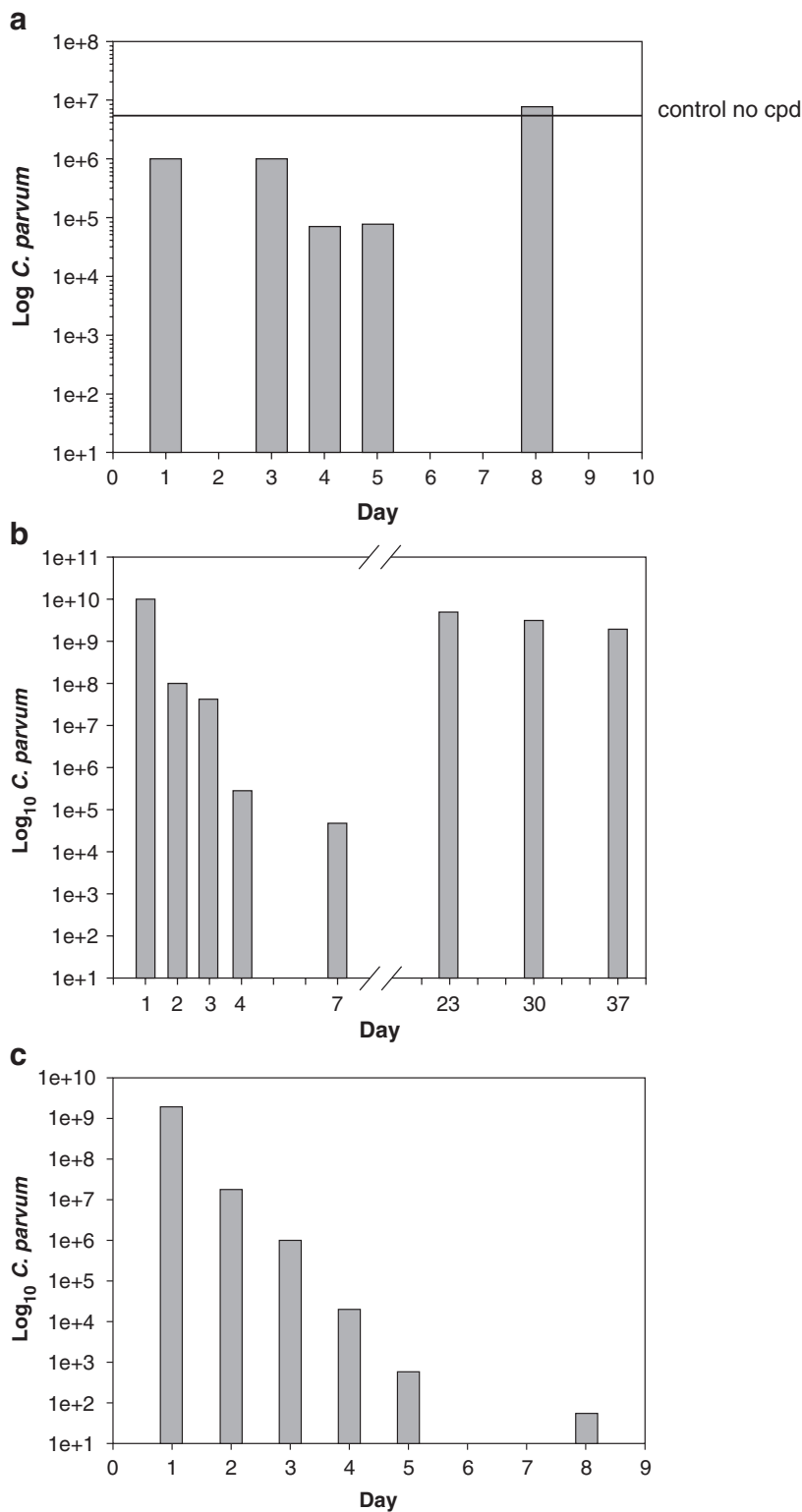
**Fig. 5** Growth of C. parvum during treatment with BKI-1294. (**a**) Four-day dosing with 20 $\mu$M BKI-1294. BKI-1294 was dosed on Days 1–4, and parasite numbers were evaluated by qRT-PCR daily out to 8 days, and no sample was removed on day 2. Parasites had an initial 1-log reduction in growth compared to controls, but recovered to control numbers 4 days past the final dose. A control bioreactor receiving only the vehicle produced an average
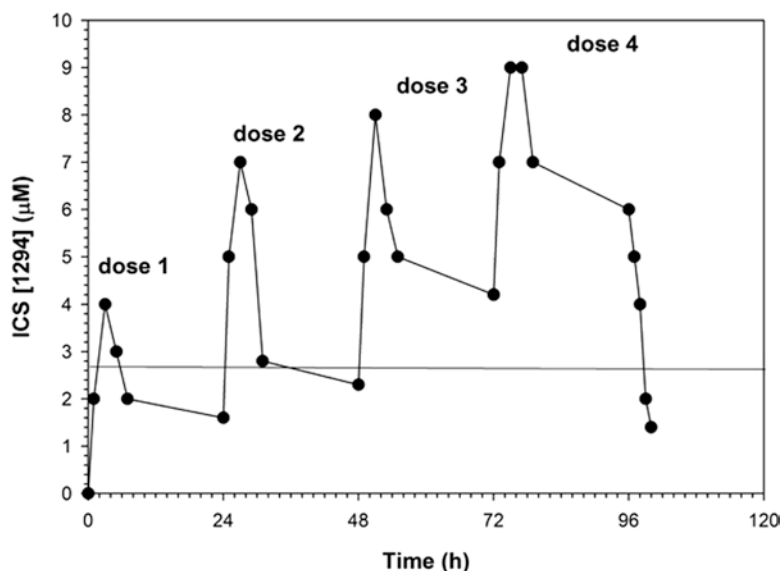
**Fig. 6** Concentration of BKI-1294 in the ICS. The concentration of BKI-1294 in the ICS of an 80 μM daily dosed HFB once daily for 4 days (corresponding to changes in BKI-1294 concentration in the ECS shown in Fig. 4). ICS samples were collected from port 5 (Fig. 1) on an hourly basis for 4 h post dosing. The concentration of BKI-1294 in the ICS increases during the first 2 h post dosing and then gradually falls to below the EC50 after 6 h on day 1, and 12 h on day 2. From day 3 on the concentration of BKI-1294 in the ECS remains above the parasite $EC_{50}$

compound across the intestinal epithelium into the plasma, showed a transient increase for the first 2 h after addition to the ECS and then demonstrated an exponential decrease to approximately 1.5 μM BKI-1294 (Fig. 6). Subsequent doses of BKI-1294 had similar decay profiles in the ICS, but maintained a concentration at or above 2.5 μM. The 4-day treatment with 80 μM BKI-1294 did not show parasite recrudescence post 20 days of the final dose (Fig. 5). However, a 3-day exposure to 80 μM is insufficient for complete removal of parasites (Fig. 5). The results obtained using the modified HFB method agree with the in vivo data where a higher than expected dose, 60 mg/kg per day for 5 days, to cure a neonatal mouse model *C. parvum* infection resulted in a 2-log reduction of parasites but failed to cure [12].

The method is a valuable addition to the tools available for the determination of test compounds for the treatment of cryptosporidiosis. It has advantages over the standard 48 h in vitro assay in static multiwell plates where drug concentration does not vary over time as it does in vivo. It can be included in any compound screening method to advance compounds selected from the preliminary $EC_{50}$ screen to provide valuable information concerning bioavailability and dosing schedules prior to in vivo analysis in animal

models thereby reducing the number of laboratory animals required for preliminary screening during drug development strategies.

## 4 Notes

1. When inoculating the cartridge initially, the user should experience minimal backpressure, after several months (>6 months) of continuous culture, an increase in backpressure can be experienced with the 3 mL ECS cartridge which can become significant hence it is not recommended for studies beyond this point.

## Acknowledgments

## References

1. Vinayak S, Pawlowic MC, Sateriale A, Brooks CF, Studstill CJ, Bar-Peled Y, Cipriano MJ, Striepen B (2015) Genetic modification of the diarrhoeal pathogen *Cryptosporidium parvum*. Nature 523(7561):477–480

2. Arnold SLM, Choi R, Hulverson MA, Schaefer DA, Vinayak S, Vidadala RSR, McCloskey MC, Whitman GR, Huang W, Barrett LK, Ojo KK, Fan E, Maly DJ Riggs MW, Striepen B, Van Voorhis WC (2017) Necessity of bumped kinase inhibitor gastrointestinal exposure in treating *Cryptosporidium* infection. J Infect Dis 216(1):55–63

3. Love MS, Beasley FC, Jumani RS, Wright TM, Chatterjee AK, Huston CD, Schultz PG, McNamara CW (2017) A high throughput phenotypic screen identifies clofazimine as a potential treatment for cryptosporidiosis. PLoS Negl Trop Dis 11(2):e0005373

4. Manjunatha UH, Vinayak S, Zambriski JA, Chao AT, Sy T, Noble CG, Bonamy GMC, Kondreddi RR, Zou B, Gedeck P, Brooks CF, Herbert GT, Sateriale A, Tandel J, Noh S, Lakshminarayana SB, Lim SH, Goodman LB, Bodenreider C, Feng G, Zhang L, Blasco F, Wagner J, Leong FJ, Striepen B, Diagana TT (2017) A *Cryptosporidium* PI(4)K inhibitor is a drug candidate for cryptosporidiosis. Nature 546(7658):376–380

5. Baydoun M, Vanneste SB, Creusy C, Guyot K, Gantois N, Chabe M, Delaire B, Mouray A, Baydoun A (2017) Three dimensional (3D) culture of adult murine colon as an in vitro model of cryptosporidiosis: proof of concept. Sci Rep 7(1):17288

6. DeCicco RePass MA, Chen Y, Lin Y, Zhou W, Kaplan DL, Ward HD (2017) Novel bioengineered three dimensional human intestinal model for long-term infection of *Cryptosporidium parvum*. Infect Immun 85(3):e00731–e00716

7. Morada M, Lee S, Gunther-Cummins L, Weiss LM, Widmer G, Tzipori S, Yarlett N (2016) Continuous culture of *Cryptosporidium parvum* using hollow fiber technology. Int J Parasitol 46(1):21–29

8. Yarlett N, Morada M (2018) Long-term *in vitro* culture of *Cryptosporidium parvum*. Bio-Protocol 8(15):e2947. https://doi.org/10.21769/BioProtoc.2947

9. Venczel LV, Arrowood MJ, Hurd M, Sobsey MD (1997) Inactivation of *Cryptosporidium parvum* oocysts and *Clostridium perfringes* spores by a mixed-oxidant disinfectant and by free chlorine. Appl Environ Microbiol 63(4):1598–1601

10. Hulverson MA, Choi R, Arnold SLM, Schaefer DA, Hemphill A, McCloskey MC, Betzer DP, Müller J, Vidadala RSR, Whitman GR, Rivas KL, Barrett LK, Hackman RC, Love MS, McNamara CW, Shaughnessy TK, Kondratiuk A, Kurnick M, Banfor PN, Lynch JJ, Van Voorhis WC (2017) Advances in bumped kinase inhibitors for human and animal therapy

for cryptosporidiosis. Int J Parasitol 47(12): 753–763

11. Zhang H, Zhu G (2015) Quantitative RT-PCR assay for high-throughput screening (HTS) of drugs against the growth of *Cryptosporidium parvum* in vitro. Front Microbiol 6:991. https://doi.org/10.3389/fmicb.2015.00991

12. Hulverson MA, Vinayak S, Choi R, Schaefer DA, Castellanos-Gonzalez A, Vidadala RSR, Brooks CF, Herbert GT, Betzer DP, Whitman GR, Sparks HN, Arnold SLM, Rivas KL, Barrett LK, White AC Jr, Maly DJ, Riggs MW, Striepen B, Van Voorhis WC, Ojo KK (2017) Bumped-kinase inhibitors for cryptosporidiosis therapy. J Infect Dis 215:1275–1284

# Chapter 20

# In Vitro Culture of *Cryptosporidium parvum* Using Stem Cell-Derived Intestinal Epithelial Monolayers

## Georgia Wilke, Yi Wang, Soumya Ravindran, Thaddeus Stappenbeck, William H. Witola, and L. David Sibley

## Abstract

*Cryptosporidium parvum* has a complex life cycle consisting of asexual and sexual phases that culminate in oocyst formation in vivo. The most widely used cell culture platforms to study *C. parvum* only support a few days of growth and do not allow the parasite to proceed past the sexual stages to complete oocyst formation. Additionally, these cell culture platforms are mostly adenocarcinoma cell lines, which do not adequately model the parasite's natural environment in the small intestine. We describe here a method to create primary intestinal epithelial cell monolayers that support long-term *C. parvum* growth. Monolayers were derived from mouse intestinal stem cells grown as spheroids and plated onto transwells, allowing for separate apical and basolateral compartments. In the apical chamber, the cell growth medium was removed to create an "air–liquid interface" that enhanced host cell differentiation and supported long-term *C. parvum* growth. The use of primary intestinal cells to grow *C. parvum* in vitro will be a valuable tool for studying host–parasite interactions using a convenient in vitro model that more closely resembles the natural niche in the intestine.

**Key words** *Cryptosporidium parvum*, Transwell, Long-term growth, Primary cell, Air-liquid interface

## 1 Introduction

A significant barrier to *Cryptosporidium* research is the lack of a cell culture system that enables long-term growth in vitro. Historically, human adenocarcinoma cells have been used to study asexual and early sexual development of the parasite [1]. These cell lines support growth of the parasite over 2–3 days, but after the parasite enters the sexual part of its life cycle, numbers rapidly diminish because oocyst formation does not occur [1–3]. This limitation greatly affects the type of studies researchers can pursue when investigating *Cryptosporidium* biology.

Recently, there have been several reports of cell culture platforms capable of supporting complete life cycle development of

*C. parvum*, a species of *Cryptosporidium* frequently associated with disease in humans and animals [4–6]. One platform utilizes a hollow fiber system to mimic the intestine by allowing for separate control of the apical and basolateral compartments [7]; this system enables the creation of an environment with redox and nutrient conditions optimal for parasite growth. However, it requires the use of expensive equipment and the hollow fiber cannot be "opened" during the experiment, making direct observation studies difficult. Also, the expense of the hollow fiber system means that experiments are not easily scalable, and perturbations to the system are often done serially, instead of doing in parallel, a major limitation for many studies. Another novel platform is a 3D intestinal model that uses silk protein as a scaffold for cell growth [8]; this model also has a lumen, creating two separate compartments. This platform involves the use of specialized equipment to create the 3D scaffolds, making it more challenging for other researchers to adopt. The two new platforms use adenocarcinoma cells (HCT-8 and Caco-2 cells), which are likely very different from the cell types *C. parvum* infects in vivo, in terms of differentiation status, metabolism, and proliferation.

To accurately reproduce in vivo host–parasite interactions in cell culture, it is desirable to utilize primary cells. Advancements in stem cell biology have made it possible to propagate intestinal stem cells as spheroids in medium containing specific growth factors [9, 10]. The required cell culture medium has become relatively easy to make due to the creation of a supportive cell line, L-WRN, that secretes Wnt, R-spondin, and noggin, creating conditioned medium that is used to support spheroid growth [9]. Cells derived from spheroids can be used to create primary intestinal epithelial cell monolayers grown on transwell membranes that recapitulate aspects of the gut, including development of specific cell lineages such as goblet and Paneth cells [11, 12]. Murine ileal monolayers derived from an early version of this method support robust *C. parvum* growth, which enabled the creation of novel antibodies to intracellular parasite stages [13]. These monolayers, however, typically only last 2–5 days, which make them unsuitable for studying long-term *C. parvum* growth. A more recent report utilized organoids grown in Matrigel and demonstrated that *C. parvum* will undergo its entire life cycle within a closed system [14]. However, the difficulty in having to microinject individual organoids, combined with the relative inaccessibility of the system, is likely to limit this platform for larger scale studies.

Recently, a protocol for creating primary intestinal monolayers that exhibit dramatic differentiation and long-term viability has been published [15]. This method relies on the inclusion of a feeder cell layer to support stem cell growth and the introduction of an air–liquid interface (ALI) at the surface of the cell monolayer. ALI has been used to enhance differentiation of airway tract epithelial cells for many years [16–18] and has also recently been

applied to intestinal epithelial cell culture, resulting in increased expression of markers associated with cell differentiation and also significant changes in cell metabolism [19, 20].

Epithelial monolayers grown with an ALI and feeder cell layer can survive for weeks [15], making them attractive candidates for a cell culture platform for *C. parvum*. We have found that primary intestinal monolayers grown under ALI conditions on transwells in L-WRN-conditioned medium support long-term *C. parvum* growth [21]. Parasite growth can last for weeks in this system without affecting host cell viability. We are providing detailed methods here for the ALI culture system with the hope that it will support further research into the biology of *C. parvum*.

