Contract/Task order number: HHSN272201100003I/HHSN27200002

Task Order C02: "Development of Continuous *in vitro* Culture System for Cryptosporidium"

Final Technical Report – Standard Operating Procedures

15 September 2017

Submitted By:
Robert Molestina
Timothy T. Stedman

American Type Culture Collection
10801 University Boulevard
Manassas, VA 20110

Contents

1 SOP 1 - Protocol for the Isolation of Primary Human Intestinal Epithelial Cells 2

2 SOP 2 - Immunofluorescence Assay of Host Cell Cultures Adhered to Glass Coverslips 3

3 SOP 3 - Isolation of Primary Intestinal Epithelium from Laboratory Mice 4

4 SOP 4 - Immunofluorescence Assay for *Cryptosporidium parvum*-infected Epithelial Cells Growing on Polycarbonate Membrane Supports (Transwells) 5

5 SOP 5 - Quantitative reverse transcriptase PCR (qRT-PCR) assay for *Cryptosporidium parvum*-infected Epithelial Cells Growing on Polycarbonate Membrane Supports (Transwells)............... 6

6 SOP 6 - Protocol for Human Intestinal Organoid Isolation 7

7 SOP 7 - Protocol for Mouse Intestinal Organoid Culture 9

8 SOP 8 - Immunofluorescence Assay of Mouse Intestinal Organoid Cultures 12

9 SOP 9 - Inoculation of Mouse Intestinal Organoids with *Cryptosporidium parvum* 13

10 SOP 10 - Immunofluorescence assay for developing stages of *Cryptosporidium* adhered to glass coverslips 16

11 SOP 11 - Transmission Electron Microscopy for axenic cultures of *Cryptosporidium* 17

12 SOP 12 - Transmission Electron Microscopy for *Cryptosporidium parvum*-infected Intestinal Epithelial Cells Growing on Polycarbonate Membrane Supports (Transwells)........... 19

1 SOP 1 - PROTOCOL FOR THE ISOLATION OF PRIMARY HUMAN INTESTINAL EPITHELIAL CELLS

1. Procure intestine by the organ procurement organization under an IRB approved protocol.
2. Remove the intestine and flush the luminal contents with cold PBS.
3. Place the flushed intestine immediately in cold DMEM containing penicillin-streptomycin antibiotics for transport.
4. Ship sample on a next flight out basis for delivery to the laboratory via courier.
5. Upon arrival, inspect the intestine for any abnormalities (i.e., tumors) and remove the unwanted tissue such as lymph nodes, fat, and blood vessels.
6. Flush the lumen with PBS without Ca^{2+} or Mg^{2+} for a minimum of three times.
7. Cut the intestinal tissue into segments, sliced longitudinally, and place in PBS solution containing 0.5 mM dithiothreitol and 1.5 mM EDTA for 30 minutes at 4°C with gentle shaking.
8. Shake solutions vigorously by hand and examine the contents of the solution under an inverted microscope to determine if the epithelium has been liberated.
9. Continue the incubation at 4°C while checking the contents every 15 minutes.
   <u>Note:</u> If sufficient epithelium has not detached after 2 hours, gently scrape the lumen to release the epithelium.
10. After sufficient epithelial release, remove the tissue and continue the incubation for an additional 30-60 minutes at 4°C with gentle shaking to continue disruption into a single cell suspension.
11. Centrifuge the suspension at 300xg for 8 minutes.
12. Re-suspend the pellet in complete warmed media and seed at 5,000 to 50,000 cells/cm^2 in a collagen-coated flask.
13. Incubate cultures at 37°C, 5% CO_2 in DMEM supplemented with 10% heat-inactivated FBS, 30 ng/ml of EGF, 0.25 U/ml insulin, and 1X penicillin/streptomycin solution .

Recommended sources of human tissue, media, and reagents

Intestine: National Disease Research Interchange http://ndriresource.org/
DMEM, penicillin/streptomycin solution, PBS, and FBS: ATCC www.atcc.org/
Dithiothreitol, EDTA, EGF, and insulin: Sigma-Aldrich www.sigmaaldrich.com/

