Standard Operating Procedures

Development of Continuous *in vitro* Culture System for *Cryptosporidium*

In Vitro Assessment for Antimicrobial Activity – Part C

Contract Number: RFP NIH-NIAID-DMID-NIH AI2009066

Task Order: HHSN272201100015I/HHSN27200001 C02

Period of Performance: September 16, 2016 to September 15, 2017

Principal Investigator:
Saul Tzipori
200 Westboro Road
North Grafton, MA 01536 Phone: 508-839-7955
Fax: 508-839-7911
Email: Saul.Tzipori@tufts.edu

Contractor's Name & Address:
Tufts University
c/o Barbara Gardner
Office of Research Administration 136 Harrison Avenue
Boston, MA 02111
Phone: 617-636-0465
FAX: 617-636-2917
Email: Barbara.gardner@tufts.edu

Submitted: September 13, 2017

Department of Infectious Disease and Global Health Cummings School of Veterinary Medicine at Tufts University	
Standard Operating Procedure	
Continuous in vitro culture of *Cryptosporidium hominis* in COLO-680N cells using hollow fiber technology System	
SOP Number: 001	**Version Number: 2.1**
Date: Aug 22, 2017	**Author: Sangun Lee**

1. Purpose and Scope

   The purpose of this SOP is to describe the procedure to establish the *Cryptosporidium* hominis culture in COLO-680N cells using a hollow fiber cartridge. This SOP describes the procedure to set up a hollow fiber cartridge, to prepare COLO-680N cells for culture, and to inoculate and maintain the *Cryptosporidium* culture long-term.

2. Materials and Equipment

   2.1. Sample Requirements

   2.1.1. Host cells

   2.1.1.1. COLO-680N cells (Human esophagus squamous cell carcinoma cell line, passage 35, Cat. 300464 CLS Cell Lines Service GmbH)

   2.1.2. Purified *Cryptosporidium* oocysts

   2.1.2.1. *C. hominis* TU502 oocysts (Excystation rate over 60%)

   2.2. Reagents

   2.2.1. Sterile water, molecular biology grade

   2.2.2. Sterile PBS

   2.2.3. Absolute ethanol (200 proof), molecular biology grade

   2.2.4. Clorox® regular bleach (The Clorox Company, Oakland, CA)

   2.2.5. Sodium taurodeoxycholate hydrate (T0557, Sigma-Aldrich Co., St. Louis, MO)

   2.2.6. Cholesterol (C4951, Sigma-Aldrich Co., St. Louis, MO)

   2.2.7. Oleic acid (O1257, Sigma-Aldrich Co., St. Louis, MO)

   2.2.8. Alpha-linolenic acid (L9530, Sigma-Aldrich Co., St. Louis, MO)

   2.2.9. Glutathione, reduced (G6013, Sigma-Aldrich Co., St. Louis, MO)

2.2.10. Taurine (T8691, Sigma-Aldrich Co., St. Louis, MO)

2.2.11. Cysteine (C7880, Sigma-Aldrich Co., St. Louis, MO)

2.2.12. Mannitol (M9546, Sigma-Aldrich Co., St. Louis, MO)

2.2.13. Thioglycolic acid (102933, MP Biomedicals)

2.2.14. Roche® Glucose strips (22-045-871)

2.2.15. Antibiotic-Antimycotic (15240-062, Invitrogen)

2.2.16. RPMI1640 (R8758, Sigma-Aldrich Co., St. Louis, MO)

2.2.17. 10% FBS

2.2.18. RPMI (I) [RPMI1640 (R8758, Sigma-Aldrich Co., St. Louis, MO) with 10% FBS and Antibiotic-Antimycotic (15240-062, Invitrogen), pH 7.4]

2.2.19. RPMI (II) [RPMI1640 (R8758, Sigma-Aldrich Co., St. Louis, MO) with 10% FBS and Antibiotic-Antimycotic (15240-062, Invitrogen) plus 100 mM HEPES (H3375, Sigma-Aldrich Co., St. Louis, MO), pH 7.8]

2.2.20. *Cryptosporidium* 5F10 monoclonal antibody (Tufts generated mAb; hybridoma supernatant that has been diluted 1:4 in PBS + 1% BSA)

2.2.21. Goat anti- mouse IgG Alexa Fluor 488 conjugated(1:1,000 dilution in PBS + 1% BSA); ThermoFisher Scientific catalog # A-11001)

2.2.22. SlowFade Gold antifade reagent with DAPI (Molecular Probes S36939)

2.2.23. E.Z.N.A.® Stool DNA kit (D4015 Omega Bio-Tek)

2.2.24. *Cryptosporidium* HSP70 primers and probes (Integrated DNA Technologies, Inc.)

Name	Sequences
HSP70 FWD	TCT GAA GGA ATG CGA ACA ACT
HSP70 REV	GGG TTT GTG ATT GCT TGT CTT T
HSP70 PRB-FAM	/56-FAM/TGG GCA GAG /ZEN/ATT GGT TGG TGA AGT /3IABkFQ/