## 2  Materials

### 2.1  Creating Irradiated 3T3 (i3T3) Cell Stocks for Feeder Cell Layer

1. Biosafety cabinet and 37 °C, 5% $CO_2$ incubator.
2. Mouse fibroblast cells (NIH/3T3, ATCC CRL-1658).
3. 37 °C water bath.
4. 15 and 50 mL polypropylene conical tubes.
5. 3T3 growth medium: For 500 mL, combine 445 mL of Dulbecco's Modified Eagle's Medium (DMEM)—high glucose with 50 mL heat-inactivated fetal bovine serum (FBS) and 5 mL 100× penicillin/streptomycin.
6. Centrifuge capable of holding 15 and 50 mL conical tubes that can spin at $200 \times g$.
7. Tissue culture supplies such as 25, 75, and 150 cm² cell culture flasks (T-25, T-75, T-150) and disposable plastic pipettes.
8. Dulbecco's Phosphate-Buffered Saline (DPBS)-EDTA: For 500 mL, add 500 μL 0.5 M EDTA pH 8.0 solution to 500 mL tissue culture grade DPBS.
9. Trypsin- Ethylene diamine tetra acetic acid (EDTA): For 100 mL, add 10 mL 10× trypsin solution to 90 mL DPBS-EDTA. Aliquot and store at −20 °C, stable for months; aliquots can be thawed and stored at 4 °C for 7–10 days.
10. Machine capable of irradiating 15 mL conical at 3000 rads (such as the Xstrahl Small Animal Radiation Research Platform (SARRP)).
11. Hemocytometer and cover glass.
12. 3T3 freezing medium: For 50 mL, combine 10 mL FBS, 2.5 mL Dimethyl sulfoxide (DMSO), and 37.5 mL 3T3 growth medium.
13. Cryovials, 1.5 mL volume.
14. −80 °C freezer.
15. Liquid nitrogen ($LN_2$) storage tank.

**2.2   Plating i3T3 cells on Transwells**

1. Biosafety cabinet.

2. Matrigel.

3. Cold Dulbecco's phosphate-buffered saline (DPBS).

4. Transwell with 0.4 μm pore polyester membrane insert.

5. Tissue culture supplies such as 15 mL conical centrifuge tubes, 1.7 mL microcentrifuge tubes.

6. Vacuum flask and sterile disposable glass Pasteur pipettes.

7. 37 °C, 5% $CO_2$ incubator.

**2.3   Preparing Conditioned Medium**

Conditioned medium was prepared according to Miyoshi and Stappenbeck [10] with slight modifications described here.

1. Biosafety cabinet.

2. L-WRN cells producing Wnt, R-Spondin and Noggin (American Type Culture Collection (ATCC) CRL-3276).

3. L cell media: To 500 mL of DMEM high glucose media, add 50 mL heat-inactivated FBS and 5 mL 100× penicillin/streptomycin.

4. Washing medium: Add 50 mL FBS, 5 mL 100× penicillin/streptomycin, 5 mL 100× L-glutamine to DMEM/F12 with 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES).

5. Primary medium: Add 6.25 mL 100× L-glutamine, 6.25 mL 100× penicillin/streptomycin, and 125 mL heat-inactivated FBS to 500 mL Advanced DMEM/F12.

6. DPBS-EDTA, Trypsin EDTA.

7. Selective agents Hygromycin B Gold and the aminoglycoside antibiotic G418.

8. FBS for conditioned medium.

9. T150 flasks.

10. Easy fill cell factory (Nalge Nunc Int., Rochester, NY).

11. Autoclaved 2 L glass media bottles.

12. Magnetic stir bars.

13. Corning 1 L storage bottles.

14. 50 mL conical centrifuge tubes.

15. Autoclavable bottle-top dispenser (5–50 mL).

16. Inlet tube for autoclavable bottle-top dispenser.

17. Centrifuge capable of holding 50 mL conical centrifuge tubes that can spin at 200 and 1800 × $g$.

18. 37 °C, 5% $CO_2$ tissue culture incubator.

*2.4 Passaging Spheroids*

1. Spheroid line established according to Miyoshi and Stappenbeck [10]; for our purposes, we used a line isolated from the ileum of 8- to 10-week-old C57BL/6 J mouse. Lines can be frozen 1–2 passages after the original derivation and thawed as needed; use single line for 20–25 passages.

2. 37 °C water bath.

3. Tissue culture supplies such as 12 well plates, disposable pipettes, 15 and 50 mL conical centrifuge tubes, 1.7 mL microcentrifuge tubes.

4. Washing medium, as described above.

5. Matrigel.

6. Biosafety cabinet.

7. Cell scrapers.

8. Centrifuge capable of holding 15 conical tubes that can spin at $200 \times g$.

9. Centrifuge capable of holding 1.7 mL microcentrifuge tubes that can spin at $200 \times g$.

10. DPBS-EDTA and trypsin-EDTA.

11. 50% Conditioned medium.

12. Primary medium, as described above.

13. Rho kinase inhibitor (ROCK inhibitor Y-27632).

14. 37 °C, 5% $CO_2$ incubator.

*2.5 Creating Primary Cell Monolayers for C. parvum Infection*

1. Biosafety cabinet.

2. Cell scrapers.

3. Tissue culture supplies such as 12 well plates, disposable pipettes, 15 and 50 mL conical centrifuge tubes.

4. Centrifuge capable of holding 15 mL conical tubes that can spin at $200 \times g$.

5. DPBS-EDTA and trypsin-EDTA.

6. 37 °C water bath.

7. Washing medium, as described above.

8. 50% Conditioned medium prepared according to Miyoshi and Stappenbeck [10]; requires L-WRN cell line (ATCC CRL-3276).

9. Primary medium: Add 6.25 mL 100× L-glutamine, 6.25 mL 100× penicillin/streptomycin, and 125 mL heat-inactivated FBS to 500 mL Advanced DMEM/F12.

10. Rho kinase inhibitor (ROCK inhibitor Y-27632).

11. Sterile cell strainers, 40 μm.

12. 37 °C, 5% $CO_2$ incubator.

| | |
|---|---|
| *2.6 Preparing and Maintaining C. parvum Oocysts for Infection* | |

1. Biosafety cabinet.

2. Purified oocysts, stored in 50 mM Tris-10 mM EDTA, pH 7.2.

3. Cold DPBS.

4. 15 mL conical centrifuge tubes.

5. Commercial laundry bleach disinfectant containing 8.25% sodium hypochlorite.

6. Centrifuge capable of holding 15 conical tubes that can spin at 800 and $1250 \times g$.

7. Centrifuge buckets with sealed biohazard caps.

8. Cold DPBS +1% Bovine serum albumin (BSA).

9. Excystation buffer: To make 5 mL, dissolve 0.75 g synthetic sodium taurocholate into 5 mL DPBS (1.5% w/v sodium taurocholate solution). May need vortexing to dissolve completely. Filter sterilize with 0.2 μm filter. Can be stored at 4 °C for several weeks.

10. 37 °C water bath.

11. Glass slides and square coverslips ($22 \times 22$ mm).

12. Swin-Lok or Swinnex filter holders, 25 mm.

13. Track-etch polycarbonate hydrophilic membranes, 25 mm circle, 1.0 μm pore size.

14. 3 or 5 mL plastic syringe.

15. Cell growth medium.

*2.7 Measuring Host and Parasite Replication in Primary Cell Monolayers by qPCR*

1. QIAamp DNA Mini Kit (Qiagen Inc., Germantown, MD).

2. Blunt pipette tips (20–200 μL pipette tips with ends snipped off).

3. 1.7 mL microcentrifuge tubes.

4. 56 °C water bath.

5. 70 °C heat block.

6. Ethanol, 200 proof.

7. Centrifuge capable of spinning microcentrifuge tubes and spin columns at $7000 \times g$.

8. Sterile $H_2O$.

9. 2× SYBR Green mix or similar qPCR reaction mix.

10. 96-well reaction plate compatible with qPCR machine.

11. Adhesive film for sealing reaction plate.

12. Centrifuge capable of spinning 96-well plates.

13. Quantitative polymerase chain reaction (qPCR) machine with 96-well block.

*2.8 Monitoring Life Cycle Progression in Primary Cell Monolayers by Microscopy*

1. DPBS.

2. Fixative such as 4% formaldehyde.

3. Permeabilization agent such as Triton X-100 or saponin.

4. Normal goat serum (NGS).

5. Fetal bovine serum (FBS).

6. Hoechst 33258 dye or other DNA stain.

7. Scalpel and forceps.

8. Mounting reagent with fluorescence antiquenching agent.

9. Glass slides and square coverslips ($22 \times 22$ mm).

# 3   Methods

*3.1 Creating Irradiated 3T3 (i3T3) Cell Stocks for Feeder Cell Layer*

This protocol is based on the standard protocols for maintaining 3T3 cells in culture and preparing standard fibroblast cell line 3T3 cells for feeder cell layers. All cell manipulation is performed inside a biosafety cabinet under sterile conditions.

1. Thaw a frozen vial of 3T3 cells (*see* **Note 1**) in 37 °C water bath. Remove vial immediately when cells have thawed.

2. Add thawed cell mixture to 15 mL conical containing 10 mL 3T3 growth medium.

3. Spin at $200 \times g$ for 3 min. Remove supernatant.

4. Resuspend pellet in 5 mL growth medium and pipette 8 to 10 times to evenly distribute cells.

5. Transfer to T-25 flask and culture in 37 °C, 5% $CO_2$ incubator.

6. When cells are approximately 90% confluent, remove the medium from the flask and wash with 10 mL DPBS-EDTA.

7. Add trypsin-EDTA to flask in a volume sufficient to cover cell monolayer (1 mL for T-25, 3 mL for T-75, 6 mL for T-150). Swirl flask to ensure complete cell coverage.

8. Keep flask at room temperature for 2–3 min. Swirl flask to see whether cells have detached.

9. Once cells have detached, add sufficient volume of growth medium to cover flask and resuspend the cells (4 mL for T-25, 6 mL for T-75, 7 mL for T-150). Serum in the medium will stop the action of the trypsin.

10. Pipette the medium over the surface of the flask three times to ensure that all cells are in suspension. Pipette up and down 8 to 10 times to create a single cell suspension.

11. Transfer cell mixture to 15 mL conical and spin at $200 \times g$ for 3 min.

12. Remove supernatant and resuspend pellet in desired amount of medium.

13. Pipette mixture up and down 5 to 10 times to ensure even distribution of cells and transfer desired amount of cell to new flasks. Swirl flasks to ensure that cells are covering the flask surface.

14. The passage ratio determines the length of time it takes the flask to reach 90% to 100% confluency. Passage ratio of 1:2.5 will reach confluency in 2 days, 1:5 in 3 days, and 1:10 in 4 days.

15. Expand the cells into the number of flasks desired for the irradiation process.

16. When final flasks become confluent, trypsinize cells as described in **steps 1–6**. The irradiation protocol can be scaled up or down depending on the number of irradiated cells required. For example, three T-T150s, once trypsinized, can be pooled into one 50 mL conical and spun down at $200 \times g$ for 3 min.

17. Remove supernatant and resuspend pellet in 10 mL growth medium and transfer cell mixture to 15 mL conical.

18. Irradiate cells at 3000 rads. We use the Xstrahl Animal Radiation Research Platform (SARRP) for this purpose.

19. Pipette cells to ensure even distribution and take 10 μL to determine cell density using a hemocytometer. Cells should not be clumpy and should appear uniformly round.

20. Spin down cells at $200 \times g$ for 3 min.

21. Resuspend pellet in sufficient volume of freezing medium to achieve $2 \times 10^6$ cells/mL.

22. Aliquot cells for freezing, 1.0 mL per cryovial. Three T-150 s typically yield 20 to 25 vials at the above cell density.

23. Move cryovials to −80 °C freezer for short-term storage (days to weeks) and then to liquid nitrogen ($LN_2$) tank for long-term storage (months).

### 3.2 Plating i3T3 Cells on Transwells

This step occurs the day before the stem cell spheroids are split and plated onto the transwells. All cell manipulation is performed inside a biosafety cabinet under sterile conditions.

1. Make 10% Matrigel solution: dilute Matrigel (*see* **Note 2**) in cold Dulbecco's Phosphate-Buffered Saline (DPBS) [keep DPBS and Matrigel on ice], pipette to ensure even distribution. Prepare sufficient volume for 100 μL of solution per transwell.

2. Add 100 μL 10% Matrigel solution to each transwell. Move plate with transwells to 37 °C incubator for 20–30 min to

allow the Matrigel to polymerize while finishing the following steps.

3. Thaw vial(s) of frozen i3T3 cells in 37 °C water bath. Once thawed, immediately add to 15 mL conical containing 10 mL 3T3 growth medium.

4. Spin down cells at $200 \times g$ for 3 min.

5. Remove supernatant and resuspend cells in 1–3 mL of growth medium.

6. Count cells using a hemocytometer. Each transwell will be plated with $8 \times 10^4$ cells in 100 μL volume; resuspend cells accordingly.

7. After transwells have been in 37 °C incubator for at least 20 min, remove and aspirate Matrigel using vacuum flask and glass pipette. Alternatively, Matrigel can be removed with pipet tip.

8. Add i3T3 cell suspension to each transwell.

9. Add 400 μL growth medium to bottom chamber of each transwell.

10. Place transwells in 37 °C, 5% $CO_2$ incubator.

*3.3 Preparing Conditioned Medium*

The protocol for this step has been published in detail previously in Miyoshi and Stappenbeck [10]. All cell manipulation is performed inside a biosafety cabinet under sterile conditions.

1. Thaw a vial of L-WRN cells in a 37 °C water bath.

2. Transfer to 25 mL of warm L-cell media and transfer into a T150 flask.

3. Allow the cells to grow in a 37 °C, 5% $CO_2$ incubator for 3 days without media change.

4. On day 3, aspirate media and wash with DPBS-EDTA.

5. Aspirate DPBS and add 1 mL trypsin-EDTA and incubate for 5 min in the 37 °C incubator.

6. Tap the flask to ensure that cells have dislodged. Check under an inverted tissue culture microscope to confirm.

7. Add 12 mL of L-cell media to wash the cells for complete cell retrieval.

8. Add the above media containing cells to 140 mL of pre-warmed L-cell media containing 750 μl of 100 mg/mL Hygromycin B Gold and 1.5 mL of 50 mg/mL G418.

9. Swirl contents and distribute 25 mL each into six T150 flasks.

10. Allow the cells to grow for 3 days in a 37 °C, 5% $CO_2$ incubator.

11. Once cells reach confluency, follow the same procedure as above for trypsinizing cells from the flasks and transferring to the EasyFill Cell factory.

12. Allow the cells to grow for 3 days in a 37 °C, 5% $CO_2$ incubator.

13. On Day 3, wash the cell factory with 1000 mL of Washing media before adding 1050 mL of Primary culture media.

14. Incubate cell factory in a 37 °C, 5% $CO_2$ incubator for 24 h.

15. Collect the conditioned medium every 24 h from the cell factory into 50 mL conical tubes.

16. Replace with fresh primary culture media.

17. Spin down the condition medium in 50 mL tubes in a centrifuge at $1800 \times g$.

18. Transfer supernatant equally to each of the four glass media bottles avoiding the cell pellet.

19. Dilute 100% conditioned media 1:1 ratio with primary culture medium generating "50% conditioned medium," that is used for cultivation below.

20. Keep on a stir plate for 30 min to ensure uniform mixing.

21. Dispense 40 mL of 50% conditioned medium into 50 mL conical tubes. Store at −20 °C.

*3.4 Passaging Spheroids*

This is a very brief summary of the protocol described in Miyoshi and Stappenbeck [10]. Growing the spheroids (and primary cell monolayers described in Subheading 3.5) requires the use of conditioned medium produced by the L-WRN cell line. The production of this medium is described above and in more detail in Miyoshi and Stappenbeck 2013; it requires the L-WRN cell line (American Type Culture Collection (ATCC) CRL-3276). The final step of preparing conditioned medium is to add the conditioned medium to an equal volume of primary medium (recipe included in Subheading 2); therefore, all conditioned medium used in these protocols is "50% conditioned medium."

1. Thaw vial of spheroids isolated and frozen according to Miyoshi and Stappenbeck [10]. Place vial in 37 °C water bath until contents are thawed (1–2 min).

2. Transfer cells to 15 mL conical containing 5 mL pre-warmed washing medium.

3. Centrifuge at $200 \times g$ for 5 min.

4. Aspirate supernatant, being careful not to disturb pellet.

5. Add 1 mL washing medium and resuspend cells by gentle pipetting. Transfer to 1.5 mL microcentrifuge tube.

6. Centrifuge at $200 \times g$ for 5 min.

7. Remove supernatant with a pipette, being careful not to disturb pellet.

8. Place tube on ice and chill for 5 min.

9. Add Matrigel to cells for desired volume. For example, if plating a 12-well plate, the volume of Matrigel/cell suspension for each well is 30 μL. Pipette cells gently, avoiding bubbles. Hold tube by rim while pipetting to avoid warming Matrigel.

10. Place tissue culture plate (e.g. a 12-well plate) on ice. This can be done inside or outside biosafety cabinet.

11. Remove lid from plate and place on sterile surface.

12. Pipette appropriate volume of Matrigel/cell suspension into the middle of each well. Carefully spread out Matrigel using pipette tip; do not let Matrigel touch sides of well.

13. Cover plate with lid, remove from ice, flip upside down (this is done to prevent the cells from touching plate bottom), and place into 37 °C incubator for 10 min. Matrigel will solidify.

14. Remove plate from incubator, flip right side-up, and add appropriate volume of 50% conditioned medium with 10 μM Rho kinase inhibitor (ROCK) inhibitor (e.g. 800 μL/well for 12-well plate).

15. Place cells in 37 °C, 5% $CO_2$ incubator.

16. Two days after plating, aspirate medium and add fresh 50% conditioned medium.

17. Three days after plating, scrape Matrigel/cell suspension into medium using a cell scraper or pipette tip. Combine wells into 15 mL conical (6 wells of a 12 well plate can be combined into 1 conical).