2 SOP 2 - IMMUNOFLUORESCENCE ASSAY OF HOST CELL CULTURES ADHERED TO GLASS COVERSLIPS

1. Aspirate growth medium from the culture well of a 24-well plate and wash cell monolayers adhered to glass coverslips three times with 0.5 mL PBS.
2. Aspirate the PBS and add 0.5 ml of 3% paraformaldehyde (PFA) in PBS. Incubate at room temperature for 15 min or overnight at 4°C.
3. Aspirate the fixative and wash coverslips twice with PBS.
4. Add 0.5 ml of 0.1% Triton X-100 in PBS and incubate at room temperature for 5 min.
5. Aspirate Triton X-100 and wash twice with PBS. After the last wash, block coverslips with 0.5 ml of 3% bovine serum albumin (BSA) in PBS for 1 hour at room temperature.
6. Cover the bottom of a 15 cm Petri dish with wet filter paper and a piece of Parafilm cut to closely fit the bottom of the dish.
7. Add 50 μL of primary antibody diluted diluted 1:500 in blocking buffer in spots on the Parafilm. Try to maintain the same orientation on the Parafilm as in the 24-well plate.
8. Using a hypodermic needle and forceps, remove coverslips carefully from the wells one at a time.
9. Invert the coverslips individually on top of the spots of primary antibody, ensuring that the entire cell-covered surface is immersed. Cover Petri dish with a lid and incubate at room temperature for of 1 hour.
10. Return the coverslips to the appropriate wells of the 24-well plate, taking care to invert each one so that the cell-covered surface is facing up. Wash three times with PBS.
11. Replace the piece of Parafilm from the Petri dish with a new one. Add 50 μL of fluorophore-conjugated secondary antibody diluted 1:2000 in blocking buffer in spots on the Parafilm. Try to maintain the same orientation on the Parafilm as in the 24-well plate.
12. Using a hypodermic needle and forceps, remove coverslips carefully from the wells one at a time.
13. Invert the coverslips individually on top of the spots of secondary antibody, ensuring that the entire cell-covered surface is immersed. Cover Petri dish with a lid and incubate at room temperature for of 1 hour in the dark.
14. Return the coverslips to the appropriate wells of the 24-well plate, taking care to invert each one so that the cell-covered surface is facing up. Wash three times with PBS.
15. Place 10 μl of mounting medium (Life Technologies ProLong Gold Antifade Reagent with DAPI) on the surface of a microscope slide.
16. Using a hypodermic needle and forceps, remove coverslips carefully from the wells one at a time. Invert the coverslips individually on top of the spots of mounting medium ensuring that the entire cell-covered surface is immersed. Store the slide in the dark at 4°C until imaging.

Recommended sources of reagents

PFA solution, Triton X-100, and BSA: Sigma-Aldrich www.sigmaaldrich.com/

Fluorophore-conjugated secondary antibody and mounting medium: ThermoFisher www.thermofisher.com/

3 SOP 3 - ISOLATION OF PRIMARY INTESTINAL EPITHELIUM FROM LABORATORY MICE

1. Euthanize a C57BL/6 mouse via IACUC approved protocol and pin the mouse via the extended extremities to reveal the abdomen.
2. Rinse the abdomen with 70% ethanol and make an incision into the peritoneal cavity and upwards to the rib cage.
3. Visualize the stomach and intestines. Use sterile scissors to cut the intestines at the duodenum and ileum areas.
4. Remove the small intestine segment with sterile forceps and remove excess connective tissue.
5. Transfer the tissue to a 100 mm petri dish containing ice cold PBS and flush the tissue segment with 5 mL of ice cold PBS containing 1X antibiotic/antimycotic solution (Gibco 15240062) using a syringe and blunt 18 g needle. Repeat the flushing with fresh PBS until all material from within the intestine is removed.
6. Cut the intestinal tissue into segments, sliced longitudinally, and place in PBS solution containing 0.5 mM dithriothreitol and 1.5 mM EDTA for 30 minutes at 4°C with gentle shaking.
7. Shake the suspension vigorously by hand and examine the contents of the suspension under an inverted microscope to determine if the epithelium has been liberated.
8. Continue the incubation at 4°C while checking the contents every 15 minutes. If sufficient epithelium has not detached after 2 hours, gently scrape the lumen to release the epithelium.
9. After sufficient epithelial release, remove the tissue and continue the incubation for an additional 30-60 minutes at 4°C with gentle shaking to continue disruption into a single cell suspension.
10. Centrifuge the suspension at 300xg for 8 minutes.
11. Re-suspend the pellet in complete warmed medium consisting of DMEM supplemented with 10% FBS, 0.25 U/mL insulin, 50ng/mL EGF, and 1X antibiotic/antimycotic solution.
12. Seed 5,000 to 50,000 cells/cm2 in a collagen coated T25 flask. Incubate at 37°C under 5% CO_2 atmosphere. Observe under an inverted microscope on a daily basis.
13. Set up cell cultures on sterile glass coverslips placed inside 24 well plates and perform IFA as indicated in SOP 2.