2.2.25. PrimeTime® Gene Expression Master Mix (1055772 Integrated DNA Technologies, Inc.)

2.2.26. TempPlate semi-skirted 96-well PCR plate (1402-9100 USA Scientific, Inc.)

2.2.27. TempPlate RT optically clear film (2978-2700 USA Scientific, Inc.)

2.3. Supplies

2.3.1. FiberCell® polysulfone fiber type cartridge (C2025D)

2.3.2. FiberCell® 33 mm reservoir cap, autoclaved (A1005)

2.3.3. Glass bottle, autoclaved, 125 ml and 250 ml

2.3.4. Roche® Diagnostics Accutrend plus meter kit (22-045-734)

2.3.5. Hemacytometer

2.3.6. Sterile luer-lock syringe 3 ml, 10 ml, 20 ml

2.3.7. Alcohol pads

2.3.8. Aluminum foil

2.3.9. Autoclave bags (7½" x 13")

2.3.10. Spray bottle containing 70% ethanol

2.3.11. Sterile large bore needles

2.3.12. Sterile 50 ml conical tubes

2.3.13. Sterile 1.5 ml microcentrifuge tubes

2.3.14. P20, P200 and P1000 Pipettors

2.3.15. Sterile pipet tips for pipettors

2.3.16. Disposable gloves

2.3.17. Lab coat

2.4. Equipment

2.4.1. Incubator with 37°C and 5% CO_2

2.4.2. FiberCell® Systems Duet pump

2.4.3. Biological safety cabinet (BSC)

2.4.4. Microcentrifuge

2.4.5. Fluorescence microscope

2.4.6. StepOne™ Plus Real-Time PCR system

2.4.7. Autoclave

2.5. Software

2.5.1. Microsoft Excel

2.5.2. GraphPad Prism

2.5.3. StepOne™ Software

3. Procedure

3.1. Module setup

The module setup is followed by manufacturer's instruction.

3.1.1. Autoclave reservoir cap with two short pieces of tubing supplied with each new cartridge and place on 125 ml sterile PBS bottle.

3.1.2. Connect the reservoir cap to the flow path of FiberCell® cartridge.

3.1.3. Perfuse PBS through the flow path circuit by pumping the compression tubing and fill the ECS with PBS.

3.1.4. Place the cartridge onto the Duet pump in 37°C incubator with 5% CO_2 and run PBS through the system at a flow rate of 15 for 24 hr.

3.1.5. Replace the old PBS with a new 125 ml sterile PBS bottle and run for 24 hr.

3.1.6. Replace the old PBS with a new 125 ml bottle of serum-free RPMI medium and run for 24 hr.

3.1.7. Replace the old serum-free medium with a new 125 ml bottle of serum-free RPMI medium and run for 24 hr.

3.1.8. Replace the old serum-free medium with a new 125 ml bottle of RPMI (II) medium and run for 24 hr.

3.1.9. The cartridge is ready for seeding COLO-680N cells.

3.2. COLO-680N cell inoculation and culture

3.2.1. Maintain the COLO-680N cells in RPMI (I) culture.

3.2.2. Prepare two 75 cm^2 flasks to culture the COLO-680N cells with RPMI (II) medium. When the cells are confluent 70-80%, harvest the cells.

3.2.3. Centrifuge the harvested cells and resuspend the cells with RPMI (II).

3.2.4. Add COLO-680N 1 – 5 x 10^6 cells in 2 ml of RPMI (II) medium into the ECS of HF cartridge prepared from 3.1.9.

3.2.5. Replace the old RPMI (I) medium with a new 50 ml bottle of RPMI (II) medium.

3.2.6. Place the cartridge onto the Duet pump in 37°C incubator with 5% CO_2 and run the medium through the system at a flow rate of 10.

3.2.7. Check glucose level in medium daily and replace the 50 ml old RPMI (II) medium with a new 80 ml bottle of RPMI (II) medium when 50% of glucose has been consumed.

3.2.8. Check glucose level in medium daily and replace the 80 ml old RPMI (II) medium with a new 125 ml bottle of RPMI (II) medium when 50% of glucose has been consumed.

3.2.9. Check glucose level in medium daily and replace the 125 ml old RPMI (II) medium with a new 125 ml bottle of RPMI (II) medium when 50% of glucose has been consumed. The cartridge is ready for inoculating *Cryptosporidium* oocysts.

3.3. Preparation of ECS supplement

3.3.1. Prepare the lipids mix, 50x

3.3.1.1. Dissolve 67 mg oleic acid, 180 mg cholesterol, and 68 mg alpha-linolenic acid in 10 ml distilled water.

3.3.1.2. Filter sterilize and store at 4°C for up to 6 months.

3.3.2. Prepare the thiol mix, 10x

3.3.2.1. Boil 20 ml distilled water.

3.3.2.2. Dispense 10 ml into a glass test tube and gas with nitrogen bubbling in it until cool.

3.3.2.3. Dissolve 20 mg each of reduced glutathione, taurine, cysteine into 10 ml water from 3.3.2.2.

3.3.2.4. Flush a sterile syringe with helium or nitrogen. Take up thiol solution and filter sterilize.

3.3.2.5. Aliquot 1 ml into a capped tube.

3.3.2.6. Cap tube tightly and keep at -20°C for up to 6 months.

3.3.3. Prepare 10 ml of ECS supplement freshly on the day of use.

3.3.3.1. Take 9 ml of RPMI (II) from a new bottle of medium.

3.3.3.2. Dissolve 5 mg thioglycolic acid, 150 mg sodium taurodeoxycholate hydrate, and 54.7 mg mannitol in 9 ml of RPMI (II).

3.3.3.3. Filter sterilize and add 200 µl of lipid mix from 3.3.1 and 1 ml of thiol mix from 3.3.2.

3.3.3.4. Adjust pH to 7.4 – 7.8.

3.4. *Cryptosporidium* preparation and inoculation into the cartridge

3.4.1. Acquire purified *Cryptosporidium* hominis, 1 x 10^6 oocysts.

3.4.2. Before inoculation, incubate the oocysts with 10% Clorox® regular bleach on ice for 10 min.

3.4.3. Wash 3 times with sterile water.

3.4.4. Resuspend the oocysts pellet into 2 ml of RPMI (II) medium.

3.4.5. Add the oocysts in 2 ml RPMI (II) medium and the freshly prepared supplement 2 ml from 3.3 into the ECS of the cartridge from 3.2.9.