18. Spin cells at $200 \times g$ for 3 min.

19. Aspirate supernatant and resuspend cells in 5 mL DPBS-EDTA.

20. Spin cells at $200 \times g$ for 5 min (longer spin time is necessary to ensure adequate exposure to DPBS-EDTA).

21. Aspirate supernatant and add 300 μL trypsin-EDTA to cells. Pipette 2 to 3 times to ensure even mix.

22. Add cells to 37 °C water bath, incubate for 1.5 min.

23. Remove cells from water bath, pipette gently 10 to 12 times. Add 5 mL washing medium.

24. Spin cells at $200 \times g$ for 3 min.

25. Aspirate supernatant and resuspend cells in 1 mL 50% conditioned medium with 10 μM ROCK inhibitor. Move cell suspension to 1.7 mL microcentrifuge tube.

26. Spin cells at $200 \times g$ for 3 min.

27. Remove supernatant with pipette tip. Place tube on ice. Let chill for 5 min.

28. Add desired volume of Matrigel to cell pellet (volume is dependent on the number of wells being plated). Spheroid lines have different optimal passage ratios; for the murine ileal lines used to grow *C. parvum*, a 1:6 passage ratio is often used. Passage ratios will need to be adjusted in response to spheroid density; murine ileal spheroids should not be maintained at a very high density or else they will not grow well. Pipette Matrigel gently to mix, avoid bubbles.

29. Plate spheroids as described in **steps 10–15**.

30. Passage spheroids every 3 days.

*3.5  Creating Primary Cell Monolayers for C. parvum Infection*

This protocol is based on the use of murine stem cells passaged as spheroids as described in Miyoshi and Stappenbeck [10]. All cell manipulation is performed inside a biosafety cabinet under sterile conditions. *See* Fig. 1a, b for summary of the time line for production of ALI cultures.

1. Scrape spheroids and Matrigel into medium using pipette tip or cell scraper. Combine wells with 5 mL plastic pipette and move cell suspension into 15 mL conical tube. Six wells of a 12-well plate can be combined in one 15 mL tube.

2. Spin spheroids down at $200 \times g$ for 3 min.

3. Remove medium and resuspend spheroids in 5 mL DPBS-EDTA. At this time, warm an aliquot of trypsin-EDTA in 37 °C water bath.

4. Spin spheroids down at $200 \times g$ for 5 min (longer spin time is necessary to ensure adequate exposure to DPBS-EDTA).

5. Remove DPBS-EDTA and add 300 µL warm trypsin-EDTA to pellet. Pipette up and down to ensure that cells are evenly exposed to trypsin.

6. Move cells to 37 °C water bath, incubate for 1.5 min.

7. Remove cells from bath and pipette 20 to 30 times to break up spheroids.

8. Add 5 mL washing medium to cells.

9. Place sterile cell strainer (40 µm) on top of 50 mL conical tube. Pre-wet strainer with 2 mL washing medium. Pipette cell mixture on top of strainer. Two 15 mL conical tubes worth of cells (equivalent to one 12-well plate of spheroids) can be combined onto one cell strainer.

10. Tap strainer against conical to force liquid through; move filtrate from 50 mL conical to 15 mL conical.

11. Spin at $200 \times g$ for 3 min.
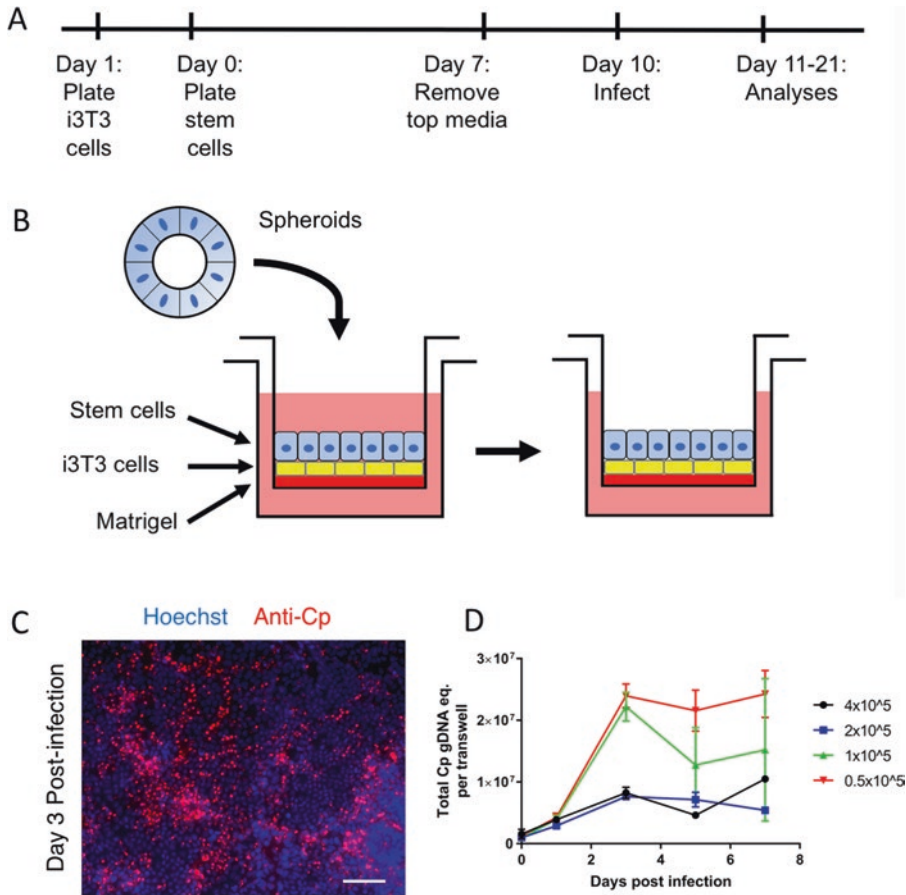
**Fig. 1** Time line and schematic for production of ALI monolayers and infection with *C. parvum*. (*a*) Time line for production of irradiated 3T3 feeder cells, plating of transwells form spheroids and construction of ALI cultures. Optimal production of monolayers is observed with submerged culture on 50% conditioned medium for 7 days, followed by removal of top medium to allow ALI to form. Optimal infection is achieved by infected at 3 days post top medium removal. (*b*) Schematic of the construction of ALI monolayers from feeder cells and spheroids (i.e. stem cell). Irradiated 3T3cells (feeder cells) are plated on transwell filters containing a layer of Matrigel. The feeder cells are overlaid with a layer of stem cells isolated from parallel cultures of spheroids. The top and bottom chambers both contain 50% conditioned medium. After 7 days, the medium is removed from the top chamber to allow development of the ALI culture. (*c*) Immunofluorescent staining of *C. parvum* infected ALI monolayers. ALI monolayers were infected on day 3 post top media removal with $2 \times 10^5$ oocysts. Monolayers were incubated with oocysts for 4 h and then washed 3 times. On 3 days post infection, monolayers were fixed and stained with pan-Cp (rabbit polyclonal raised against *C. parvum* oocysts and sporozoites) and Hoechst. Scale bar = 50 μm. (*d*) Lower seeding density improves *C. parvum* growth. Stem cells were seeded at different densities. Seven days post-seeding, top media was removed to create ALI cultures. Three days post top media removal, monolayers were infected with $2 \times 10^5$ oocysts. Monolayers were incubated with oocysts for 4 h and then washed 3 times. DNA samples were collected every other day. Graph depicts means ± S.D. for qPCR measurements of *C. parvum* in DNA samples where each data point represents the mean ± S.D. of two transwells

12. Remove washing medium and resuspend cells in 1–3 mL of 50% conditioned medium with 10 µM ROCK inhibitor (Y-27632, *see* **Note 3**). Count cells using a hemocytometer. Cells will be very clumpy. Each transwell will be plated with $5 \times 10^4$ cells in 200 µL medium, resuspend cells accordingly (*see* **Note 4**).

13. Remove transwells plated with i3T3 cells from 37 °C incubator; confirm i3T3 monolayer is uniform by examining with an inverted light microscope equipped with phase contrast optics (4× objective, 10× ocular).

14. Remove 3T3 growth medium from top chamber of compartment using pipette tip (do not use vacuum flask because aspiration will disturb the monolayer).

15. Add stem cells to transwells.

16. Remove 3T3 growth medium from bottom chamber of transwell compartment and add 400 µL conditioned medium with 10 µM ROCK inhibitor.

17. Move transwells to 37 °C, 5% $CO_2$ incubator.

18. Change medium in top and bottom chambers of transwells every 2 days. Add 200 and 400 µL 50% conditioned medium with 10 µM ROCK inhibitor to the top and bottom chambers, respectively. Remove medium from top chamber with pipette tip only; aspiration can disturb cell layer. Cells can tolerate a 2-day gap between feedings; medium can be changed on a Monday, Wednesday, Friday schedule with no ill effects.

19. After 7 days, remove medium from top chamber with pipette tip, and do not replace. This is "day 0 of top media removal" and the creation of the ALI.

20. Continue to change medium in bottom chamber 3 times weekly (*see* **Note 5**).

21. On day 3 post top media removal, remove liquid/mucus from top chamber of transwells (*see* **Note 6**). Resuspend desired infectious dose of *C. parvum* oocysts or sporozoites in 30 µL 50% conditioned medium per transwell. Add oocysts or sporozoites to transwell.

22. After incubation period (1–2 h for sporozoites, 3–4 h for oocysts), remove liquid and wash with DPBS if required for assay.

***3.6   Preparing and Maintaining C. parvum Oocysts for Infection***

We have used the AUCP-1 isolate of *C. parvum* for growth in the air–liquid interface (ALI) system (*see* **Note 7**) and other lines would need to be tested for efficiency. It is a requirement of our biosafety protocol to use plastic caps on the centrifuge buckets when spinning the oocysts. Only open the caps inside a biosafety cabinet. Autoclave the buckets and caps after the protocol in case

of accidental contamination. All pipette tips and other material contaminated with oocysts should be disposed off in a container that can be sealed before removing from the biosafety cabinet and placing in biohazard waste. An absorbent mat pad can be placed in biosafety cabinet during this procedure in case of spills.

1. Store purified oocysts at 4 °C in 50 mM Tris-10 mM EDTA, pH 7.2.

2. Before infection, dilute $1 \times 10^8$ oocysts into 1 mL cold DPBS in a 15 mL conical. Place on ice.

3. Add 1 mL of 40% commercial bleach (diluted in cold DPBS) to oocysts. Pipette multiple times to ensure even mix.

4. Bleach oocysts on ice for 10 min.

5. Spin at $800 \times g$ for 3 min. Remove bleach, resuspend pellet in cold DPBS +1% BSA. Move oocyst mixture to new 15 mL conical.

6. Spin at $800 \times g$ for 3 min. Remove supernatant and resuspend pellet in cold DPBS +1% BSA. Repeat centrifugation and washing **step 3** times to remove all traces of bleach.

7. After final centrifugation step, resuspend oocysts in cold DPBS +1% BSA at a concentration of $1 \times 10$ [8]/mL. Bleached oocysts can be stored at 4 °C for up to 2 weeks. After 2 weeks, the excystation rate will decline.

8. Oocysts can be diluted into cell culture medium at desired concentration for infection.

9. If sporozoites are needed for infection, add required amount of oocysts to the same volume of excystation buffer in a 15 mL conical. For example, if 500 µL of oocysts ($0.5 \times 10^8$ oocysts total) is required, add the oocysts to 500 µL excystation buffer. Final concentration of sodium taurocholate in solution will be 0.75% w/v.

10. Incubate oocysts in 37 °C water bath for 45–60 min. Check excystation rate by pipetting 10 µL onto glass slide, cover sporozoites with glass coverslip, and look under inverted light microscope equipped with phase contrast optics (20× objective, 10× ocular). Sporozoites are best seen at ≥400× combined magnification. Excystation rate should be high (80–100%) by 60 min. Lower excystation rates indicate that the viability of the oocysts is declining and the subsequent growth in vitro will not be optimal.

11. After incubation period is over, remove plunger from 3 or 5 mL plastic syringe and place upright in biosafety cabinet. Attach syringe to plastic filter holder containing filter membrane with 1 µm pore size. Place filter with syringe on top of 15 mL conical.

12. Pipet 1 mL of cell growth medium into syringe. Pipet excysted oocysts into syringe. Insert plunger into syringe and push contents through filter. Remove filter with syringe and place aside. Filter can be carefully disassembled and dropped in an autoclave-safe container filled with $H_2O$ and autoclaved. Autoclave cycle must not include any drying time, because this can warp the filters. Membrane can be discarded after autoclaving.

13. Spin filtered sporozoites at $1250 \times g$ for 3 min.

14. Remove supernatant and resuspend sporozoites in desired volume of cell growth medium. For infecting ALI monolayers, resuspend sporozoites in 50% conditioned medium (without ROCK inhibitor), 30 μL per transwell.

15. To confirm that oocysts have been completely removed from inoculum, check filtered sporozoites under an inverted tissue culture microscope (20× objective, 10× ocular) by pipetting onto glass slide and covering with glass coverslip. Infected monolayers can also be fixed on the same day of infection and stained with an antibody that recognizes *C. parvum* oocysts.

### 3.7 Measuring Host and Parasite Replication in Primary Cell Monolayers by qPCR

This method relies on measuring parasite replication by quantifying *C. parvum* genomic DNA equivalents. Host viability can be assessed by measuring host genomic DNA equivalents from the same sample. The following is a modified protocol from the Qiagen QIAamp DNA mini kit. Buffers tissue lysis buffer (ATL), lysis buffer (AL), AW1, AW2, and AE are provided in the kit.

1. Add 100 μL of Buffer ATL to transwell. Thoroughly scrape cells into buffer with blunt pipette tip; be careful not to puncture transwell membrane. Lysed cells will be sticky and clumpy.

2. Move entire contents of transwell (will likely be more than 100 μL due to presence of mucus/liquid in top chamber) to 1.7 mL microcentrifuge tube. Add 20 μL proteinase K. Vortex to mix.

3. Incubate in 56 °C water bath for 3–24 h.

4. Briefly centrifuge tube to remove drops from inside of lid.

5. Add 200 μL Buffer AL to sample, vortex briefly to mix. A white precipitate is likely to form. Briefly centrifuge tube to remove drops from inside the lid.

6. Incubate in 70 °C heat block for 10 min.

7. Add 200 μL ethanol to sample, vortex briefly to mix. Briefly centrifuge tube to remove drops from inside the lid.

8. Move sample from microcentrifuge tube to spin column. When pipetting sample, be careful not to wet the rim of the spin column. Spin at $7000 \times g$ for 1 min.

9. Place spin column in new collection tube, discard tube containing filtrate.

10. Add 500 μL Buffer AW1 to spin column (be sure not to wet the rim). Centrifuge at $7000 \times g$ for 1 min.

11. Place spin column in new collection tube, discard tube containing filtrate.

12. Add Buffer AW2 to spin column (be sure not to wet the rim). Centrifuge at maximum speed for 3 min.

13. Place spin column in clean 1.7 mL microcentrifuge tube and discard the tube containing filtrate. Add 100 μL Buffer AE to the spin column, incubate at room temperature for 1 min, then centrifuge at $7000 \times g$ for 1 min.

14. Dilute DNA sample tenfold with sterile $H_2O$.

15. To make primer mix for qPCR reaction mix: make 200 μM master stocks of forward and reverse primers in sterile $H_2O$. Combine equal volume of forward and reverse master stocks to make 100 μM master mix. Dilute 100 μM master mix in sterile $H_2O$ to make 5 μM working mix.

16. To make qPCR reaction mix: 9.0 μL 2× SYBR Green mix (or equivalent), 1.8 μL 5 μM primer mix, 5.2 μL $H_2O$ (16 μL total volume). Scale up volume for number of wells needed. Pipet mix into required number of wells of 96-well qPCR reaction plate.

17. Add 2 μL of diluted DNA sample to well. Seal plate with optical film. Centrifuge briefly to ensure that DNA and reaction mix are combined.

18. Primers to measure *C. parvum* GAPDH: forward--GAAGAT-GCGCTGGGAACAAC, reverse--CGGATGGCCATACCTGTGAG.

19. Primers to measure mouse GAPDH: forward--GCCAT-GAGTGGACCCTTCTT, reverse--GAAAACACGGGGGCAATGAG.

20. qPCR thermocycler protocol (*see* Table 1).

21. Place reaction plate in qPCR machine and run thermocycler protocol.

22. In order to correlate $C_t$ values with actual genomic DNA numbers, it is necessary to make a standard for *C. parvum* and mouse cells. For *C. parvum*, DNA from a known number of oocysts can be extracted using the Qiagen QIAamp DNA Mini Kit and diluted to make a standard. For example, DNA from $1 \times 10^7$ oocysts can be extracted. After extraction, the number of gDNA equivalents will be $4 \times 10^7$ per 100 μL (since each oocyst contains 4 sporozoites and the DNA is eluted in a 100 μL volume) or $4 \times 10$ [5]/μL. Create a dilution series (1:2

**Table 1**
**qRT-PCR thermal cycling protocol**

| Reaction step | | Temperature | Duration |
|---|---|---|---|
| Priming | | 95 °C | 2 min |
| Three-step thermal cycles (40×) | Denaturing | 95 °C | 10 s |
| | Annealing | 60 °C | 20 s |
| | Extension | 72 °C | 30 s(read fluorescence) |
| Melt curve | | 95 °C | 15 s |
| | | 60–95 °C | 15 s |
| Hold | | 4 °C | |

to 1:16, for a total of 5 values including the undiluted sample to make up the curve). For mouse cells, a known number of cells can be lysed after the cells are quantified in Subheading 2.2, **step 12** and a dilution series set up in the same way as for *C. parvum*.

23. Because the DNA sample was diluted 10× before running the qPCR reaction, the number of gDNA equivalents determined to be present in 2 μL of sample needs to be multiplied by 10 to be accurate.