Recommended source of C57BL/6 mice: Charles River Laboratories www.criver.com/

4 SOP 4 - IMMUNOFLUORESCENCE ASSAY FOR *CRYPTOSPORIDIUM PARVUM*-INFECTED EPITHELIAL CELLS GROWING ON POLYCARBONATE MEMBRANE SUPPORTS (TRANSWELLS)

1. Remove the transwell with sterile forceps, aspirate the culture medium, and transfer to a well with 0.6 ml of PBS. Add 0.2 ml of PBS to the upper compartment to wash the host cell monolayer.
2. Aspirate the PBS from the upper compartment and add fresh PBS to wash. Repeat twice.
3. Remove the transwell with sterile forceps, aspirate the PBS, and transfer to a well with 0.6 ml of 3% paraformaldehyde in PBS. Add 0.2 ml of the same fixative to the upper compartment and incubate at room temperature for 15 min. Sample may be left at 4^oC overnight.
4. Remove the transwell with forceps, aspirate the fixative, and transfer to a well with 0.6 ml of PBS. Add 0.2 ml of PBS to the upper compartment to wash.
5. Aspirate the PBS from the upper compartment and add fresh PBS to wash. Repeat twice.
6. Remove the transwell with forceps, aspirate the PBS, and transfer to a well with 0.6 ml of 0.1% Triton X-100 in PBS. Add 0.2 ml of 0.1% Triton X-100 to the upper compartment and incubate at room temperature for 5 min.
7. Wash the transwell as described in steps 4 and 5. After the last wash, block cells in the upper compartment with 0.2 ml of 3% bovine serum albumin (BSA) in PBS for 1 hour at room temperature.
8. Aspirate BSA and add 0.2 ml of primary antibody diluted 1:500 or 1:1000 in blocking buffer to the upper compartment. Incubate for 1 h at room temperature.
9. Wash the transwell as described in steps 4 and 5.
10. Add 0.2 ml of fluorophore-conjugated secondary antibody diluted 1:2000 in blocking buffer to the upper compartment. Incubate for 1 h at room temperature.
11. Wash the transwell as described in steps 4 and 5. After the last wash, remove the transwell and invert over a paper towel. Carefully remove the polycarbonate membrane with a scapel.
12. Place 10 µl of mounting medium (Life Technologies ProLong Gold Antifade Reagent with DAPI) on the center of a round coverslip. Using forceps, place the cell monolayer side of the membrane over the drop of mounting medium. Invert the coverslip and membrane on the surface of a clean microscope slide. Store the slide in the dark at 4^oC until imaging.

Recommended source of transwells: Corning www.corning.com/

5 SOP 5 - QUANTITATIVE REVERSE TRANSCRIPTASE PCR (QRT-PCR) ASSAY FOR *CRYPTOSPORIDIUM PARVUM*-INFECTED EPITHELIAL CELLS GROWING ON POLYCARBONATE MEMBRANE SUPPORTS (TRANSWELLS)

1. Remove the transwell with sterile forceps, aspirate the culture medium, and transfer to a new 6-well plate.
2. Add 0.6 ml of RLT buffer (Qiagen) to the cell monolayer. Let stand for 1 minute at room temperature and transfer lysate to a sterile microfuge tube. Lysate may be stored at -80°C if not processed immediately.
3. Proceed with RNA isolation according to the Qiagen RNeasy kit manual.
4. Analyze the purity and concentration of RNA by spectrophotometry using the NanoDrop 1000 system (Thermo Scientific, Pittsburgh, PA). Stored RNA at -80°C.
5. Perform qRT-PCR in duplicate 20 µl volumes using the iTaq Universal Sybr Green One-step kit (Biorad, Hercules CA).
6. Set up each reaction in duplicate using 0.1 µg of RNA and 0.3 µM of each forward and reverse primer.
7. Run the qRT-PCR reactions in a Biorad CFX96 Real time PCR system consisting of a reverse transcription stage at 50 °C for 10 min, followed by an initial PCR activation step of 1 min at 95 °C then 40 cycles of 95 °C for 30 s and 59°C for 30 s.
8. Perform a melt-curve analysis using the instrument's default setting.
9. For calibration of the standard curve, perform ten-fold dilutions (10^6 copies to 10 copies) of *C. parvum* DNA (ATCC PRA-67DQ) that had been quantified using droplet digital PCR (ddPCR).