3.4.6. Place the cartridge onto the Duet pump in 37°C incubator with 5% CO_2 and run through the system at a flow rate of 10.

3.5. Maintenance of cartridges

3.5.1. Incubate the cartridge onto the Duet pump at a flow rate of 10 in 37°C incubator with 5% CO_2.

3.5.2. Replace the old RPMI (II) medium with a new 125 ml of RPMI (II) medium on Monday and Wednesday. Replace the old medium with a new 250 ml of RPMI (II) medium on Friday.

3.5.3. Measure the glucose level and pH of the old medium on Monday, Wednesday, and Friday.

3.5.4. Every Wednesday, harvest 1 ml of medium from the ECS of the cartridge and add the freshly prepared supplement 2 ml from 3.3 into the ECS of the cartridge.

3.5.5. If glucose consumption is low and the floating cells are abnormally increased, prepare the fresh COLO-680N cells and add into the ECS.

3.6. Preparation of test samples

3.6.1. Centrifuge 1 ml oocyst harvest (from 3.5.4) at 13,000 x g for 5 min.

3.6.2. Remove the supernatant and resuspend in 200 µl of water.

3.6.3. Use 50 µl for immunofluorescence staining and 50 µl for DNA extraction.

3.6.4. Store the remaining 100 µl at 4°C.

3.7. Immunofluorescence staining of oocysts

3.7.1. Transfer 50 µl of harvest (from 3.6.3) into a new 1.5 ml tube.

3.7.2. Add 950 µl PBS to sample into the tube.

3.7.3. Vortex for 5 sec and centrifuge at 13,000 x g for 5 min.

3.7.4. Carefully remove supernatant – do not disturb the pellet.

3.7.5. Add 50 µl 5F10 *Cryptosporidium* mAb to the tube and mix well by pipetting up and down.

3.7.6. Incubate tubes at room temperature for 30 min.

3.7.7. Add 950 µl PBS and vortex for 5 sec.

3.7.8. Centrifuge tubes at 13,000 x g for 5 min.

3.7.9. Carefully remove supernatant – do not disturb the pellet.

3.7.10. Add 50 µl goat anti-mouse Ab conjugated with Alexa Fluor 488 into the tube and mix well by pipetting up and down.

3.7.11. Incubate tubes at room temperature for 30 min in the dark.

3.7.12. Add 950 µl PBS and vortex for 5 sec (keep in the dark).

3.7.13. Centrifuge tubes at 13,000 x g for 5 min (keep in the dark).

3.7.14. Carefully remove supernatant – do not disturb the pellet.

3.7.15. Add 950 µl water and vortex for 5 sec (keep in the dark).

3.7.16. Centrifuge tubes at 13,000 x g for 5 min (keep in the dark).

3.7.17. Carefully remove supernatant – do not disturb the pellet.

3.7.18. Add 50 µl sterile H_2O and resuspend the pellet by pipetting up and down.

3.7.19. Use 10 µl for oocyst counting and 10 µl for oocyst observation using a fluorescence microscope.

3.8. Oocyst counting on hemacytometer

The samples should be kept in dark during the procedure.

3.8.1. Prepare hemacytometer with a cover glass.

3.8.2. Pipet 10 µl of stained oocysts (from 3.7.16) onto hemacytometer (keep in dark).

3.8.3. Count oocysts in the 4 corner squares of ’16 squares’ using fluorescence microscope.

3.8.4. Calculate # of oocyst per ml.

(# Oocysts counted in the 4 corner squares) x (1/4) x (10^4) x (1/5) = (# Oocysts/ml)

ex. (19+21+24+20) x (1/4) x (10^4) x (1/5) = 42000 oocysts /ml.

3.9. Oocyst observation using a fluorescence microscope

The samples should be kept in dark during the procedure

3.9.1. Pipet 10 µl of stained oocysts (from 3.7.16) and smear on a slide glass.

3.9.2. Let air dry.

3.9.3. Immerse with anti-fade reagent with DAPI.

3.9.4. Place a cover glass on the slide glass.

3.9.5. Observe the oocysts using a fluorescence microscope.

3.10. DNA extraction

The procedure uses E.Z.N.A. Stool DNA kit following by manufacturer's instruction.

3.10.1. Transfer 50 µl of harvest (from 3.6.3) into a new 1.5 ml tube.

3.10.2. Add 50 µl of sterile water.

3.10.3. Do 5 freeze-thaw cycles.

3.10.4. Transfer 100 µl in a 1.5 ml microcentrifuge tube containing 100 mg Glass Beads X. Place the tube on ice (total volume ~200 µl)

3.10.5. Add 270 µl SLX-Mlus Buffer. Vortex at maximum speed for 10 min or until the samples is thoroughly homogenized.

3.10.6. Add 30 µl DS buffer and 10 µl Proteinase K solution. Vortex or pipet up and down to mix thoroughly.

3.10.7. Incubate at 70°C for 10 min. Vortex twice during incubation.

3.10.8. Add 100 µl SP2 buffer. Vortex at maximum speed for 30 sec. (total ~610 µl).

3.10.9. Let sit on ice for 5 min.

3.10.10. Centrifuge at 13,000 x g for 5 min.

3.10.11. Transfer 200 µl supernatant to a new 1.5 ml tube. Do not disturb the pellet.

3.10.12. Add 100 µl HTR reagent. Vortex at maximum speed for 10 sec. HTR reagent must be thoroughly resuspended and pipetted with end-cut tip.