*3.8 Monitoring Life Cycle Progression in Primary Cell Monolayers by Fluorescence Microscopy*

1. Move transwells to 24-well plate. Add 500 μL PBS to bottom chamber of transwell.

2. Fix transwells in desired fixative. For example, 4% formaldehyde for 10 min.

3. Wash transwells 2× with PBS. Use pipette to remove liquid from transwell because aspiration may disturb cell layer. If there is a significant mucus layer present (likely if the transwells are at day 7 or later of top media removal), the mucus layer can be removed through pipetting.

4. After fixing, transwells can be stored at 4 °C for up to 10 days before staining (helpful in experiments that span over multiple days).

5. Permeabilize and block cells with desired agents. For example, 0.1% Triton X-100 for 3–5 min or 0.05% saponin for 10 min. If using Triton X-100, dilute in PBS containing 1% BSA. This buffer can be used throughout the staining protocol as a blocking buffer and as a vehicle for primary and secondary antibodies. If using saponin, block with 0.05% saponin in PBS containing 5% normal goat (NGS) and 5% fetal bovine serum (FBS). For antibody dilution and wash steps, drop the saponin concentration down to 0.01% in PBS containing 1% NGS.

6. After blocking, dilute primary antibody in buffer and stain for 60 min at room temperature or overnight at 4 °C.

7. Wash 3× with buffer.

8. Dilute secondary antibody (for example, goat anti-mouse IgG conjugated to Alexa Fluor dyes) in buffer and stain for 60 min at room temperature. Protect from light.

9. Wash 3× with buffer.

10. Stain with 1 μg/mL Hoechst 33258 (1:1000 dilution of stock solution) for 10–30 min. Protect from light.

11. Wash 1× with PBS.

12. Dot 30–50 μL mounting medium fluorescence antiquenching agent onto glass slide. Cut membrane from transwell insert with scalpel. Using forceps, place membrane onto mounting medium, cell side up.

13. Place square coverslip over membrane and push firmly down to remove air pockets. If there is an air pocket that will not go away, check membrane for plastic "curlicues" that can be created during the cutting process—these will stop the coverslip from lying flush with the membrane. Two membranes can fit under one coverslip, but this increases the chance the coverslip will not lie flush with the membranes and air pockets can develop overnight as the mounting medium dries. It is important to thoroughly press down on the coverslip to remove all bubbles. Excess mounting medium will be pushed out and can be wiped off.

14. Protect slides from light and let dry overnight at room temperature.

15. Examine using a light microscope equipped with epifluorescence illumination and appropriate filters. An example of fluorescent staining of *C. parvum* grown in ALI monolayers is shown in Fig. 1c.

## 4    Notes

1. Stocks of 3T3 cells should be kept at low passage numbers (<10). Check appearance of 3T3 cells; unhealthy cells will grow slowly and have vacuolated cytoplasm.

2. Matrigel typically arrives from the manufacturer in glass bottles containing 10 mL and should be stored at −20 °C. To prepare Matrigel aliquots, thaw Matrigel overnight on ice in a polystyrene foam container in a 4 °C fridge. Remove cap and pipette Matrigel up and down 3 times using 10 mL pipette (only draw up 9 mL to avoid creation of bubbles). Aliquot Matrigel in 1 mL aliquots in 1.7 mL microcentrifuge tubes and store at

−20 °C. Move aliquots to 4 °C to thaw. Do not keep aliquots in "warm spots" in fridge such as the door because they may solidify. Aliquots can be kept at 4 °C for several weeks, but it is best to use quickly. Frozen aliquots can be thawed rapidly (30–60 min) by placing in an ice slurry or placing on top of crushed ice. Do not thaw aliquots in 37 °C water bath or room temperature because the Matrigel will solidify. Solidified Matrigel can be refrozen at −20 °C and will thaw into a liquid, though it is not clear if this negatively affects the product. When handling thawed aliquots of Matrigel, do not grip tube with hand, because this will cause it to solidify. Keep the aliquots on ice as much as possible. Always dilute Matrigel into cold DPBS; if the DPBS is warm, the Matrigel will solidify upon contact and will not create a homogenous solution.

3. Y-27632 arrives from the manufacturer as a powder that can be stored at room temperature. Add sterile $H_2O$ to the powder to make 10 mM stock solution. Aliquot 50–150 μL amounts and store at −20 °C. Aliquots can be thawed and refrozen. Do not store aliquots at 4 °C.

4. The amount of spheroids required for different numbers of transwells can vary slightly from run-to-run. Fifteen to 20 wells (12-well plate format) of healthy spheroids should give enough cells to plate 48 transwells at the $5 \times 10^4$ seeding density. If it takes significantly more wells, the spheroids are likely to be too sparse. The $5 \times 10^4$ seeding density was determined to be optimal for *C. parvum* growth (*see* Fig. 1d); higher seeding densities lead to decreased cell growth. If *C. parvum* is not growing well, try a dilution series of different seeding densities.

5. It is important to observe the color of the medium when feeding the cells, since it is an indicator of viability. Four to 6 days after plating the stem cells, the medium should become bright yellow in between feedings. After the ALI is created (7 days after plating the stem cells), the medium will continue to turn yellow between feedings for 3 to 5 days, but the color change will gradually become less dramatic. Eventually, the medium will remain pink in between feedings. If the medium is remaining pink before the creation of the ALI, it is likely the cells are unhealthy and the monolayer is too sparse. Additionally, there will be some liquid and mucus that form in the top chamber after the start of the ALI; if there is copious liquid in the top chamber in addition to pink medium, these are signs the monolayer is dead.

6. The day of infection strongly affects the outcome of infection. Infecting too early after the creation of the ALI (i.e. day 0) leads to poor growth and cell death. Infecting too late (day 7

and later) leads to poor growth, possibly due to the presence of the mucus layer, which may act as a physical barrier to the oocysts and sporozoites. Optimal growth is seen when cultures are infected at day 3 after medium removal.

7. In our experience, it is preferable that the oocysts are purified from cow feces using Sheather's sugar flotation and discontinuous sucrose density gradient centrifugation; oocysts purified using cesium chloride gradients are less viable and exhibit lower excystation and infection rates in the ALI system.

## Acknowledgments

## References

1. Arrowood MJ (2002) In vitro cultivation of *Cryptosporidium* species. Clin Microbiol Rev 15(3):390–400

2. Flanigan TP, Aji T, Marshall R, Soave R, Aikawa M, Kaetzel C (1991) Asexual development of *Cryptosporidium parvum* within a differentiated human enterocyte cell line. Infect Immun 49(1):234–239

3. Gut J, Petersen C, Nelson R, Leech J (1991) *Cryptosporidium parvum*: in vitro cultivation in Madin-Darby canine kidney cells. J Protozool 38(6):72S–73S

4. Sow SO, Muhsen K, Nasrin D, Blackwelder WC, Wu Y, Farag TH, Panchalingam S, Sur D, Zaidi AK, Faruque AS, Saha D, Adegbola RA, Alonso PL, Breiman RF, Bassat Q, Tamboura B, Sanogo D, Onwuchekwa U, Manna B, Ramamurthy T, Kanungo S, Ahmed S, Qureshi S, Quadri F, Hossain A, Das SK, Antonio M, Hossain MJ, Mandomando I, Nhampossa T, Acácio S, Omore R, Oundo JO, Ochieng JB, Mintz ED, O'Reilly CE, Berkeley LY, Livio S, Tennant SM, Sommerfelt H, Nataro JP, Ziv-Baran T, Robins-Browne RM, Mishcherkin V, Zhang J, Liu J, Houpt ER, Kotloff KL, Levine MM (2016) The Burden of *Cryptosporidium* diarrheal disease among children < 24 months of age in moderate/high mortality regions of Sub-Saharan Africa and South Asia, utilizing data from the global enteric multicenter study (GEMS). PLoS Negl Trop Dis 10(5):e0004729. https://doi.org/10.1371/journal.pntd.0004729

5. Chalmers RM, Smith R, Elwin K, Clifton-Hadley FA, Giles M (2011) Epidemiology of anthroponotic and zoonotic human cryptosporidiosis in England and Wales, 2004–2006. Epidemiol Infect 139(5):700–712. https://doi.org/10.1017/S0950268810001688

6. Cacciò SM, Chalmers RM (2016) Human cryptosporidiosis in Europe. Clin Microbiol Infect 22(6):471–480. https://doi.org/10.1016/j.cmi.2016.04.021

7. Morada M, Lee S, Gunther-Cummins L, Weiss LM, Widmer G, Tzipori S, Yarlett N (2016) Continuous culture of *Cryptosporidium parvum* using hollow fiber technology. Int J Parasitol 46(1):21–29. https://doi.org/10.1016/j.ijpara.2015.07.006

8. DeCicco RePass MA, Chen Y, Lin Y, Zhou W, Kaplan DL, Ward HD (2017) Novel bioengineered three-dimensional human intestinal model for long-term infection of *Cryptosporidium parvum*. Infect Immun 85(3):e00731–e00716

9. Miyoshi H, Ajima R, Luo C, Yamaguchi TP, Stappenbeck TS (2012) Wnt5a potentiates

TGF-beta signaling to promote colonic crypt regeneration after tissue injury. Science 338(6103):108–113

10. Miyoshi H, Stappenbeck TS (2013) In vitro expansion and genetic modification of gastrointestinal stem cells in spheroid culture. Nat Protoc 8(12):2471–2482

11. Moon C, VanDussen KL, Miyoshi H, Stappenbeck TS (2014) Development of a primary mouse intestinal epithelial cell monolayer culture system to evaluate factors that modulate IgA transcytosis. Mucosal Immunol 7(7):818–828

12. VanDussen KL, Marinshaw JM, Shaikh N, Miyoshi H, Moon CS, Tarr PI, Ciorba MA, Stappenbeck TS (2014) Development of an enhanced human gastrointestinal epithelial culture system to facilitate patient-based assays. Gut 64(6):911–920. https://doi.org/10.1136/gutjnl-2013-306651

13. Wilke G, Ravindran S, Funkhouser-Jones L, Barks J, Wang Q, VanDussen KL, Stappenbeck TS, Kuhlenschmidt TB, Kuhlenschmidt MS, Sibley LD (2018) Monoclonal antibodies to intracellular stages of *Cryptosporidium parvum* define life cycle progression in vitro. mSphere 3(3):e00124–e00118. https://doi.org/10.1128/mSphere.00124-18

14. Heo I, Dutta D, Schaefer DA, Iakobachvili N, Artegiani B, Sachs N, Boonekamp KE, Bowden G, Hendrickx APA, Willems RJL, Peters PJ, Riggs MW, O'Connor R, Clevers H (2018) Modelling *Cryptosporidium* infection in human small intestinal and lung organoids. Nat Microbiol 3(7):814–823. https://doi.org/10.1038/s41564-018-0177-8

15. Wang X, Yamamoto Y, Wilson LH, Zhang T, Howitt BE, Farrow MA, Kern F, Ning G, Hong Y, Khor CC, Chevalier B, Bertrand D, Wu L, Nagarajan N, Sylvester FA, Hyams JS, Devers T, Bronson R, Lacy DB, Ho KY, Crum CP, McKeon F, Xian W (2015) Cloning and variation of ground state intestinal stem cells. Nature 522(7555):173–178. https://doi.org/10.1038/nature14484

16. Whitcutt MJ, Adler KB, Wu R (1988) A biphasic chamber system for maintaining polarity of differentiation of cultured respiratory tract epithelial cells. Vitro Cell Dev Biol 24(5): 420–428

17. Pezzulo AA, Starner TD, Scheetz TE, Traver GL, Tilley AE, Harvey BG, Crystal RG, McCray PB, Zabner J (2011) The air-liquid interface and use of primary cell cultures are important to recapitulate the transcriptional profile of in vivo airway epithelia. Am J Physiol Lung Cell Mol Physiol 300(1):L25–L31. https://doi.org/10.1152/ajplung.00256.2010

18. O'Boyle N, Sutherland E, Berry CC, Davies RL (2017) Temporal dynamics of ovine airway epithelial cell differentiation at an air-liquid interface. PLoS One 12(7):e0181583. https://doi.org/10.1371/journal.pone.0181583

19. Nossol C, Diesing AK, Walk N, Faber-Zuschratter H, Hartig R, Post A, Kluess J, Rothkötter HJ, Kahlert S (2011) Air-liquid interface cultures enhance the oxygen supply and trigger the structural and functional differentiation of intestinal porcine epithelial cells (IPEC). Histochem Cell Biol 136(1):103–115. https://doi.org/10.1007/s00418-011-0826-y

20. Klasvogt S, Zuschratter W, Schmidt A, Kröber A, Vorwerk S, Wolter R, Isermann B, Wimmers K, Rothkötter HJ, Nossol C (2017) Air-liquid interface enhances oxidative phosphorylation in intestinal epithelial cell line IPEC-J2. Cell Death Discov Feb 27(3):17001. https://doi.org/10.1038/cddiscovery.2017

21. Georgia Wilke, Lisa J. Funkhouser-Jones, Yi Wang, Soumya Ravindran, Qiuling Wang, Wandy L. Beatty, Megan T. Baldridge, Kelli L. VanDussen, Bang Shen, Mark S. Kuhlenschmidt, Theresa B. Kuhlenschmidt, William H. Witola, Thaddeus S. Stappenbeck, L. David Sibley, (2019) A Stem-Cell-Derived Platform Enables Complete Cryptosporidium Development In Vitro and Genetic Tractability. Cell Host & Microbe

# Chapter 21

## Two- and Three-Dimensional Bioengineered Human Intestinal Tissue Models for *Cryptosporidium*

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## Abstract

Conventional cell cultures utilizing transformed or immortalized cell lines or primary human epithelial cells have played a fundamental role in furthering our understanding of *Cryptosporidium* infection. However, they remain inadequate with respect to their inability to emulate in vivo conditions, support long-term growth, and complete the life cycle of the parasite. Previously, we developed a 3D silk scaffold-based model using transformed human intestinal epithelial cells (IECs). This model supported *C. parvum* infection for up to 2 weeks and resulted in completion of the life cycle of the parasite. However, transformed IECs are not representative of primary human IEC.

Human intestinal enteroids (HIEs) are cultures derived from crypts that contain Lgr5+ stem cells isolated from human biopsies or surgical intestinal tissues; these established multicellular cultures can be induced to differentiate into enterocytes, enteroendocrine cells, goblet cells, Paneth cells, and tuft cells. HIEs better represent human intestinal structure and function than immortalized IEC lines. Recently, significant progress has been made in the development of technologies to culture HIEs in vitro. When grown in a 3D matrix, HIEs provide a spatial organization resembling the native human intestinal epithelium. Additionally, they can be dissociated and grown as monolayers in tissue culture plates, permeable supports or silk scaffolds that enable mechanistic studies of pathogen infections. They can also be co-cultured with other human cells such as macrophages and myofibroblasts. The HIEs grown in these novel culture systems recapitulate the physiology, the 3D architecture, and functional diversity of native intestinal epithelium and provide a powerful and promising new tool to study *Cryptosporidium*–host cell interactions and screen for interventions ex vivo. In this chapter, we describe the 3D silk scaffold-based model using transformed IEC co-cultured with human intestinal myofibroblasts and 2D and 3D HIE-derived models of *Cryptosporidium*, also co-cultured with human intestinal myofibroblasts.

**Key words** Three dimensional, Intestinal, Tissue, Organoid, Enteroid, Stem cell, Monolayer, Drug screen, Permeable support, Transwell, Silk scaffold

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Daviel Cardenas, Seema Bhalchandra, and Hymlaire Lamisere contributed equally to this work.

## 1  Introduction

*Cryptosporidium*, an intestinal apicomplexan parasite, is a major cause of diarrhea and death in young children and immunocompromised hosts in resource-limited countries [1]. Pathogenic mechanisms underlying *Cryptosporidium* infection and host responses are poorly understood due, in part, to a lack of appropriate human model systems, which accurately recapitulate the complex multicellular intestinal epithelium. Existing models of *C. parvum* infection in vitro employ transformed cancer-derived IECs or primary human intestinal epithelial cells on 2D [2–4] or 3D culture platforms [5, 6]. However, most of these culture models can only support short-term (<5 days) infection and do not allow continuous propagation of the parasite or completion of its life cycle [7]. Additionally, they are not representative of native intestinal tissue in many ways since they lack the cellular diversity encountered in the intestinal epithelium. Previously, we developed a 3D-silk scaffold-based model for *C. parvum* using Caco-2 and HT29, transformed, IEC which supported infection for up to 2 weeks and permitted completion of the life cycle of the parasite [5]. However, these IEC lines are not representative of native intestinal tissue in many ways since they lack the cellular diversity encountered in the intestinal epithelium and display variations in protein and gene expression [8, 9]. Therefore, an alternative source of primary human epithelial cells is needed to model physiological 3D human intestinal tissue ex vivo.

Recently, it was shown that intestinal crypts (containing leucine-rich repeat-containing G-protein-coupled receptor 5 positive (Lgr5$^+$) stem cells) obtained from human surgical or endoscopic biopsies can be cultured indefinitely as multicellular intestinal epithelial cultures that form self-organizing 3D units called intestinal organoids or enteroids (HIEs) [10–12]. HIEs accurately recapitulate the cell complexity and functionality that are observed in vivo and can differentiate into enterocytes, goblet cells, enteroendocrine cells, and Paneth cells [12, 13]. M cells, which are specialized cells located in the Peyers' patches of the intestinal epithelium and are important antigen presenting cells, can also be differentiated from HIEs by supplementing the medium with receptor activator of NFκB ligand (RankL) [14]. In addition, differentiated HIEs polarize forming brush border microvilli apically, display intact tight junctions and basolateral expression of NA$^+$/K$^+$ ATPase, NKCC1, β-catenin, and E-cadherin [11, 12, 15].