6 SOP 6 - PROTOCOL FOR HUMAN INTESTINAL ORGANOID ISOLATION

A. Media and reagents

1. Prepare chelating buffer fresh. Buffer consists of 2% sorbitol, 1% sucrose, 1% bovine serum albumin fraction V (BSA), 1X gentamicin/amphotericin solution in PBS without Ca^{2+} and Mg^{2+}. Filter sterilize.
2. Prepare Wnt-3A-conditioned medium in house using the L Wnt-3A cell line. Supplement the cell line growth medium with 2 mM glutamine, 10 mM HEPES, 100 U/ml penicillin, 100 g/ml streptomycin, 1X N2 and 1X B27 supplements (Life Technologies), and 1% BSA. Filter sterilize.
3. Prepare human minigut medium as follows: supplement Advanced DMEM/F12 medium (Life Technologies) with 2 mM glutamine, 10 mM HEPES, 100 U/mL penicillin, 100 g/mL streptomycin, 1X N2 supplement, 1X B27 supplement, and 1% BSA. Filter sterilize.
4. Prepare complete human minigut medium as follows: supplement human minigut medium with 50% Wnt-3A-conditioned medium, 1 µg/ml R-spondin 1, 100 ng/ml Noggin, 50 ng/mL EGF, 500 nM A-83-01, 10 µM SB202190, 10 nM [Leu]15-Gastrin 1, 10 mM Nicotinamide and 1 mM N-Acetylcysteine.
   NOTE: Complete human minigut media can be stored up to 2 days at 4°C.

B. Crypt isolation

1. Prepare all the reagents before beginning the isolation. Thaw the basement membrane matrix on ice and pre-incubate a 24-well plate in a CO_2 incubator at 37°C.
2. Wash the tissue with ice-cold PBS without Ca^{2+} and Mg^{2+}. Proceed until the content of the PBS is clear.
3. Secure the tissue on a silicone-coated glass Petri dish using pins. Fill the dish with ice-cold PBS. Stretch and pin the tissue flat with the mucosal side facing up.
4. Under a dissecting microscope, remove the overlying mucosa from the submucosa and connective tissue using sterile micro-dissecting scissors and fine point curved forceps.
5. Stretch and pin the dissected mucosa flat on the silicone-coated glass Petri dish with mucosal side facing up. Discard the remaining submucosa and connective tissue.
6. Gently scrape the surface of the mucosa with curved forceps to remove the villi.
7. Wash the mucosa 3-4 times with ice-cold chelation buffer to remove villi and debris.
8. Cover the mucosa with freshly prepared 2mM EDTA chelation buffer.
9. Place the Petri dish on ice and shake gently for 30 min on a horizontal orbital shaker.
10. Wash the tissue 3-4 times with ice-cold chelation buffer without EDTA. After washing, leave the mucosa in ice-cold chelation buffer.
11. Process the mucosa under a dissecting microscope using curved and fine forceps. Gently scrape the mucosa to release intestinal crypts using the curved forceps.
12. Gently remove the crypt suspension from the petri dish using a pipette and transfer to a 50 ml conical tube.

13. Filter the crypts suspension through a 150 µm mesh 2 times. Check the flow-through for crypt enrichment under a microscope.
14. Centrifuge the crypt suspension 5 min at 50 x*g*, 4°C. Discard the supernatant.
15. Resuspend the pellet in 5 ml ice-cold chelation buffer.
16. Count the number of crypts and transfer the volume needed for plating to a 5 ml round-bottom tube. Use 200 to 500 crypts per well of a 24-well plate to establish enteroids.
17. Centrifuge the crypt fraction for 10 min at 150 x*g* and 4°C. Remove the supernatant.
18. Use crypts for subsequent culture.

C. Crypt culture

1. Using pre-chilled pipet tips, resuspend the crypt pellet in basement membrane matrix.
2. Apply 50 µl of crypt suspension in basement membrane matrix per well on the pre-warmed 24 well plate.
3. Place the 24-well plate in a 37°C, 5% CO_2 incubator for 30 min to allow a complete polymerization of the basement membrane matrix.
4. Overlay the basement membrane matrix with 500 µl of complete human minigut medium supplemented with 2.5 µM CHIR99021 and 2.5 µM Thiazovivin.
5. Incubate the plate in a 37°C, 5% CO_2 incubator.
6. Replace the medium with fresh complete human minigut medium every 2 days.

Reference: Mahe, M.M., et al. 2015. J. Vis. Exp. 97:doi: 10.3791/52483.