3.10.13. Let sit at room temperature for 2 min.

3.10.14. Centrifuge at 13,000 x g for 2 min.

3.10.15. Transfer 125 µl supernatant to a new 1.5 ml tube.

3.10.16. Add 125 µl BL buffer and 125 µl 100% ethanol. Vortex at maximum speed for 10 sec.

3.10.17. Insert a HiBind DNA mini column into a 2 ml collection tube.

3.10.18. Transfer the entire sample from step 3.10.16 to the HiBind DNA mini column.

3.10.19. Centrifuge at maximum speed for 1 min.

3.10.20. Transfer the HiBind DNA mini column into a new 2 ml collection tube.

3.10.21. Add 500 µl VHB buffer.

3.10.22. Centrifuge at 13,000 x g for 30 sec.

3.10.23. Discard the filtrate and reuse the collection tube.

3.10.24. Add 700 µl DNA wash buffer.

3.10.25. Centrifuge at 13,000 x g for 1 min.

3.10.26. Discard the filtrate and reuse the collection tube.

3.10.27. Add 700 µl DNA wash buffer.

3.10.28. Centrifuge at 13,000 x g for 1 min.

3.10.29. Discard the filtrate and reuse the collection tube.

3.10.30. Centrifuge at 13,000 x g for 2 min.

3.10.31. Transfer the column into a clean 1.5 ml tube.

3.10.32. Add 100 µl Elution buffer heated to 65°C.

3.10.33. Let sit at room temperature for 2 min.

3.10.34. Centrifuge at 13,000 x g for 1 min.

3.10.35. Store DNA at -20°C until Real-Time PCR.

3.11. Real time PCR

3.11.1. Prepare the Cryptosporidium DNA standard.

3.11.1.1. Add 10^6, 10^5, 10^4, 10^3, 10^2 oocysts of *Cryptosporidium* TU502 into COLO-680N 10^6 cells, respectively.

3.11.1.2. Extract the DNA using the procedure described in 3.10.

3.11.1.3. Store at -20°C until Real-Time PCR.

3.11.2. Prepare HSP70 primer/probe mix.

3.11.2.1. Prepare the primer/probe mix of HSP70 FWD and HSP70 REV to be 5 µM and HSP70 PRB-FAM to be 2.5 µM at final conc. Otherwise, order the premix from vendor and prepare the mix by the manufacturer's direction.

3.11.2.2. Store at 4°C (for short-term use) or -20°C.

3.11.3. Prepare the PCR mix in a 96-well plate.

3.11.3.1. Prepare the PCR mix of DNA samples (from 3.10.35) and standard DNA (from 3.11.1). Per well, add the following in the table. Each sample is tested in triplicate. The PCR mix can be prepared as 3x volume (30 µl) and 10 µl distributed into each of 3 wells.

Components	**Volume**
PrimeTime Master Mix (2x)	5 µl
DNA (samples or standard)	2 µl
HSP70 primer/probe mix (3.11.2)	1 µl
Water	2 µl

3.11.3.2. Seal the plate with TempPlate RT optically clear film.

3.11.3.3. Centrifuge briefly.

3.11.4. Load the plate in the StepOne Real-Time PCR instrument.

3.11.5. Open the StepOne software and set up the plate setup and run method.

Step	Cycles	Temp (°C)	Time (Min:Sec)
Polymerase activation	1	95°C	3:00
Amplification	45		
Denaturation		95°C	0:15
Annealing/Extension		60°C	1:00

3.11.6. Run the instrument

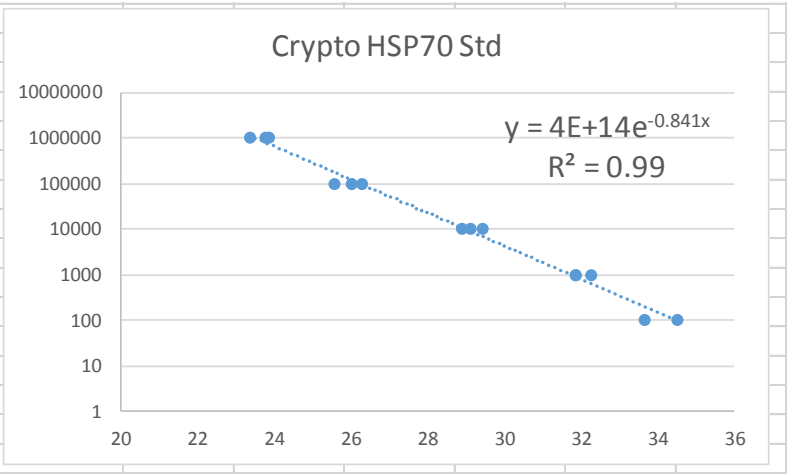
3.11.7. Data Analysis

3.11.7.1. Extract the raw data from the StepOne software to MS Excel.

3.11.7.2. Generate the standard curve using the C_T data of standard samples in MS Excel.

Example of standard curve (Exponential trend line with the equation and R-squared value).

CT	Quantity
23.36539	1000000
23.76603	1000000
23.86398	1000000
25.59637	100000
26.00708	100000
26.30232	100000
28.88531	10000
29.14804	10000
29.43307	10000
31.8645	1000
32.29043	1000
31.87642	1000
34.52393	100
33.6643	100
	100



3.11.7.3. Calculate DNA amounts equivalent to *Cryptosporidium* oocysts from C_T of samples using the equation of standard curve.