Recently, intestinal and lung organoids have been shown to support the growth of *C. parvum* [16]. Intestinal organoids or human intestinal enteroids (HIEs) provide a novel way to study host–microbe interactions in a number of different formats [12, 15, 17]. Their 3D architecture preserves the physiological nature

of intestinal epithelia, enabling them to be utilized as model systems for evaluating the dynamic processes occurring at the host–microbe interface. HIEs form 3D units naturally in culture but can also be mechanically dissociated and seeded as monolayers onto tissue culture plates, transwell inserts, or bioengineered silk scaffolds as 2D and 3D models, respectively, grown with or without other cell types such as human intestinal myofibroblasts (H-InMyoFib) [18–22]. These culture platforms have distinct apical/basolateral cell surfaces allowing easy access to the apical surface for infection. Since cell-seeding densities can be standardized, these systems permit reproducible and rapid large-scale analyses of the interaction between pathogens and host cells. Thus, by incorporating HIE with or without co-culture with other human cells, into two-dimensional (2D) and 3D model systems, we can better study host–*Cryptosporidium* interactions [5, 12, 23], can accurately analyze gene expression during infection, and discover and test new interventions against this pathogen.

Here, we describe methods for growing monolayers from IECs and HIEs, 2D and 3D models with or without co-culture with other human cells, to study aspects of *Cryptosporidium* replication and pathophysiology that have not been fully assessed previously. The HIEs grown in these novel culture systems recapitulate the physiology, the 3D architecture, and functional diversity of native intestinal epithelium and provide a powerful and promising new tool to study *Cryptosporidium*–host cell interactions and screen for interventions in vitro or ex vivo.

## 2 Materials

All materials are sterile, and all procedures performed in a biosafety cabinet under biosafety level 2 conditions, unless otherwise stated.

### 2.1 Stock Reagents, Buffers, Working Solutions, and Kits

1. 4′,6-diamidino-2-phenylindole (DAPI): Prepare a 300 nM solution by diluting a 1000× stock in PBS.

2. Antibiotic–antimycotic: 10,000 U/mL penicillin, 10,000 μg/mL streptomycin, and 25 μg/mL amphotericin B.

3. Blocking/permeabilization solution: 0.5% Triton X-100, 5% normal goat serum in PBS.

4. Bovine serum albumin (BSA): 5% solution in PBS to block nonspecific binding and a 1% solution in PBS to dilute antibodies.

5. CellTiter 96 MTT Assay kit (Promega Corp., Madison, WI).

6. Collagen type I: 80% 2 mg/mL collagen type I, 10% 10× DMEM, 10% CMGF+.

7. Collagen type IV: prepared as a 1 mg/mL solution in 0.6% acetic acid, which is then diluted 1:30 in water.

8. Commercial bleach: contains between 5 and 6% (by weight) sodium hypochlorite.

9. Crypt-a-Glo Antibody (Ab) (Waterborne, Inc., New Orleans, LA).

10. Dimethyl sulfoxide (DMSO).

11. Ethylenediaminetetraacetic acid (EDTA): 0.5 mM in water is used for dissociating HIEs.

12. Fetal bovine serum (FBS).

13. Fluoro-Gel II (with DAPI), slide mounting medium with fluorescence quenching inhibitor (Electron Microscopy Sciences, Inc., Hatfield, PA).

14. Glutaraldehyde: 2.5% in water is used for scaffold fixation for scanning electron microscopy (SEM).

15. Human transferrin: 10 μg/mL is used as a supplement for complete DMEM.

16. Matrigel, growth factor reduced (GFR), phenol-free (Corning Inc., Corning, NY) (*see* **Note 2**).

17. Methylene blue: used at 0.2% in PBS to locate HIEs in OCT.

18. Nano-Glo Luciferase Assay System (Promega).

19. Nitazoxanide: make a stock solution of 65 mM in DMSO and dilute with differentiation medium to 20 μM or as needed.

20. No-Fade mounting medium (Waterborne, Inc., New Orleans, LA).

21. Normal goat serum (NGS): used at 5% in PBS to block non-specific binding and at 1% in PBS for diluting antibodies.

22. Optimal Cutting Temperature (OCT) embedding medium.

23. Pancreatin: used at a concentration of 0.5 mg/mL in CMGF− to dissociate HIEs.

24. Paraformaldehyde (PFA): make a 4% solution in PBS while heating to 60 °C in a glass beaker on a stir plate in a ventilated hood. The pH is raised by adding 1 N NaOH dropwise from a pipet until the solution clears. Once the paraformaldehyde is dissolved, the solution should be cooled and filtered. Recheck the pH and adjust it with small amounts of dilute HCl to approximately pH 6.9. The solution can be aliquoted and frozen or stored at 2–8 °C for up to one month.

25. Poly-L-lysine: used at a concentration of 100 μg/mL in PBS.

26. Polydimethylsiloxane (PDMS): mix base reagent and curing reagent in a mass ratio of 10:1 (Dow Corning, Midland MI).

27. RNeasy Plus Mini Kit (Qiagen Inc., Valencia, CA).

28. Recovery Cell Culture Freezing medium (Thermo Fisher Scientific Inc., Waltham, MA).

29. Sodium hydroxide (NaOH): 1–5 N solution in water is used to adjust pH.

30. Sodium taurocholate: 0.75% sodium taurocholate in PBS is used for excystation of oocysts.

31. Sporo-Glo Ab (Waterborne, Inc.).

32. Staining solution: 0.05% Tween 20, 5% NGS in PBS.

33. Triton/BSA: 0.5% Triton X-100, 5% BSA in PBS.

34. Trypsin–EDTA: 0.25% trypsin, 0.5 mM EDTA in water.

35. Y-27632, Rho-associated protein kinase (ROCK) inhibitor.

*2.2  Media*

1. Complete Dulbecco's Modified Eagle Medium (DMEM): DMEM supplemented with 10% FBS, 10 μg/mL human transferrin, and 1× antibiotics and antimycotics.

2. Smooth Muscle Growth Medium-2 (SmGM-2) (Lonza, Walkersville, MD).

3. Complete Medium with Growth Factors (CMGF+): (*see* Table 1, **Note 1**).

4. Complete Medium without Growth Factors (CMGF–) (*see* Table 1, **Note 1**).

5. Differentiation medium: CMGF+ without L-WNT3a, R-spondin, nicotinamide, and SB202190; reduce Noggin to 5%.

6. High Wnt 3a growth medium: (1 part CMGF+ to 1 part L-WNT3Aa conditioned medium).

*2.3  Cell Lines and Supplements*

1. HT29-MTX (European Collection of Authenticated Cell Cultures, Public Health England, Salisbury, UK).

2. Caco-2 cells (ATCC, Manassas, VA).

3. Primary human intestinal myofibroblasts (H-InMyoFib) (Lonza).

4. L-WNT3a cells (ATCC).

5. R-Spondin: obtained from Dr. Calvin J. Kuo under a Materials Transfer Agreement with Stanford University.

6. Noggin: A gift from Dr. Gijs van den Brink (University of Amsterdam) [24].

*2.4  Parasites*

1. *C. parvum* (Iowa isolate) oocysts: stored at 4 °C in PBS with antibiotics and antimycotics and used within 3 months (Bunch Grass Farms, Deary, ID).

2. Transgenic *C. parvum* expressing nanoluciferase (NLuc) gene obtained as previously described [25].

**Table 1**
**Composition of complete media for HIE maintenance and differentiation**

| Reagent | Final concentration | Origin |
|---|---|---|
| Complete media without growth factors (CMGF−) | | |
| Advanced DMEM/F12 | N/A | Invitrogen |
| GlutaMAX-1 | 2 mM | Invitrogen |
| HEPES | 10 mM | Invitrogen |
| Penicillin/streptomycin | 100 U/ml | Invitrogen |
| Complete media with growth factors (CMGF+) | | |
| CMGF− | N/A | N/A |
| L-WNT3a-conditioned media | 50% | ATCC |
| R-Spondin-conditioned media | 20% | A gift from Dr. Calvin J. Kuo (Stanford University) |
| Noggin-conditioned media | 10% | A gift from Dr. Gijs van den Brink (University of Amsterdam) |
| B27 | 1× | Invitrogen |
| N2 | 1× | Invitrogen |
| N-acetylcysteine | 1 mM | Sigma-Aldrich |
| Mouse recombinant EGF | 50 ng/mL | Invitrogen |
| [Leu15]-Gastrin I | 10 nM | Sigma-Aldrich |
| Nicotinamide | 10 mM | Sigma-Aldrich |
| A-83-01 | 500 nM | Tocris |
| SB202190 | 10 μM | Sigma-Aldrich |

Table modified and reproduced from Ref. [21] with permission from Springer Nature Methods in Molecular Biology

# 3    Methods

***3.1    3D Silk Scaffold-Based System Using Classical, Cancer-Derived, Transformed Intestinal Epithelial Cell (IEC) Lines and Intestinal Myofibroblasts***

1. Caco-2 and HT29-MTX cells are grown in complete DMEM.
2. Primary human intestinal myofibroblasts (H-InMyoFib) are grown in SmGM-2.
3. All cell lines are cultured in T-75 or T-175 cm$^2$ tissue culture flasks at 37 °C, 5% $CO_2$ in a humidified atmosphere and harvested with trypsin–EDTA prior to seeding.

*3.1.1    Growth and Maintenance of IEC Lines*

| | |
|---|---|
| *3.1.2 Freezing Stocks of IEC* | 1. Ideally, cell viability should be in excess of 90% in order to achieve a good recovery after freezing. |
| | 2. Treat a T75 (or T175) flask of adherent Caco-2 or HT29-MTX cells with 4 (or 10) mL, respectively, of trypsin–EDTA and incubate at 37 °C for 1–2 min or until cells detach. |
| | 3. Add a volume of IEC growth medium containing 10% FBS equivalent to twice the volume of trypsin–EDTA to inactivate the enzyme. |
| | 4. Remove a small aliquot of cells (100–200 μL) and perform a cell count using a hemocytometer. Centrifuge the remaining culture at $200 \times g$ for 5 min. |
| | 5. Resuspend the pelleted cells at a concentration of $2–4 \times 10^6$ cells per mL in Recovery Cell Culture Freezing Medium (*see* **Note 2**). |
| | 6. Pipet 1 mL aliquots of cells into cryogenic vials that have been labeled with the cell line name, passage number, cell concentration, and date. |
| | 7. Place cryogenic vials inside a passive freezing container. Fill container with isopropyl alcohol and place at −80 °C overnight. Frozen cryogenic vials should be transferred to the vapor phase of a liquid nitrogen storage container and the locations of vials recorded. |
| *3.1.3 Reviving Cryopreserved IECs* | 1. Remove a frozen stock of IECs from liquid nitrogen storage and immediately transfer to a 37 °C water bath. |
| | 2. Thaw cells rapidly (<1 min) by gently swirling the vial in a 37 °C water bath. |
| | 3. Transfer vial to a biosafety cabinet. Before opening, wipe the outside of the vial with 70% ethanol. |
| | 4. Transfer thawed cells into 10 mL of warm complete DMEM growth medium in a 15 mL conical tube. |
| | 5. Centrifuge cell suspension at $200 \times g$ for 5 min at RT. |
| | 6. Check the clarity of the supernatant and visibility of a complete pellet. Aseptically decant the supernatant without disturbing the cell pellet. |
| | 7. Gently resuspend cells in 4 or 10 mL complete DMEM growth medium for T75 or T175 flasks, respectively, transfer them into a tissue culture flask, and incubate at 37 °C, 5% $CO_2$. |
| *3.1.4 Generation of 3D Silk Scaffolds* | 1. 3D silk scaffolds are prepared from silk fibroin protein extracted from *Bombyx mori* silkworm cocoons as described previously [26]. |
| | 2. To prepare silk scaffolds with hollow channels, special cylindrical molds are cast from PDMS (Fig. 1). |
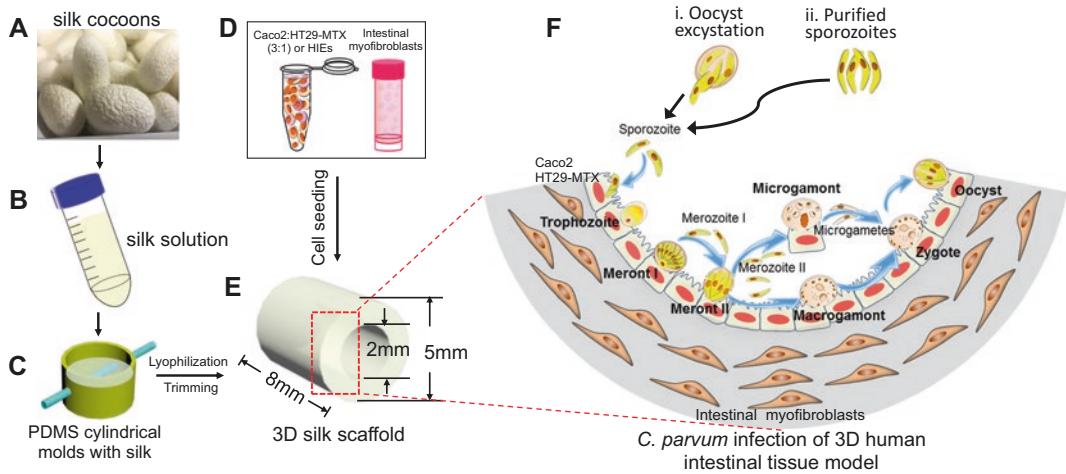
**Fig. 1** Bioengineered 3D human intestinal tissue model. (**a** and **b**) Silk cocoons (**a**) are processed to yield a viscous silk solution (**b**). (**c**) The silk solution is poured into cylindrical molds, and a wire is inserted to develop a lumen equivalent. (**d** and **e**) Caco-2 and HT29-MTX or human intestinal enteroid (HIE) cells (**d**) are seeded into the lumen (**e**), while the porous bulk space is seeded with H-InMyoFibs. (**f**) The Caco-2 and HT29-MTX or HIE cells in the lumen are infected with *C. parvum* oocysts or purified sporozoites. Intracellular development through asexual and sexual cycles occurred to complete the life cycle with the formation of oocysts. Figure modified and reproduced from Ref. [5] with permission from the American Society for Microbiology

3. Insert a Teflon-coated stainless-steel wire, diameter, 2 mm (McMaster-Carr, Douglasville, GA) through the cross section of the cylinder to develop a hollow channel in the silk scaffold.

4. Allow the PDMS to cure at 60 °C for 2 h.

5. Pour a 4–5% (w/v) viscous silk solution into the PDMS molds.

6. Freeze molds at −20 °C overnight, then transfer to a lyophilizer for drying.

7. Autoclave the dried silk scaffolds to induce β-sheet conformation.

8. Soak in distilled water overnight, and then trim along the axis of the hollow channel to obtain a cuboid 5 by 5 by 8 mm.

9. The resulting scaffold is 8 mm in length and has a hollow channel space, 2 mm in diameter and surrounded by a bulk space containing interconnected pores.

*3.1.5 Preparing Silk Scaffolds for Seeding*

1. Transfer scaffolds to a petri dish using forceps and aspirate excess fluid gently (use a 2 mL pipet tip) until the scaffolds turn white in color.

2. For every scaffold to be seeded, prepare 400 μL of collagen type I solution. To neutralize the pH, add 5 N NaOH dropwise and swirl slowly until the solution turns a peach color.

Care must be taken not to overshoot the end point of titration (pH 8.0), in which case the solution will turn bright pink in color. Keep collagen type I solution on ice until ready to use.

3. Introduce 200 μL of collagen type I solution into the lumen of a scaffold from one end. Add another 200 μL of collagen type I solution from the other end. Dispense any remaining collagen type I gel on the outer walls of the scaffold.

4. Using forceps, insert Teflon-coated stainless steel into each scaffold to create a lumen as described previously [26].

5. Place the petri dish containing scaffolds into a 5% $CO_2$ incubator at 37 °C for at least 15 min for the collagen type I to set. Do not wait longer than 1.5 h.

*3.1.6 Seeding IEC onto Silk Scaffolds*

1. Harvest Caco-2, HCT29-MTX, and H-InMyoFib cells by treating with trypsin–EDTA for 2 min at 37 °C as described in Subheading 3.1, "Freezing stocks of IEC."

2. Resuspend $2 \times 10^5$ H-InMyoFib cells per mL of collagen type I solution.

3. Introduce H-InMyoFib cells into the porous bulk space of the scaffold using a micropipette (20–200 μL).

4. Using forceps carefully transfer a Teflon-coated stainless-steel wire into the opening of the collagen type I-coated scaffold; to leave the hollow channels open for seeding with Caco-2/HT29-MTX cells in the next step.

5. After 20–25 min of gelation at 37 °C, carefully remove the Teflon-coated stainless-steel wire from the scaffold to create a hollow channel (lumen).

6. Using a micropipette (20–200 μL), seed the hollow channel of the scaffold with Caco-2/HT29-MTX cells (3:1) at a density of $4 \times 10^6$ cells/mL.

7. Flip the scaffolds upside down and incubate for 1 h at 37 °C, 5% $CO_2$ to facilitate cell adherence.

8. During the incubation, drip a small amount of SmGM-2 medium onto the scaffolds to keep them moistened.

9. Incubate seeded scaffolds in 24-well plates with SmGM-2 culture medium (complete DMEM and SmGM-2 at a 1:1 ratio) and incubate for up to 2 weeks (changing the medium every other day) at 37 °C, 5% $CO_2$ prior to infection with *C. parvum*.