3.11.7.4. Make a plot of the calculated DNA amounts using GraphPad Prism.

Department of Infectious Disease and Global Health Cummings School of Veterinary Medicine at Tufts University	
Standard Operating Procedure	
Continuous in vitro culture of *Cryptosporidium parvum* in HCT-8 cells using hollow fiber technology System	
SOP Number: 002	**Version Number: 2.1**
Date: Aug 22, 2017	**Author: Sangun Lee**

1. Purpose and Scope

   The purpose of this SOP is to describe the procedure to establish the *Cryptosporidium* parvum culture in HCT-8 cells using a hollow fiber cartridge. This SOP describes the procedure to set up a hollow fiber cartridge, to prepare HCT-8 cells for culture, and to inoculate and maintain the *Cryptosporidium* culture for long-term.

2. Materials and Equipment

   2.1. Sample Requirements

   2.1.1. Host cells

   2.1.1.1. HCT-8 cells

   2.1.2. Purified *Cryptosporidium* oocysts

   2.1.2.1. *C. parvum* MD oocysts (Excystation rate over 60%)

   2.2. Reagents

   2.2.1. Sterile water, molecular biology grade

   2.2.2. Sterile PBS

   2.2.3. Absolute ethanol (200 proof), molecular biology grade

   2.2.4. Clorox® regular bleach (The Clorox Company, Oakland, CA)

   2.2.5. Sodium taurodeoxycholate hydrate (T0557, Sigma-Aldrich Co., St. Louis, MO)

   2.2.6. Calcium panthothenate (C8731, Sigma-Aldrich Co., St. Louis, MO)

   2.2.7. 4-aminobenzoic acid (A9878, Sigma-Aldrich Co., St. Louis, MO)

   2.2.8. Folic acid (F7876, Sigma-Aldrich Co., St. Louis, MO)

   2.2.9. L-ascorbic acid (A0278, Sigma-Aldrich Co., St. Louis, MO)

2.2.10. L-glutamine (G3126, Sigma-Aldrich Co., St. Louis, MO)

2.2.11. HEPES (H3375, Sigma-Aldrich Co., St. Louis, MO)

2.2.12. D-glucose (G8270, Sigma-Aldrich Co., St. Louis, MO)

2.2.13. Heparin (H3393, Sigma-Aldrich Co., St. Louis, MO)

2.2.14. Cholesterol (C4951, Sigma-Aldrich Co., St. Louis, MO)

2.2.15. Oleic acid (O1257, Sigma-Aldrich Co., St. Louis, MO)

2.2.16. Alpha-linolenic acid (L9530, Sigma-Aldrich Co., St. Louis, MO)

2.2.17. Glutathione, reduced (G6013, Sigma-Aldrich Co., St. Louis, MO)

2.2.18. Taurine (T8691, Sigma-Aldrich Co., St. Louis, MO)

2.2.19. Cysteine (C7880, Sigma-Aldrich Co., St. Louis, MO)

2.2.20. Mannitol (M9546, Sigma-Aldrich Co., St. Louis, MO)

2.2.21. Thioglycolic acid (102933, MP Biomedicals)

2.2.22. Roche® Glucose strips (22-045-871)

2.2.23. Horse serum (16050-122, Invitrogen)

2.2.24. Antibiotic-Antimycotic (15240-062, Invitrogen)

2.2.25. MEM (11095-114, Invitrogen)

2.2.26. MEM (I) [MEM (11095-114, Invitrogen) with 10 horse serum (16050-122, Invitrogen) and antibiotic-Antimycotic (15240-062, Invitrogen), pH 7.4]

2.2.27. 1N NaOH

2.2.28. *Cryptosporidium* 5F10 monoclonal antibody (Tufts generated mAb; hybridoma supernatant that has been diluted 1:4 in PBS + 1% BSA)

2.2.29. Goat anti- mouse IgG Alexa Fluor 488 conjugated (1:1,000 dilution in PBS + 1% BSA); ThermoFisher Scientific catalog # A-11001)

2.2.30. SlowFade Gold antifade reagent with DAPI (Molecular Probes S36939)

2.2.31. E.Z.N.A.® Stool DNA kit (D4015 Omega Bio-Tek)

2.2.32. *Cryptosporidium* HSP70 primers and probes (Integrated DNA Technologies, Inc.)

Name	**Sequences**
HSP70 FWD	TCT GAA GGA ATG CGA ACA ACT
HSP70 REV	GGG TTT GTG ATT GCT TGT CTT T
HSP70 PRB-FAM	/56-FAM/TGG GCA GAG /ZEN/ATT GGT TGG TGA AGT /3IABkFQ/

2.2.33. PrimeTime® Gene Expression Master Mix (1055772 Integrated DNA Technologies, Inc.)

2.2.34. TempPlate semi-skirted 96-well PCR plate (1402-9100 USA Scientific, Inc.)

2.2.35. TempPlate RT optically clear film (2978-2700 USA Scientific, Inc.)

2.3. Supplies

2.3.1. FiberCell® polysulfone fiber type cartridge (C2025D)
2.3.2. FiberCell® 33 mm reservoir cap, autoclaved (A1005)
2.3.3. Glass bottle, autoclaved, 125 ml, 250 ml
2.3.4. Roche® Diagnostics Accutrend plus meter kit (22-045-734)
2.3.5. Hemacytometer
2.3.6. Sterile luer-lock syringe 3 ml, 10 ml, 20 ml
2.3.7. Alcohol pads
2.3.8. Aluminum foil
2.3.9. Autoclave bags (7½" x 13")
2.3.10. Spray bottle containing 70% ethanol
2.3.11. Sterile large bore needles
2.3.12. Sterile 50 ml conical tubes
2.3.13. Sterile 1.5 ml microcentrifuge tubes
2.3.14. P20, P200 and P1000 Pipettors
2.3.15. Sterile pipet tips for pipettors
2.3.16. Disposable gloves
2.3.17. Lab coat

2.4. Equipment

2.4.1. Incubator with 37°C and 5% CO_2
2.4.2. FiberCell® Systems Duet pump
2.4.3. Biological safety cabinet (BSC)
2.4.4. Microcentrifuge
2.4.5. Fluorescence microscope
2.4.6. StepOne™ Plus Real-Time PCR system
2.4.7. Autoclave

2.5. Software

2.5.1. Microsoft Excel
2.5.2. GraphPad Prism

2.5.3. StepOne™ Software

3. Procedure

3.1. Module setup

The module setup is followed by manufacturer's instruction.

3.1.1. Autoclave reservoir cap with two short pieces of tubing supplied with each new cartridge and place on 125 ml sterile PBS bottle.

3.1.2. Connect the reservoir cap to the flow path of FiberCell® cartridge.

3.1.3. Perfuse PBS through the flow path circuit by pumping the compression tubing and fill the ECS with PBS.

3.1.4. Place the cartridge onto the Duet pump in 37°C incubator with 5% CO_2 and run PBS through the system at a flow rate of 10 for 24 hr.

3.1.5. Replace the old PBS with a new 125 ml sterile PBS bottle and run for 24 hr.

3.1.6. Replace the old PBS with a new 125 ml bottle of serum-free MEM medium and run for 24 hr.

3.1.7. Replace the old serum-free medium with a new 125 ml bottle of serum-free MEM medium and run for 24 hr.

3.1.8. Replace the old serum-free medium with a new 125 ml bottle of MEM (I) medium and run for 24 hr.

3.1.9. The cartridge is ready for seeding HCT-8 cells.

3.2. Preparation of MEM (IV)

MEM (IV), the modified medium of MEM plus additional supplements is prepared for providing the culture condition of *C. parvum*.

3.2.1. In a one liter bottle of MEM, dissolve the following chemicals:0.29 g L-glutamine, 23.8 g HEPES, 4.5 g D-glucose,0.035 g L-ascorbic acid, 0.04 g 4-aminobenzoic acid, 0.02 g calcium pantothenate, and 0.01 g folic acid.

3.2.2. Adjust pH 7.8 with 1N NaOH.

3.2.3. Add 58 mg heparin.

3.2.4. Filter sterilize.

3.2.5. Add 10% horse serum and 10 ml of antibiotics (15240-062, Invitrogen).

3.3. HCT-8 cell inoculation and culture

3.3.1. Maintain HCT-8 cells culture in MEM (I) medium.

3.3.2. Prepare two 75 cm^2 flasks to culture the HCT-8 cells with MEM (IV) medium. When the cells are 70-80% confluent, harvest the cells.

3.3.3. Centrifuge the harvested cells and resuspend the cells with MEM (IV) prepared in 3.2.

3.3.4. Add HCT-8 cells ($1 – 5 \times 10^6$) in 2 ml of MEM (IV) medium into the ECS of HF cartridge prepared from 3.1.9.

3.3.5. Replace the old MEM (IV) medium with a new 50 ml bottle of MEM (IV) medium.

3.3.6. Place the cartridge onto the Duet pump in 37°C incubator with 5% CO_2 and run the medium through the system at a flow rate of 10.

3.3.7. Check glucose level in medium daily and replace the 50 ml old MEM (IV) medium with a new 80 ml bottle of MEM (IV) medium when 50% of glucose has been consumed.

3.3.8. Check glucose level in medium daily and replace the 80 ml old MEM (IV) medium with a new 120 ml bottle of MEM (IV) medium when 50% of glucose has been consumed.

3.3.9. Check glucose level in medium daily and replace the 120 ml old MEM (IV) medium with a new 120 ml bottle of MEM (IV) medium when 50% of glucose has been consumed. The cartridge is ready for inoculating *Cryptosporidium* oocysts.

3.4. Preparation of ECS supplement

3.4.1. Prepare the lipids mix, 50x.

3.4.1.1. Dissolve 67 mg oleic acid, 180 mg cholesterol and 68 mg alpha-linolenic acid in 10 ml distilled water.

3.4.1.2. Filter sterilize and store at 4°C for up to 6 months.

3.4.2. Prepare the thiol mix, 10x

3.4.2.1. Boil 20 ml distilled water.

3.4.2.2. Dispense 10 ml into a glass test tube and gas with nitrogen bubbling in it until cool.

3.4.2.3. Dissolve 20 mg each of glutathione, taurine, and cysteine into 10 ml water from 3.3.2.2.

3.4.2.4. Flush a sterile syringe with helium or nitrogen. Take up thiol solution and filter sterilize.

3.4.2.5. Aliquot 1 ml into a capped tube.

3.4.2.6. Cap tube tightly and keep at -20°C for up to 6 months.

3.4.3. Prepare 10 ml of ECS supplement freshly on the day of use.

3.4.3.1. Take 9 ml of MEM (IV) from a new bottle of medium.

3.4.3.2. Dissolve 5 mg thioglycolic acid, 150 mg sodium taurodeoxycholate hydrate, and 54.7 mg mannitol into 9 ml of MEM (IV).

3.4.3.3. Filter sterilize and add 200 µl of lipid mix from 3.4.1 and 1 ml of thiol mix from 3.4.2.

3.4.3.4. Adjust pH to 7.4 – 7.8.

3.5. *Cryptosporidium* preparation and inoculation into the cartridge

3.5.1. Acquire purified *Cryptosporidium* parvum oocysts (1×10^6).

3.5.2. Before inoculation, incubate the oocysts with 10% Clorox® regular bleach on ice for 10 min.

3.5.3. Wash 3 times with sterile water.

3.5.4. Resuspend the oocyst pellet into 2 ml of MEM (IV) medium.

3.5.5. Add the oocysts in 2 ml MEM (IV) medium and the freshly prepared supplement 2 ml from 3.4 into the ECS of the cartridge from 3.3.9.