*3.1.7 Infection of 3D IEC-Based Silk Scaffolds*

Preparation of *C. parvum* Oocysts for Infection

1. Take $1 \times 10^7$ oocysts per scaffold to be infected. Add an extra 10% of oocysts to account for loss during processing. Centrifuge oocysts at $16,000 \times g$ for 3 min at 4 °C in a microcentrifuge and discard the supernatant.

2. For excystation, resuspend $1 \times 10^7$ oocysts (per scaffold) in 1 mL of 10% commercial bleach in PBS and incubate on ice for 10 min.

3. Centrifuge at $16{,}000 \times g$ for 3 min at 4 °C and discard the supernatant.

4. Wash the oocyst pellet in 1 mL of PBS, centrifuge at $16{,}000 \times g$ for 3 min at 4 °C, and discard the supernatant. Repeat this step once to remove all traces of bleach.

5. Resuspend oocysts in 1 mL of 0.75% sodium taurocholate in PBS and incubate for 10 min at 15 °C in a dry block incubator.

6. Centrifuge at $16{,}000 \times g$ for 3 min at 4 °C and discard supernatant.

7. Resuspend the pellet in 1 mL of PBS, centrifuge at $16{,}000 \times g$ for 3 min at 4 °C, and discard the supernatant.

8. Resuspend the pellet in 1 mL of differentiation medium, centrifuge at $16{,}000 \times g$ for 3 min at 4 °C, and discard the supernatant.

9. Resuspend final pellet in enough warm differentiation medium to give 40 µL per scaffold to be infected.

10. Dilute an aliquot of oocyst suspension 1:100 in differentiation medium and count using a hemocytometer.

11. If necessary, adjust the volume to give $1 \times 10^7$ oocysts per 40 µL per scaffold.

12. Infect scaffolds following the protocol in Subheading 3.1 "Infection of 3D IEC-based silk scaffolds."

13. Leftover oocyst suspension can be incubated for 1 h at 37 °C to determine the excystation rate (determined by counting numbers of intact and excysted oocysts using a hemocytometer).

*C. parvum* Infection of 3D IEC-Based Scaffold Model with Pretreated Oocysts

1. Using forceps carefully transfer scaffold(s) to a petri dish. Avoid squeezing the spongy silk scaffold during this process.

2. Gently remove medium from inside the lumen of the scaffold using a micropipette (20–200 µL) without touching the walls so as to avoid dislodging the seeded cells within.

3. Carefully dispense 40 µL of oocyst suspension ($10^7$ total oocysts) into the scaffold lumen without touching the seeded cell layer within. To keep a steady hand while aspirating and dispensing suspensions into the lumen, it helps to rest the upper end of the pipet shaft on the raised edge of the petri dish.

4. Cover the petri dish and incubate for 2 h at 37 °C, 5% $CO_2$ to allow excystation of the oocysts and attachment of the sporozoites.

5. After 2 h, use forceps to carefully transfer each scaffold to a well of a 24-well plate containing 2 mL of warm differentiation medium.

6. Incubate at 37 °C, 5% $CO_2$ for the duration of infection.

7. Keep an uninfected control scaffold for each time point or as required.

**Isolation of *C. parvum* Sporozoites for Infection**

1. Resuspend oocysts in 1 mL of PBS and allow them to excyst by incubating for 1 h at 37 °C.

2. Monitor excystation as described in Subheading 3.1 "Preparation of *C. parvum* oocysts for infection."

3. Once ~80% of oocysts have excysted, centrifuge the excystation mixture at $500 \times g$ for 3 min at 4 °C to pellet oocyst walls (shells) and unexcysted oocysts. Sporozoites will remain in suspension.

4. Filter sporozoites through a 3 μm polycarbonate membrane to remove remaining unexcysted oocysts and empty shells. Check an aliquot of the filtrate under the microscope to ensure there are no visible oocysts. Filter again if necessary.

5. Centrifuge purified sporozoites at $16,000 \times g$ for 5 min at 4 °C.

6. Resuspend in 500 μL of culture medium and count the number of sporozoites using a hemocytometer. Adjust the volume to obtain $1 \times 10^5$ sporozoites/μL.

7. To ensure that no oocysts are present in the filtrate, check by immunofluorescence using an oocyst-specific mAb (Crypt-a-Glo) and a sporozoite-reactive rat polyclonal antibody (Sporo-Glo). Spot aliquots of the filtrate onto poly-L-lysine coated 8-well slides. Once the spots are dry, fix filtrate in ice-cold methanol for 30 min at RT, then wash off the fixative with excess PBS. Incubate with Crypt-a-Glo or Sporo-Glo for 30 min at RT according to the manufacturer's instructions. Place a drop of mounting medium on each well containing the fixed sporozoites prior to sealing with a coverslip. Secure the coverslip with clear nail polish. Proceed for imaging by differential interference contrast (DIC) or fluorescence microscopy.

***C. parvum* Infection of 3D IEC-Based Scaffolds with Purified Sporozoites**

1. Using forceps carefully transfer a scaffold to a petri dish. Avoid squeezing the spongy silk scaffold during this process.

2. Gently remove medium from inside the lumen using a micropipette (20–200 μL) without touching the walls so to avoid dislodging the seeded cells within.

3. Dispense 40 µL of purified sporozoites ($4 \times 10^6$) into the lumen of the scaffolds.

4. Incubate the infected scaffolds for 2 h at 37 °C, 5% $CO_2$.

5. After 2 h, use forceps to carefully transfer each scaffold to a well of a 24-well plate containing 2 mL warm differentiation medium.

6. Incubate at 37 °C 5% $CO_2$ for the duration of infection, changing the medium every other day.

**Processing Infected 3D IEC-Based Scaffolds for Confocal Microscopy**

1. At each time point, remove scaffold from culture plate and transfer to a new 24-well plate.

2. Add 1 mL of freshly prepared 4% PFA and incubate for 45 min at RT.

3. Remove PFA and wash three times with PBS.

4. Cut the scaffold lengthwise into two halves using a pair of small scissors (*see* **Note 3**).

5. Submerge the scaffold halves (lumen face down) in 1–2 mL of 0.5% Triton X-100, 5% BSA in PBS for 10–15 min to permeabilize the cells within the scaffold.

6. Wash three times with PBS.

7. Block nonspecific binding with 5% BSA for 30 min to 2 h at RT.

8. Wash three times with PBS.

9. Incubate scaffold with primary anti-*C. parvum* antibody of choice or Sporo-Glo (Waterborne Inc.) in 1% BSA (lumen face down) in a closed, moist chamber overnight at 4 °C.

10. Wash three times with PBS.

11. If using primary anti-*C. parvum* antibody, incubate the scaffold with secondary antibody prepared in 1% BSA in a closed, moist chamber for 1.5 h at RT.

12. Wash three times with PBS.

13. Counterstain scaffold with 300 nM DAPI for 30 min to 1 h at RT. Keep protected from light in a closed, moist chamber.

14. Remove DAPI solution, wash scaffolds three times with PBS, and store in PBS supplemented with 50% glycerol for up to 4 weeks at 4 °C.

15. Before imaging, cut each half lengthwise into two additional pieces, so the arch of the lumen is minimized for better imaging. Leave in PBS until ready to image. Place the scaffold strips lumen face down on microscope slides during imaging.

16. Image using a confocal microscope at 20× magnification.

| | |
|---|---|
| Processing Infected 3D IEC-Based Scaffolds for Scanning Electron Microscopy | 1. Fix the infected and uninfected scaffolds in 2.5% glutaraldehyde as described [22]. |

1. Fix the infected and uninfected scaffolds in 2.5% glutaraldehyde as described [22].

2. Prepare the samples by progressive dehydration in a graded series of ethanol concentrations (30%, 50%, 75%, 95%, and twice in 100% for 30 min at each concentration).

3. Dry the samples by critical point drying with a liquid $CO_2$ dryer.

4. Coat with 5 nm of platinum/palladium using a sputter coater and image using a scanning electron microscope (e.g., Zeiss UltraPlus or Zeiss Ultra 55 (Carl Zeiss SBE, LLC, Thornwood, NY) at a voltage of 2–3 kV).

*3.1.8   Quantification of C. parvum Infection in 3D IEC-Based Scaffolds [5]*

Extraction of RNA from Scaffolds

1. At each time point, remove the luminal contents from each scaffold and reserve.

2. Add 40 µL of trypsin–EDTA solution into the lumen and incubate the scaffold for 10 min at 37 °C, 5% $CO_2$.

3. Collect the luminal contents following trypsinization, then flip the scaffold 180°, and repeat trypsinization step with an additional 40 µL of trypsin–EDTA solution. Collect all the cells released and reserve.

4. Combine all reserved contents and centrifuge at 16,000 × *g* for 2 min at 4 °C.

5. At each time point, extract RNA from three infected scaffolds using the RNeasy Plus Mini Kit per manufacturer's protocol.

6. Remove contaminating genomic DNA by treatment with TURBO DNase kit (Thermo Fisher Scientific, Inc.) per manufacturer's protocol.

7. Quantify RNA yield and purity using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.).

RT-qPCR and Statistical Analysis

Infection in IEC monolayers can be monitored by reverse transcription, quantitative PCR (RT-qPCR) using *C. parvum* 18S rRNA-specific gene primers [12], and QuantiTect SYBR Green Master Mix (Qiagen).

1. For each sample, use 5 ng of RNA to synthesize cDNA using standard kits.

2. Prepare qPCR reactions in 96-well PCR plates and perform qPCR using a standard machine.

3. Reaction mixtures are heated to 95 °C for 15 min and then subjected to 40 thermal cycles (94 °C for 30 s, 52 °C for 30 s, and 72 °C for 30 s) of PCR amplification.

4. After amplification, perform melting curve analysis between 55 and 95 °C to assess the specificity of the reactions.

5. Perform three replicate reactions for each sample and repeat the qPCR assay twice for a total of six technical replicates.

6. Determine the 18S cDNA copy number by comparison with a standard curve obtained by qPCR of DNA extracted from $10^2$ to $10^7$ oocysts using a QIAamp DNA Mini Kit (Qiagen) as described previously [5].

7. Since each *C. parvum* genome contains five copies of the 18S rRNA gene and each oocyst contains four sporozoites [27], the oocyst number is multiplied by 20 to obtain the approximate total number of 18S ribosomal cDNA copies.

8. Statistical analyses are performed using the Kruskal–Wallace test followed by the Dunn's multiple comparisons test.

**3.2 Human Intestinal Enteroid (HIE) Culture-Based Models**

*3.2.1 Growth and Maintenance of HIEs*

1. Passage HIEs at a ratio of 1:2 to 1:3 every 6–7 days.

2. Aspirate medium from wells without disturbing the Matrigel plug containing HIEs.

3. Add 500 μL of ice-cold CMGF− to each well and mechanically break up Matrigel plug by gently pipetting up and down with a micropipette (100–1000 μL) pipet (*see* **Note 2**).

4. Further disrupt the HIEs by passing 2–3 times through a 25G × 5/8″ needle attached to a 1 mL syringe. Transfer the contents of up to 10 wells into a 15 mL conical tube. Add an additional two volumes of cold CMGF− to dissolve the Matrigel.

5. Centrifuge tubes at $200 \times g$ for 5 min at 4 °C and discard supernatant.

6. Resuspend the cell pellet in enough Matrigel to give 30 μL/well. Use cold pipet tips to prevent the Matrigel from congealing and avoid introducing bubbles into the suspension.

7. Using cold micropipette (20–200 μL) tips, take 30 μL of Matrigel suspension and place in the center of each well in a 24-well plate.

8. Transfer plate to a 37 °C $CO_2$ incubator for 5–10 min to allow the Matrigel to set.

9. Add 500 μL of growth medium at RT to each well of HIEs and culture in a 37 °C, 5% $CO_2$ incubator for 6–7 days, changing the growth medium every other day (Mondays, Wednesdays, and Fridays) (*see* **Note 4**).

10. If downstream analysis or infection is desired, culture HIEs in growth medium for 4 days and then switch to differentiation medium for 3–5 days.

11. Change the differentiation medium every other day until use.

*3.2.2   Cryogenic Preservation and Revival of HIEs*

Cryogenic Preservation of HIEs

1. After 6–7 days of culture, HIEs can be prepared for long-term storage in liquid nitrogen.

2. Aspirate the medium from wells without disturbing the Matrigel plug. Add 500 µL of cold CMGF– to each well and mechanically break up the Matrigel by passing through a micropipette (100–1000 µL) tip several times.

3. Transfer the contents of up to 10 wells into an ice-cold 15 mL conical tube and add an additional two volumes of cold CMGF– to dissolve the Matrigel.

4. Centrifuge tube at $200 \times g$ for 5 min at 4 °C and discard the supernatant.

5. Resuspend the pellet in enough freezing medium to give 500 µL for every two wells of HIEs that were processed. Place 500 µL into each cryovial. For easy identification, use color-coded vials for different lines of HIEs.

6. Transfer cryovials into a cell freezing container and place overnight at −80 °C. The next day transfer vials into liquid nitrogen for long-term storage.

Reviving Frozen Stocks of HIEs from Liquid Nitrogen

1. Thaw enough Matrigel overnight at 4 °C to give 120 µL per cryovial to be thawed.

2. Add 10 mL of CMGF– into a 15 mL conical tube and keep on ice.

3. Take vial(s) from liquid nitrogen and immediately hold under tap water at RT until contents are thawed.

4. Transfer the contents in the vial(s) to the 15 mL tube containing 10 mL of ice-cold CMGF–.

5. Centrifuge HIEs at $200 \times g$ for 5 min at 4 °C and discard the supernatant without disturbing the pellet.

6. Using cold micropipette tips, resuspend the pellet in 120 µL of Matrigel per cryovial and place 30 µL in the center of as many wells as needed of a 24-well plate.

7. Carefully place plate in a 37 °C, 5% $CO_2$ incubator for 5–10 min to allow Matrigel to set.

8. Add 500 µL of growth medium at RT to each well of HIEs and culture in a 37 °C, 5% $CO_2$ incubator for 6–7 days, changing the growth medium every other day (*see* **Note 5**).

*3.3   Growth and Infection of 3D HIEs in Culture*

Grow and maintain HIE cultures as in Subheading 3.2.

*3.3.1   Growth of 3D HIEs in Culture*

*3.3.2  C. parvum Infection
of 3D HIE in Culture*

Preparing Oocysts
for Infection

1. Take $5 \times 10^5$ oocysts for every well of HIEs to be infected.

2. Centrifuge oocysts at $16{,}000 \times g$ for 3 min at 4 °C in a micro-centrifuge and discard the supernatant.

3. Resuspend the pellet in 1 mL of 10% commercial bleach in PBS and incubate on ice for 10 min.

4. Centrifuge the oocysts at $16{,}000 \times g$ for 3 min at 4 °C in a microcentrifuge.

5. Resuspend the pellet in 1 mL of PBS and centrifuge at $16{,}000 \times g$ for 3 min at 4 °C. Repeat this wash step one more time.

6. Resuspend oocysts in 1 mL of 0.75% sodium taurocholate in PBS and incubate for 10 min at 15 °C.

7. Centrifuge at $16{,}000 \times g$ for 3 min at 4 °C and discard the supernatant.

8. Resuspend in 1 mL of ice-cold CMGF− and centrifuge at $16{,}000 \times g$ for 3 min at 4 °C. Repeat this wash step one more time.

9. Resuspend the final oocyst pellet in 200 µL of CMGF−.

Infection of 3D HIEs

1. Prepare a solution of 0.5 mg/mL pancreatin in CMGF− solution.

2. For each well of HIEs to be infected, use two wells of 4-day-old differentiated or undifferentiated HIE cultures (*see* **Note 6**).

3. Aspirate medium from 4-day-old HIE cultures without disturbing the Matrigel plug and add 500 µL of cold CMGF− to each well.

4. Mechanically break up the Matrigel by pipetting up and down with a micropipette (100–1000 µL). Add an additional two volumes of ice-cold CMGF− to dissolve the Matrigel. Transfer HIE suspension to a chilled 15 mL conical tube.

5. Centrifuge the tube at $200 \times g$ for 5 min at 4 °C. Discard the supernatant and add more CMGF− to wash the pellet. Repeat centrifugation step.

6. Carefully discard the supernatant without disturbing the HIE pellet.

7. Resuspend the pellet in the 200 µL of pretreated oocyst suspension.

8. Add 200 µL of 0.5 mg/mL pancreatin in CMGF− to the HIE/oocyst suspension, bringing the final concentration of pancreatin to 0.25 mg/mL.

9. Further break open the HIEs by pipetting up and down ~20 times with a micropipette (100–1000 µL).

10. Incubate the 15 mL tube containing the oocyst/HIE mix for 2 h at 37 °C to allow for excystation and attachment of the parasite to HIEs. Leave the cap loose to allow for gas exchange.

11. After 2 h of incubation, centrifuge the tube at $200 \times g$ for 5 min at 4 °C and discard the supernatant.

12. Resuspend the pellet into enough Matrigel to give 30 μL per well of a 24-well plate. Add 30 μL droplets to each well and incubate plate for 5–10 min at 37 °C to allow Matrigel to set.

13. Add 500 μL of differentiation medium per well and incubate the plate in a 37 °C, 5% $CO_2$ incubator.

14. Harvest the HIEs at different time points post infection to isolate RNA or make OCT blocks. Two wells of HIEs are required for preparing one OCT block.

*3.3.3 Processing of Infected 3D HIE for Frozen Sections and Immunofluorescence Assays (IFA)*

Preparing OCT Blocks for Cryosectioning

1. Wash HIE cultures to be processed with 500 μL of ice-cold PBS.

2. Add 500 μL of ice-cold PBS per well and transfer to a 1.5 mL microcentrifuge tube.

3. Centrifuge tube(s) at $200 \times g$ for 5 min at 4 °C and carefully discard the supernatant without disturbing the pellet.

4. Add 500 μL of freshly made 4% PFA to the tube(s) and incubate for 20 min at RT.

5. After incubation, wash the pellet twice with 500 μL of cold PBS.

6. Resuspend the pellet in 50 μL of 0.2% methylene blue in PBS and incubate for 30 min at RT.

7. Add PBS to fill up the tube and centrifuge at $200 \times g$ for 5 min at 4 °C.

8. Wash the pellet with PBS twice more, or until the excess methylene blue is no longer present in the wash and discard the supernatant.

9. Add a drop of OCT compound to the center of a cryomold.

10. Submerge a plastic disposable inoculation loop into the OCT and then carefully scoop out the HIE pellet from the tube (*see* **Note 7**).

11. Transfer the whole clump of cells into the center droplet of the mold, place the mold onto dry ice immediately, and wait for the droplet to solidify. Then, add more OCT to fill up the mold.

12. Immediately store the molds overnight at −80 °C or until ready for sectioning.

13. Using a cryostat, prepare 5 μm sections from the OCT block for IFA.

14. Store blocks and prepared slides containing cut sections at −80 °C until ready for IFA.

**Processing Sections of Infected 3D HIE for Immunofluorescence Assays (IFA)**

Cryosections on slides can be processed by IFA to identify *C. parvum*-infected cells as described previously [25] and to examine other structural and nonstructural cellular components.

1. Prepare the blocking/permeabilization solution (0.5% Triton X-100, 5% normal goat serum in PBS) and the staining solution (0.05% Tween 20, 5% normal goat serum in PBS).

2. Remove slides from −80 °C and let them thaw for 30 min at RT before starting IFA.

3. Using a hydrophobic barrier (PAP) pen, draw a circle around the HIE sections and allow to dry. Solutions will be placed within these circles during staining.

4. Enough solution to completely cover the section without spilling over the PAP pen edge is needed (~75–100 µL for one section, ~150 µL for two sections next to each other).

5. Add blocking/permeabilization solution to each slide and incubate for 1 h at 37 °C. After blocking the sections, a smaller volume is necessary to cover them (~75 µL for a single section).

6. Place slides in a covered moist chamber.

7. Use staining solution to prepare the desired dilutions of primary antibodies. Cover the cryosections with primary antibody solution but avoid flooding the slide.

8. Place slides in a covered moist chamber and incubate overnight at 4 °C.

9. Wash the tissue sections three times with wash buffer by placing slides into a glass slide chamber, adding the wash buffer, and gently letting them rock on a rocking platform or rotator for ~5 min each wash.

10. Add the desired secondary antibodies diluted in staining solution.

11. Incubate the slides for 2 h at RT protected from light.

12. Wash tissue sections three times in wash buffer as in **step 8**.

13. Place a drop of mounting medium over the section and seal with a coverslip. Secure the coverslip to the glass slide with clear nail polish.

14. Proceed for imaging by differential interference contrast (DIC) or fluorescence microscopy.

**Processing Infected 3D HIE for Paraffin Embedding and Immunohisto chemistry.**

Where desired, immunohistochemistry can also be employed for staining sections of fixed HIEs as described previously [19].

Quantification of *C. parvum*
Infection in 3D HIEs

1. Extract RNA from 3D HIEs as described in Subheading 3.1.

2. Perform RT-qPCR and analyze the data statistically as in Subheading 3.1.

### 3.4 3D Silk Scaffold-Based Model System Using HIE

Prepare 3D silk scaffolds following the steps in Subheading 3.1 "Preparing silk scaffolds for seeding."

*3.4.1 Preparing 3D Silk Scaffolds for Seeding*

*3.4.2 Seeding of HIE onto 3D Silk Scaffolds*

Cell Separation and Dissociation of HIEs

1. For seeding one scaffold, collect three wells of HIEs (each containing at least 300–400 HIEs) cultured in growth medium for 6–7 days.

2. Carefully aspirate medium around the Matrigel plug containing HIEs from each well, leaving the plug intact.

3. Add 500 μL of cold 0.5 mM EDTA and mechanically break up the Matrigel by gently pipetting up and down 10 times with a micropipette (100–1000 μL) to help release HIEs from the Matrigel.

4. Transfer entire contents of up to 10 wells into a chilled 15 mL tube kept on ice.

5. Centrifuge HIEs at $200 \times g$ for 5 min at 4 °C and discard the supernatant.

6. Resuspend the pellet in 0.5 mL of trypsin–EDTA and incubate for 4 min at 37 °C.

7. Inactivate the trypsin by adding 1 mL of DMEM containing 10% FBS.

8. Dissociate the HIEs by vigorously pipetting up and down ~50 times using a micropipette (100–1000 μL). Pipet against the side of the tube to avoid making bubbles while dissociating the HIEs.

9. Place a 40 μm cell strainer on top of a 50 mL conical tube and wet the membrane by passing 1 mL of DMEM containing 10% FBS through it.

10. Using a micropipette (100–1000 μL), pass the cells through the cell strainer and into the 50 mL conical tube.

11. Discard the cell strainer containing any cell clumps and centrifuge the cells that passed through the cell strainer at $200 \times g$ for 5 min at 4 °C.

12. Resuspend the cell pellet in an appropriate volume of growth media containing 10 μM Y-27632.

Cell Seeding in 3D Silk
Scaffolds

1. Remove petri dish containing collagen type I-coated scaffold from the $CO_2$ incubator.

2. Using forceps, slide out the Teflon-coated wires and remove any excess solution or debris from the lumen using a micropipette (20–200 µL).

3. Add 35 µL of the homogenous HIE cell suspension into the lumen of each scaffold (*see* **Note 8**).

4. Incubate petri dish containing scaffold at 37 °C, 5% $CO_2$ for at least 45 min to allow cells to attach.

5. Repeat **steps 1–12** of "Cell separation and dissociation of HIEs" above to be able to seed the other half of the scaffold.

6. Flip the scaffold over and seed the second half by adding 35 µL of cell suspension into the lumen of each scaffold (ensure cell suspension is homogenous before adding).

7. Incubate the scaffold at 37 °C, 5% $CO_2$ for 1 h to allow cell attachment before transferring them into a 24-well plate using forceps.

8. Add 1.5 mL of growth medium with 10 µM Y-27632 to each well containing a seeded scaffold.

9. For downstream analysis or infection of scaffolds, switch to differentiation medium after 24 h. Incubate for 3–5 days, changing the differentiation media every other day until ready for infection.

*Infection of 3D HIE-based silk scaffolds with oocysts and purified sporozoites*

See Subheading 3.1.

*Processing of infected 3D HIE-based scaffolds for confocal microscopy*

See Subheading 3.1 "Processing infected 3D IEC-based scaffolds for confocal microscopy."

*Processing of infected 3D HIE-based scaffolds for scanning electron microscopy*

See Subheading 3.1 "Processing infected 3D IEC-based scaffolds for scanning electron microscopy."

*Quantification of C. parvum infection in 3D HIE-based scaffolds*

See Subheading 3.1 "Quantification of *C. parvum* infection in 3D IEC-based scaffolds."

*3.5 Screening for C. parvum Interventions in HIE-Derived Monolayers in Multiwell Tissue Culture Plates*

*3.5.1 Preparation of HIE for Growth as Monolayers on Multiwell Plates*

1. Coat each well to be seeded of a 96-well tissue culture plate with 100 µL of ice-cold 33 µg/mL collagen type IV (*see* **Note 9**).

2. Incubate plate for 1.5–2 h at 37 °C, 5% $CO_2$.

3. Wash HIE cultures grown in 24-well plates with 500 µL of cold 0.5 mM EDTA in PBS, disrupt Matrigel plug with a micropipette (100–1000 µL) tip, and transfer to a 15 mL conical tube.

4. Centrifuge at 300 × *g* for 5 min at 4 °C and discard the supernatant.

5. Wash the pellet in 500 μL of trypsin–EDTA and incubate for 4 min at 37 °C. Use 1 mL of trypsin–EDTA when pooling 6–10 wells of HIE.

6. Add 1 mL of CMGF– + 10% FBS to inactivate trypsin.

7. Vigorously pipet the cell suspension ~50 times using a micropipette (100–1000 μL) to further dissociate the cells.

8. Place a 40 μm cell strainer over a 50 mL conical tube and wash membrane with 1 mL of CMGF– + 10% FBS.

9. Pass cell suspension through strainer using a micropipette (100–1000 μL) and discard the strainer.

10. Dilute a ~10 μL aliquot of HIE cells 1:10–1:20 in PBS and determine cell count using a hemocytometer.

11. Take the needed volume of cells to give $1 \times 10^5$ cells per well of a 96-well plate, centrifuge at $400 \times g$ for 5 min at RT and discard the supernatant (*see* **Note 10**).

12. Resuspend the pellet in enough growth medium supplemented with 10 μM Y-27632 to give a final volume of 100 μL per well.

13. Remove collagen type IV solution from wells.

14. Add 100 μL of cell suspension to each well.

15. 48 h later, remove growth medium from wells and replace with 100 μL of differentiation medium.

16. Change the differentiation medium every other day.

*3.5.2 Infection of HIE-Derived Monolayers with Transgenic C. parvum and Treatment with Interventions*

1. After 4 days in differentiation medium, HIE-derived monolayers are ready to be infected. Take 5000 transgenic *C. parvum* oocysts per well to be infected of a 96-well plate and place in a 1.5 mL microcentrifuge tube (*see* **Note 11**).

2. Centrifuge the tube at $16,000 \times g$ for 3 min at 4 °C in a microcentrifuge and discard the supernatant.

3. Resuspend the pellet in 1 mL of 10% v/v commercial bleach in PBS and incubate on ice for 10 min.

4. Centrifuge the tube at $16,000 \times g$ for 3 min at 4 °C in a microcentrifuge and discard the supernatant.

5. Resuspend the pellet in 1 mL of PBS.

6. Centrifuge the tube at $16,000 \times g$ for 3 min at 4 °C in a microcentrifuge and discard the supernatant.

7. Resuspend the pellet in 1 mL of 0.75% w/v sodium taurocholate in PBS and incubate at 15 °C for 10 min in a benchtop hot/cold dry block incubator.

8. Centrifuge the tube at $16,000 \times g$ for 3 min at 4 °C in a microcentrifuge and discard the supernatant.

9. Resuspend the pellet in 1 mL of PBS.

10. Centrifuge the tube at $16,000 \times g$ for 3 min at 4 °C in a micro-centrifuge and discard the supernatant.

11. Resuspend the pellet in enough differentiation medium to give a final volume of 100 µL per well.

12. Add 100 µL to each well and allow the infection to proceed for 2 h at 37 °C, 5% $CO_2$.

13. Remove the medium from the wells and wash once with warm PBS to remove any sporozoites that did not invade.

14. Add 100 µL of differentiation medium containing the drugs, antibodies, or biologics to be tested at the desired concentrations to each well and incubate for 72 h at 37 °C, 5% $CO_2$ (*see* **Note 12**).

15. Change the differentiation medium containing test compounds every 48 h.

*3.5.3 Luciferase Assay to Monitor Infection*

1. After 72 h of incubation at 37 °C, 5% $CO_2$, remove the medium from all the wells of the 96-well plate using a multi-channel pipettor (*see* **Note 13**).

2. Add 100 µL of Nano-Glo Luciferase Assay Buffer to each well.

3. Incubate the plate for 10 min at 37 °C, 5% $CO_2$ to lyse the cells.

4. Remove the plate from the incubator and add 100 µL of Nano-Glo Luciferase Assay Substrate mixed 1:50 with Assay Buffer to each well, bringing the final volume to 200 µL per well. Keep reagents and plate protected from light.

5. Mix the samples thoroughly while avoiding bubble formation and transfer to a white 96-well plate.

6. Measure luminescence using a microplate reader (*see* **Note 14**).

*3.5.4 Toxicity Testing*

1. After 4 days in differentiation medium, treat HIE monolayers in 96-well plate(s) with compound(s) to be tested at desired concentrations and incubate at 37 °C, 5% $CO_2$.

2. 72 h post treatment, add 15 µL of CellTiter 96 MTT Assay Dye Solution to each well of the plate using a multichannel pipettor.

3. Incubate the plate at 37 °C, 5% $CO_2$ for 1–4 h to allow MTT to be reduced to formazan (*see* **Note 15**).

4. Remove the plate from the incubator and add 100 µL of CellTiter 96 MTT Assay Solubilization Solution to each well.

5. Incubate the plate at RT for 1 h (*see* **Note 16**).

6. Mix the samples thoroughly while avoiding bubble formation.
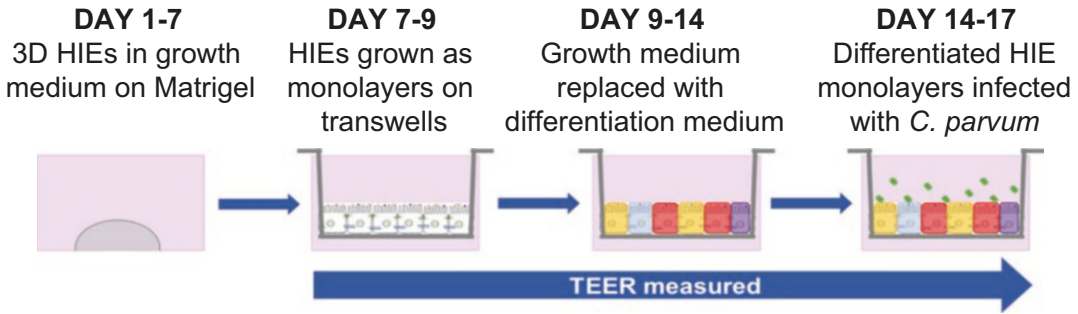
**Fig. 2** *C. parvum* infection of human intestinal enteroid (HIE) monolayers in 2D human intestinal tissue model. HIEs are grown in Matrigel in 24-well plates in growth medium for 7 days. HIEs are dissociated into single cells and grown as a monolayer on transwell inserts in growth medium for 3 days after which the growth medium is changed to differentiation medium for 5 days and the apical surface infected with *C. parvum.* Transepithelial electrical resistance (TEER) is measured from days 7–17. Figure modified and reproduced from Ref. [21] with permission from Springer Nature Methods in Molecular Biology

7. Measure absorbance at 570 nm using a microplate reader. A reference wavelength of 630–750 nm may be used. The absorbance may also be measured at a wavelength range of 550–600 nm.

**3.6 2D Model for Growth of Polarized HIE Monolayers on Transwell Inserts (Fig. 2)**

*3.6.1 Preparation of Transwells for Seeding*

1. Place the transwell inserts in a 24-well plate (*see* **Note 17**).

2. Coat the upper compartment of each transwell insert with 100 µL of 33 µg/mL Collagen type IV solution.

3. Add 300 µL of water to the 24-well plates containing the transwell inserts (lower compartment).

4. Incubate the 24-well plate with transwells for 1 h at 37 °C, 5% $CO_2$.

5. After 1 h, remove the liquid from the upper and lower compartments of the transwells.

6. Incubate the 24-well plate with transwells for 30 min at 37 °C, 5% $CO_2$ to allow them to dry.

*3.6.2 Growth and Differentiation of HIE Monolayers on Transwells*

1. Collect HIE from a 24-well plate. Each well should have at least 50 HIEs cultured in growth medium for 6–7 days.

2. Remove the growth medium from all wells and place 500 µL of cold 0.5 mM EDTA in every other well. Work on ice or at 4 °C.

3. Pipet up and down to break up the Matrigel.

4. Transfer the contents into the next well; break up the Matrigel in that well and so on.

5. Place the cells in a 50 mL conical tube on ice.

6. After HIEs from all the wells have been collected, centrifuge the tube at $300 \times g$ for 5 min at 4 °C and discard the supernatant.

7. Add 1 mL of warm trypsin–EDTA for every four wells of HIEs collected in **step 1** and incubate for 4 min at 37 °C, 5% $CO_2$.

8. At this point, remove the transwells from the incubator.

9. Inactivate the trypsin by adding an equal amount of CMGF– containing 10% FBS to the cells.

10. Dissociate the cells by pipetting vigorously up and down 50 times using a micropipette (100–1000 µL).

11. Place a 40 µm cell strainer on top of a 50 mL conical tube. Wet the cell strainer membrane with CMGF– containing 10% FBS.

12. Pipet the cells onto the strainer. The cells should pass through by gravity. Discard the strainer and any material that remained in it. Centrifuge the cells at $300 \times g$ for 5 min at RT and discard the supernatant.

13. Resuspend the HIE pellet in 3 mL of growth medium.

14. Using a hemocytometer, count the total number of cells.

15. Add 600 µL of growth medium containing 10 µM Y27632 into the lower compartment of the transwell (i.e., the well of the 24-well plate containing the transwell insert).

16. Add $5–7.5 \times 10^5$ total cells to the upper compartment of each transwell and bring the volume of growth medium containing 10 µM Y27632 to 300 µL for each upper compartment of the transwell. Incubate the cells at 37 °C, 5% $CO_2$.

17. The next day, measure the TEER of the monolayer.

18. Two days from the original seeding time, change the growth medium containing 10 µM Y27632 to an equal volume of differentiation medium. Change the differentiation medium every other day.