3.5.6. Place the cartridge onto the Duet pump in 37°C incubator with 5% CO_2 and run through the system at a flow rate of 10.

3.6. Maintenance of cartridges

3.6.1. Incubate the cartridge onto the Duet pump at a flow rate of 10 in 37°C incubator with 5% CO_2.

3.6.2. Replace the old MEM (IV) medium with fresh 125 ml of MEM (IV) medium on Monday and Wednesday. Replace the old medium with fresh new 250 ml of MEM (IV) medium on Friday.

3.6.3. Measure the glucose level and pH of medium of the old medium on Monday, Wednesday, and Friday.

3.6.4. Every Wednesday, harvest 1 ml of medium from the ECS of the cartridge and add freshly prepared supplement 2 ml from 3.3 into the ECS of the cartridge.

3.6.5. If glucose consumption is low and the floating cells are abnormally high, prepare fresh HCT-8 cells and add into the ECS.

3.7. Preparation of test samples

3.7.1. Centrifuge 1 ml oocyst harvest (from 3.6.4) at 13,000 x g for 5 min.

3.7.2. Remove the supernatant and resuspend in 200 µl of water.

3.7.3. Use 50 µl for immunofluorescence staining and 50 µl for DNA extraction.

3.7.4. Store the remaining 100 µl at 4°C.

3.8. Immunofluorescence staining of oocysts

3.8.1. Transfer 50 µl of harvest (from 3.7.3) into a new 1.5 ml tube.

3.8.2. Add 950 µl PBS to sample into the tube.

3.8.3. Vortex for 5 sec and centrifuge at 13,000 x g for 5 min.

3.8.4. Carefully remove supernatant – do not disturb the pellet.

3.8.5. Add 50 µl 5F10 *Cryptosporidium* mAb to the tube and mix well by pipetting up and down.

3.8.6. Incubate tubes at room temperature for 30 min.

3.8.7. Add 950 µl PBS and vortex for 5 sec.

3.8.8. Centrifuge tubes at 13,000 x g for 5 min.

3.8.9. Carefully remove supernatant – do not disturb the pellet.

3.8.10. Add 50 µl goat anti-mouse Ab conjugated with Alexa Fluor 488 into the tube and mix well by pipetting up and down.

3.8.11. Incubate tubes at room temperature for 30 min in the dark.

3.8.12. Add 950 µl PBS and vortex for 5 sec (keep in the dark).

3.8.13. Centrifuge tubes at 13,000 x g for 5 min (keep in the dark).

3.8.14. Carefully remove supernatant – do not disturb the pellet.

3.8.15. Add 950 µl water and vortex for 5 sec (keep in the dark).

3.8.16. Centrifuge tubes at 13,000 x g for 5 min (keep in the dark).

3.8.17. Carefully remove supernatant – do not disturb the pellet.

3.8.18. Add 50 µl sterile H_2O and resuspend the pellet by pipetting up and down.

3.8.19. Use 10 µl for oocyst counting and 10 µl for oocyst observation using a fluorescence microscope.

3.9. Oocyst counting on hemacytometer

The samples should be kept in dark during the procedure.

3.9.1. Prepare hemacytometer with a cover glass.

3.9.2. Pipet 10 µl of stained oocysts (from 3.8.16) onto hemacytometer (keep in dark).

3.9.3. Count oocysts in the 4 corner squares of ’16 squares’ using a fluorescence microscope.

3.9.4. Calculate # of oocyst per ml.

(# Oocysts counted on 4 corner squares) x (1/4) x (10^4) x (1/5) = (# Oocysts/ml)

ex. (19+21+24+20) x (1/4) x (10^4) x (1/5) = 42,000 oocysts /ml.

3.10. Oocyst observation using a fluorescence microscope

The samples should be kept in dark during the procedure.

3.10.1. Pipet 10 µl of stained oocysts (from 3.8.16) and smear on a slide glass.

3.10.2. Let air dry.

3.10.3. Immerse with anti-fade reagent with DAPI.

3.10.4. Place a cover glass on the slide glass.

3.10.5. Observe the oocysts using a fluorescence microscope.

3.11. DNA extraction

The procedure uses E.Z.N.A. Stool DNA kit following by manufacturer’s instruction

3.11.1. Transfer 50 µl of harvest (from 3.7.3) into a new 1.5 ml tube.

3.11.2. Add 50 µl sterile water.

3.11.3. Do 5 freeze-thaw cycles.

3.11.4. Transfer 100 µl in a 1.5 ml microcentrifuge tube containing 100 mg Glass Beads X. Place the tube on ice (total volume ~200 µl).

3.11.5. Add 270 µl SLX-Mlus Buffer. Vortex at maximum speed for 10 min or until the samples is thoroughly homogenized.

3.11.6. Add 30 µl DS buffer and 10 µl Proteinase K solution. Vortex or pipet up and down to mix thoroughly.

3.11.7. Incubate at 70°C for 10 min. Vortex twice during incubation.

3.11.8. Add 100 µl SP2 buffer. Vortex at maximum speed for 30 sec. (total ~610 µl).

3.11.9. Let sit on ice for 5 min.

3.11.10. Centrifuge at 13,000 x g for 5 min.

3.11.11. Transfer 200 µl supernatant to a new 1.5 ml tube. Do not disturb the pellet.

3.11.12. Add 100 µl HTR reagent. Vortex at maximum speed for 10 sec. HTR reagent must be thoroughly resuspended and pipetted with an end-cut pipet tip.

3.11.13. Let sit at room temperature for 2 min.

3.11.14. Centrifuge at 13,000 x g for 2 min.

3.11.15. Transfer 125 µl supernatant to a new 1.5 ml tube.

3.11.16. Add 125 µl BL buffer and 125 µl 100% ethanol. Vortex at maximum speed for 10 sec.

3.11.17. Insert a HiBind DNA mini column into a 2 ml collection tube.

3.11.18. Transfer the entire sample from step 3.11.16 to the HiBind DNA mini column.

3.11.19. Centrifuge at maximum speed for 1 min.

3.11.20. Transfer the HiBind DNA mini column into a new 2 ml collection tube.

3.11.21. Add 500 µl VHB buffer.

3.11.22. Centrifuge at 13,000 x g for 30 sec.

3.11.23. Discard the filtrate and reuse the collection tube.

3.11.24. Add 700 µl DNA wash buffer.

3.11.25. Centrifuge at 13,000 x g for 1 min.

3.11.26. Discard the filtrate and reuse the collection tube.

3.11.27. Add 700 µl DNA wash buffer.

3.11.28. Centrifuge at 13,000 x g for 1 min.

3.11.29. Discard the filtrate and reuse the collection tube.

3.11.30. Centrifuge at 13,000 x g for 2 min.

3.11.31. Transfer the column into a clean 1.5 ml tube.

3.11.32. Add 100 µl Elution buffer heated to 65°C.

3.11.33. Let sit at room temperature for 2 min.

3.11.34. Centrifuge at 13,000 x g for 1 min.

3.11.35. Store DNA at -20°C until Real-Time PCR.

3.12. Real time PCR

3.12.1. Prepare the Cryptosporidium DNA standard.

3.12.1.1. Add 10^6, 10^5, 10^4, 10^3, 10^2 oocysts of *Cryptosporidium* TU502 into COLO-680N 10^6 cells, respectively.

3.12.1.2. Extract the DNA using the procedure described in 3.11.

3.12.1.3. Store at -20°C until Real-Time PCR.

3.12.2. Prepare HSP70 primer/probe mix.

3.12.2.1. Prepare the primer/probe mix of HSP70 FWD and HSP70 REV to be 5 µM and HSP70 PRB-FAM to be 2.5 µM at final conc. Otherwise, order the premix from vendor and prepare the mix by the manufacturer's direction.

3.12.2.2. Store at 4°C (for a short-term use) or -20°C.

3.12.3. Prepare the PCR mix in a 96-well plate.

3.12.3.1. Prepare the PCR mix of DNA samples (from 3.11.35) and standard DNA (from 3.12.1). Per well, add the following components in the table. Each sample is tested in triplicate. The PCR mix can be prepared as 3x volume (30 µl) and 10 µl distributed into each of 3 wells.

Components	**Volume**
PrimeTime Master Mix (2x)	5 µl
DNA (samples or standard)	2 µl
HSP70 primer/probe mix (3.11.2)	1 µl
Water	2 µl

3.12.3.2. Seal the plate with TempPlate RT optically clear film.

3.12.3.3. Centrifuge briefly.

3.12.4. Load the plate into the StepOne Real-Time PCR instrument.

3.12.5. Open the StepOne software and set up the plate setup and run method.

Step	**Cycles**	**Temp (°C)**	**Time (Min:Sec)**
Polymerase activation	1	95°C	3:00
Amplification	45		
Denaturation		95°C	0:15
Annealing/Extension		60°C	1:00

3.12.6. Run the instrument

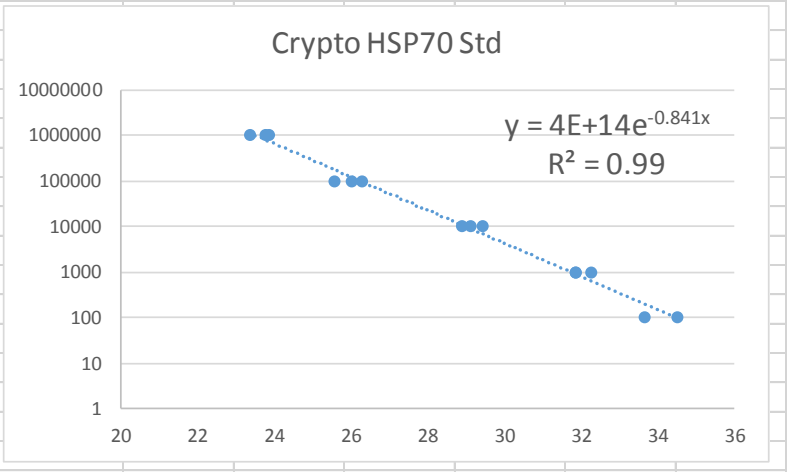
3.12.7. Data Analysis

3.12.7.1. Extract the raw data from the StepOne software to MS Excel.

3.12.7.2. Generate the standard curve using the C_T data of standard samples in MS Excel.

Example of standard curve (Exponential trend line with the equation and R-squared value).

Cт	Quantity
23.36539	1000000
23.76603	1000000
23.86398	1000000
25.59637	100000
26.00708	100000
26.30232	100000
28.88531	10000
29.14804	10000
29.43307	10000
31.8645	1000
32.29043	1000
31.87642	1000
34.52393	100
33.6643	100
	100



3.12.7.3. Calculate DNA amounts equivalent to *Cryptosporidium* oocysts from C_T of samples using the equation of standard curve.

3.12.7.4. Make a plot of the calculated DNA amounts using GraphPad Prism.