*3.6.3 C. parvum Infection of HIE Monolayers*

1. Resuspend the desired number of oocysts in 1 mL of PBS.

2. Centrifuge the oocysts at $16,000 \times g$ for 3 min at 4 °C.

3. Discard the supernatant and keep the oocysts on ice.

4. Add 1 mL of 10% bleach (mix by vortex) and incubate on ice for 10 min.

5. Centrifuge at $16000 \times g$ for 3 min at 4 °C and discard the supernatant.

6. Wash the pellet twice with 1 mL of PBS by centrifugation at $16,000 \times g$ for 3 min at 4 °C.

7. Discard the supernatant, resuspend the pellet in 1 mL of 0.75% sodium taurocholate, and incubate in a dry block incubator for 10 min at 15 °C.

8. Centrifuge at $16000 \times g$ for 3 min at 4 °C and discard the supernatant.

9. Wash the pellet in 1 mL of PBS by centrifugation at $16,000 \times g$ for 3 min at 4 °C two times.

10. Discard the supernatant and resuspend the oocysts in differentiation medium. Count the number of oocysts using a hemocytometer.

11. Add oocysts to the upper compartment of the transwells at a multiplicity of infection (MOI) of 0.1–1.

*3.6.4 Monitoring Monolayer Integrity via Transepithelial Electrical Resistance (TEER) Measurements*

Perform all TEER measurements in a sterile environment. Before measurements, make sure the voltohmmeter and electrodes are calibrated according to the manufacturer's instructions.

1. Place the electrodes in 70% ethanol for 10 min to sanitize. Allow the HIE-derived monolayers grown on the transwells to come to RT. Keep some growth medium at RT ready to rinse the electrodes. Lastly, have a transwell (with medium only) ready.

2. Rinse the electrode in the growth medium at RT and carefully place the long side of the electrode in the bottom compartment and the short side in the top compartment of the transwell. Measure the resistance (ohms) of the blank sample. Keep your hands steady to get a reproducible reading.

3. Measure the TEER of the first well; rinse the electrodes in the growth medium at RT and move on to the next well. Do this for every sample in the 24-well plate (*see* **Notes 18** and **19**).

4. After all the TEER values have been recorded, place the 24-well plates containing transwells back in the incubator or take desired time points for downstream assays (IFA, RT-qPCR, or Western blots).

5. Place the electrodes in 70% ethanol for 10 min, and then dry and store in a clean area.

*3.6.5 Quantification of C. parvum Infection of HIE-Derived Monolayers by RT-qPCR*

1. At the desired time point postinfection, remove the medium from 1 transwell to a 1.5 mL microcentrifuge tube.

2. Add 100 μL of trypsin–EDTA to the upper compartment of the same transwell and incubate for 1–2 min at 37 °C.

3. Pipet up and down a few times to loosen the monolayer and transfer to the same microcentrifuge tube.

4. Place another 100 μL of trypsin–EDTA into the same transwell and scrape the membrane carefully using a micropipette (100–1000 μL) tip and transfer to same microcentrifuge tube.

5. Add 1 mL of CMGF– + 10% FBS to inactivate the trypsin in the microcentrifuge tube.

6. Centrifuge at $2350 \times g$ for 5 min at 4 °C and discard the supernatant.

7. Resuspend pellet in 1 mL of PBS, centrifuge again, and discard the supernatant.

8. Store the pellet at –80 °C.

9. Extract RNA and quantify infection by RT-qPCR using a standard curve as described in Subheading 3.1 "Quantification of *C. parvum* infection in 3D HIE."

*3.6.6 Processing HIE-Derived Monolayers for IFA and Confocal Microscopy*

1. For IFA and confocal microscopy, wash each transwell three times with 300 µL of PBS.

2. Add 300 µL of freshly made 4% PFA to the top and bottom chambers of the transwells.

3. Incubate for 15 min at RT.

4. After 15 min, remove the PFA from the transwells and wash three times with 300 µL of PBS (*see* **Note 20**).

5. Incubate with 300 µL of 0.25% Triton X-100 in PBS for 10 min at RT.

6. Wash the transwells twice with 300 µL 0.1% Tween 20 in PBS at RT.

7. Wash the transwells twice with PBS.

8. Leave the transwells overnight at 4 °C in 300 µL of 5% NGS in both the top and lower chamber.

9. Wash the transwells twice with 300 µL of PBS.

10. Dilute the primary antibody in 1% NGS and add 100 µL to the top chamber of the transwells. Add 300 µL of PBS to the bottom chamber of the transwells.

11. Incubate for 1 h at RT (or overnight at 4 °C, depending on antibody specifications).

12. Wash the transwells three times with 1 mL of PBS.

13. Dilute the secondary antibody in 1% NGS and add 100 µL to the top chamber of the transwells and 300 µL of PBS to the bottom chamber of the transwells.

14. Incubate for 1 h at RT.

15. Next, add a drop of Fluoro-Gel II (with DAPI) to a glass slide.

16. Use clean blade and fine forceps to cut out the membranes of the transwells carefully. Place the transwell membranes facing up onto the slide and add another drop of Fluoro-Gel II (with DAPI) onto the membrane.

17. Place a cover slip on the membrane and use a Kimwipe or paper towel to clean around the edges of the slide.

18. Seal the edges of the cover slip with clear nail polish.

19. Store slides in the dark at 4 °C until ready to be imaged via confocal microscopy.

## 4   Notes

1. Conditioned media from L-WNT3a, R-Spondin 1, and Noggin can be aliquoted and stored at −20 °C for 1 month. Avoid freeze and thaw cycles. CMGF+ can be kept at −20 °C for up to 2 weeks. CMGF− and differentiation media can be kept at 4 °C for up to 1 month.

2. Matrigel is stored long term at −20 °C. When working with Matrigel, thaw aliquots overnight at 4 °C and keep on ice to avoid a temperature increase. Matrigel will quickly solidify at warmer temperature, therefore when routinely passaging cells using Matrigel, all tubes and pipet tips must be prechilled at 4 °C before plating and kept on ice during plating.

3. Make one single cut down the center (avoid chopping motions). Make the second cut on the opposite side, so now you have two lengths with an open lumen that can be stained with different antibodies as required.

4. HIEs from jejunum and duodenum are grown in CMGF+ growth medium. HIEs from ileum are grown in High Wnt3a growth medium.

5. Newly thawed HIEs are initially slow to grow. Passage them one or two times after thawing before setting up an experiment with them.

6. Either undifferentiated or differentiated HIEs may be used for infections. Infection in differentiated HIEs lasts only for 5 days, whereas infection of undifferentiated HIEs lasts up to 3 weeks.

7. Submerging the inoculation loop into the OCT first helps to keep the HIEs together and prevents them from adhering to the loop.

8. Each scaffold requires 35 μL of cell suspension per side (top and bottom) of the lumen. For example, for seeding 6 scaffolds, resuspend the cell pellet in 420 μL of growth medium containing 10 μM Y-27632.

9. Stock collagen type IV is prepared as a 1 mg/mL solution in 0.6% acetic acid, which is then diluted 1:30 in water.

10. Use one well of a 7-day-old HIE culture for every three wells of 96-well plate to be seeded; final seeding density should be approximately $1 \times 10^5$ cells per well. Do not pool more than 10 wells per 15 mL conical tube.

11. A transgenic *C. parvum* line that expresses a nanoluciferase reporter gene is used for infections. Purified transgenic oocysts are kept in PBS supplemented with antibiotic–antimycotic (10,000 U/mL penicillin, 10,000 μg/mL streptomycin, and 25 μg/mL amphotericin B) at 4 °C for up to 3 months.

12. 20 μM nitazoxanide may be used as a positive control (the only drug that is FDA approved for treatment of *C. parvum* in immunocompetent hosts [28]). Make a nitazoxanide stock solution at a concentration of 65 mM in DMSO and dilute with differentiation medium as needed.

13. Use a multichannel pipettor and reagent reservoirs for all subsequent steps in this section.

14. The luminescence emitted is directly proportional to the number of parasites in the well.

15. The amount of formazan formation is proportional to the number of live cells in the well. Decreased formazan formation is indicative of cytotoxicity.

16. Incubation at 37 °C will accelerate solubilization and is recommended for assays with a large quantity of formazan formation. After solubilization, plates may be stored at 4 °C for several days.

17. Transwell inserts can be made of polycarbonate or polyethylene with pore sizes of 0.4 μm or 3 μm. Use 0.4 μm polycarbonate transwell inserts.

18. Be sure to not cross contaminate uninfected wells with infected wells.

19. When samples are switched from growth medium to differentiation medium, be sure to use differentiation medium to rinse the electrodes.

20. At this point, store the transwells in PBS at 4 °C or continue with protocol.

## Acknowledgments

## References

1. Zhou W, Chen Y, Roh T, Lin Y, Ling S, Zhao S, Lin JD, Khalil N, Cairns DM, Manousiouthakis E, Tse M, Kaplan DL (2018) Multifunctional bioreactor system for human intestine tissues. ACS Biomater Sci Eng 4(1):231–239. https://doi.org/10.1021/acsbiomaterials.7b00794

2. Castellanos-Gonzalez A, Cabada MM, Nichols J, Gomez G, White AC Jr (2013) Human primary intestinal epithelial cells as an improved in vitro model for *Cryptosporidium parvum* infection. Infect Immun 81(6):1996–2001. https://doi.org/10.1128/IAI.01131-12

3. Varughese EA, Bennett-Stamper CL, Wymer LJ, Yadav JS (2014) A new in vitro model using small intestinal epithelial cells to enhance infection of *Cryptosporidium parvum*. J Microbiol Meth 106:47–54. https://doi.org/10.1016/j.mimet.2014.07.017

4. Miller CN, Josse L, Brown I, Blakeman B, Povey J, Yiangou L, Price M, Cinatl J Jr, Xue WF, Michaelis M, Tsaousis AD (2018) A cell culture platform for *Cryptosporidium* that enables long-term cultivation and new tools for the systematic investigation of its biology. Int J Parasitol 48(3–4):197–201. https://doi.org/10.1016/j.ijpara.2017.10.001

5. DeCicco RePass MA, Chen Y, Lin Y, Zhou W, Kaplan DL, Ward HD (2017) Novel bioengineered three-dimensional human intestinal model for long-term infection of *Cryptosporidium parvum*. Infect Immun 85(3):e00731-16. https://doi.org/10.1128/IAI.00731-16

6. Morada M, Lee S, Gunther-Cummins L, Weiss LM, Widmer G, Tzipori S, Yarlett N (2016) Continuous culture of *Cryptosporidium parvum* using hollow fiber technology. Int J Parasitol 46(1):21–29. https://doi.org/10.1016/j.ijpara.2015.07.006

7. Bhalchandra S, Cardenas D, Ward HD (2018) Recent breakthroughs and ongoing limitations in *Cryptosporidium* research. F1000Res 7:F1000. https://doi.org/10.12688/f1000research.15333.1

8. Larregieu CA, Benet LZ (2013) Drug discovery and regulatory considerations for improving in silico and in vitro predictions that use Caco-2 as a surrogate for human intestinal permeability measurements. AAPS J 15(2):483–497. https://doi.org/10.1208/s12248-013-9456-8

9. Sun D, Lennernas H, Welage LS, Barnett JL, Landowski CP, Foster D, Fleisher D, Lee KD, Amidon GL (2002) Comparison of human duodenum and Caco-2 gene expression profiles for 12,000 gene sequences tags and correlation with permeability of 26 drugs. Pharm Res 19(10):1400–1416

10. Koo BK, Clevers H (2014) Stem cells marked by the R-spondin receptor LGR5. Gastroenterology 147(2):289–302. https://doi.org/10.1053/j.gastro.2014.05.007

11. Zachos NC, Kovbasnjuk O, Foulke-Abel J, In J, Blutt SE, De Jonge HR, Estes MK, Donowitz M (2015) Human enteroids/colonoids and intestinal organoids functionally recapitulate normal intestinal physiology and pathophysiology. J Biol Chem 291(8):3759–3766. https://doi.org/10.1074/jbc.R114.635995

12. Yu H, Hasan NM, In JG, Estes MK, Kovbasnjuk O, Zachos NC, Donowitz M (2017) The contributions of human mini-intestines to the study of intestinal physiology and pathophysiology. Annu Rev Physiol 79:291–312. https://doi.org/10.1146/annurev-physiol-021115-105211

13. Sato T, Vries RG, Snippert HJ, van de Wetering M, Barker N, Stange DE, van Es JH, Abo A, Kujala P, Peters PJ, Clevers H (2009) Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. Nature 459(7244):262–265. https://doi.org/10.1038/nature07935

14. Rouch JD, Scott A, Lei NY, Solorzano-Vargas RS, Wang J, Hanson EM, Kobayashi M, Lewis M, Stelzner MG, Dunn JC, Eckmann L, Martin MG (2016) Development of functional microfold (M) cells from intestinal stem cells in primary human enteroids. PLoS One 11(1):e0148216. https://doi.org/10.1371/journal.pone.0148216

15. Foulke-Abel J, In J, Kovbasnjuk O, Zachos NC, Ettayebi K, Blutt SE, Hyser JM, Zeng XL, Crawford SE, Broughman JR, Estes MK, Donowitz M (2014) Human enteroids as an ex-vivo model of host-pathogen interactions in the gastrointestinal tract. Exp Biol Med 239(9):1124–1134. https://doi.org/10.1177/1535370214529398

16. Heo I, Dutta D, Schaefer DA, Iakobachvili N, Artegiani B, Sachs N, Boonekamp KE, Bowden G, Hendrickx APA, Willems RJL, Peters PJ, Riggs MW, O'Connor R, Clevers H (2018) Modelling *Cryptosporidium* infection in human small intestinal and lung organoids. Nat Microbiol 3(7):814–823. https://doi.org/10.1038/s41564-018-0177-8

17. Ramani S, Crawford SE, Blutt SE, Estes MK (2018) Human organoid cultures: transformative new tools for human virus studies. Curr Opin Virol 29:79–86. https://doi.org/10.1016/j.coviro.2018.04.001

18. Mahe MM, Aihara E, Schumacher MA, Zavros Y, Montrose MH, Helmrath MA, Sato T, Shroyer NF (2013) Establishment of gastrointestinal epithelial organoids. Curr Prot Mouse Biol 3:217–240. https://doi.org/10.1002/9780470942390.mo130179

19. Saxena K, Blutt SE, Ettayebi K, Zeng XL, Broughman JR, Crawford SE, Karandikar UC, Sastri NP, Conner ME, Opekun AR, Graham DY, Qureshi W, Sherman V, Foulke-Abel J, In J, Kovbasnjuk O, Zachos NC, Donowitz M, Estes MK (2015) Human intestinal enteroids: a new model to study human rotavirus infection, host restriction, and pathophysiology. J Virol 90(1):43–56. https://doi.org/10.1128/JVI.01930-15

20. In J, Foulke-Abel J, Zachos NC, Hansen AM, Kaper JB, Bernstein HD, Halushka M, Blutt S, Estes MK, Donowitz M, Kovbasnjuk O (2016) Enterohemorrhagic reduce mucus and intermicrovillar bridges in human stem cell-derived colonoids. Cell Mol Gastroenterol Hepatol 2(1):48–62 e43. https://doi.org/10.1016/j.jcmgh.2015.10.001

21. Zou WY, Blutt SE, Crawford SE, Ettayebi K, Zeng XL, Saxena K, Ramani S, Karandikar UC, Zachos NC, Estes MK (2017) Human intestinal enteroids: new models to study gastrointestinal virus infections. Meth Mol Biol. https://doi.org/10.1007/7651_2017_1

22. Chen Y, Zhou W, Roh T, Estes MK, Kaplan DL (2017) In vitro enteroid-derived three-dimensional tissue model of human small intestinal epithelium with innate immune responses. PLoS One 12(11):e0187880. https://doi.org/10.1371/journal.pone.0187880

23. Zachos NC, Kovbasnjuk O, Foulke-Abel J, In J, Blutt SE, de Jonge HR, Estes MK, Donowitz M (2016) Human enteroids/colonoids and intestinal organoids functionally recapitulate normal intestinal physiology and pathophysiology. J Biol Chem 291(8):3759–3766. https://doi.org/10.1074/jbc.R114.635995

24. Heijmans J, van Lidth de Jeude JF, Koo BK, Rosekrans SL, Wielenga MC, van de Wetering M, Ferrante M, Lee AS, Onderwater JJ, Paton JC, Paton AW, Mommaas AM, Kodach LL, Hardwick JC, Hommes DW, Clevers H, Muncan V, van den Brink GR (2013) ER stress causes rapid loss of intestinal epithelial stemness through activation of the unfolded protein response. Cell Rep 3(4):1128–1139. https://doi.org/10.1016/j.celrep.2013.02.031

25. Vinayak S, Pawlowic MC, Sateriale A, Brooks CF, Studstill CJ, Bar-Peled Y, Cipriano MJ, Striepen B (2015) Genetic modification of the diarrhoeal pathogen *Cryptosporidium parvum*. Nature 523(7561):477–480. https://doi.org/10.1038/nature14651

26. Chen Y, Lin Y, Davis KM, Wang Q, Rnjak-Kovacina J, Li C, Isberg RR, Kumamoto CA, Mecsas J, Kaplan DL (2015) Robust bioengineered 3D functional human intestinal epithelium. Sci Rep 5:13708. https://doi.org/10.1038/srep13708

27. Le Blancq SM, Khramtsov NV, Zamani F, Upton SJ, Wu TW (1997) Ribosomal RNA gene organization in *Cryptosporidium parvum*. Mol Biochem Parasitol 90(2):463–478

28. Cabada MM, White AC Jr (2010) Treatment of cryptosporidiosis: do we know what we think we know? Curr Opin Infect Dis 23(5):494–499. https://doi.org/10.1097/QCO.0b013e32833de052

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