Recommended sources of cell lines, media and reagents

L Wnt-3A cell line and Cell Matric Basement Membrane: ATCC www.atcc.org/
Advanced DMEM/F12 medium, 1X N2, and 1X B27 supplements: ThermoFisher www.thermofisher.com/
Human recombinant R-Spondin: Peprotech www.peprotech.com/
Human recombinant Noggin: R&D Systems www.rndsystems.com/
EGF, SB202190, [Leu]15-Gastrin 1, Nicotinamide, and N-Acetylcysteine: Sigma-Aldrich www.sigmaaldrich.com/
A-83-01: Tocris Bioscience www.tocris.com/
CHIR99021 and Thiazovivin: StemGent www.stemgent.com/

7 SOP 7 - PROTOCOL FOR MOUSE INTESTINAL ORGANOID CULTURE

A. Mouse Small Intestinal Crypt Tissue Collection Protocol

1. Euthanize a mouse via IACUC approved method.
2. Pin the mouse via the extended extremities to reveal the abdomen.
3. Rinse the abdomen with 70% ethanol.
4. Make an incision in the abdomen into the peritoneal cavity and upwards to the rib cage.
5. Visualize the stomach and intestines.
6. Use scissors to cut the intestines at the duodenum and ileum areas.
7. Remove the small intestine segment with sterile forceps and remove excess connective tissue.
8. Transfer the tissue to a 100 mm petri dish containing ice cold PBS.
9. Flush the tissue segment with 5 mL of ice cold PBS containing 1X antibiotic/antimycotic solution (Gibco 15240062) using a syringe and blunt 18 g needle. Repeat the flushing with fresh PBS until all material from within the intestine is removed.
10. Transfer the intestine to a 50 ml conical tube containing 50 ml of ice cold PBS containing 1X antibiotic/antimycotic solution. Place the tube on ice.
11. If the intestine floats, gently shake the tube until the tissue segment sinks and settles to the bottom of the tube.

Notes:

- Typical target length of intestine is 10 cm.
- If the intestine is damaged at any point discard and do not continue.
- Ensure the intestine is thoroughly flushed to remove waste material and ensure no air is present.

12. Transfer the intestine to a 100 mm dish containing ice cold PBS.
13. Using angled dissection scissors, cut the intestine length wise into a flat sheet.
14. Scrape the inner surface to remove villus.
15. Transfer tissue to a new dish containing fresh ice cold PBS.
16. Cut the tissue into ~2 mm x 2 mm pieces and transfer to a 50 ml conical tube containing 15 ml of ice cold PBS.
17. Pipet the tissue segments up and down 3-5 times with a 10 ml serological pipet.
18. Allow pieces to settle by gravity.
19. Aspirate the supernatant and add 15 ml fresh ice cold PBS to tube.
20. Repeat steps 6-8 until the supernatant is no longer cloudy.
21. Aspirate the supernatant and add 25 ml room temperature Non-Enzymatic Cell Dissociation Solution.
22. Place the tube on a rocker for 20 min at room temperature.
23. Return the tube to BSC and allow pieces to settle by gravity. Aspirate the supernatant.
24. Re-suspend pieces in 10 ml ice cold PBS containing 0.1% BSA. Allow the pieces to settle by gravity.

25. Collect the supernatant and pass through a 70 micron cell strainer into a 50 ml tube. Place tube on ice.
26. Repeat steps 13 and 14 three additional times and pool the supernatant.
27. Spin tube at 300 x g for 5 minutes at 4°C.
28. Discard the supernatant.
29. Re-suspend the pellet in 10 ml ice cold PBS containing 0.1% BSA and transfer to a 15 ml conical tube.
30. Spin the tube at 200 x g for 5 minutes at 4°C.
31. Aspirate the supernatant.
32. Re-suspend in 10 ml ice cold DMEM:F-12.
33. Count the crypts using a hemocytometer and inverted microscope.

Notes:

- Complete removal of villus is vital for downstream culture purity.
- If tissue sticks to pipets, pre-wet with PBS.
- Do not store crypts, plate immediately.

B. Mouse Small Intestinal Organoid Generation Protocol

1. Place a 24-multiwell plate in a cell culture incubator for 15 minutes.
2. Transfer 25-50 crypts to be plated per well to a microfuge tube. Spin at 200 x g for 5 minutes at 4°C.
3. Aspirate all the supernatant.
4. Re-suspend the crypts in 100% ice cold Cell Matrix Basement Membrane Gel (ATCC ACS-3035), in 50 µl per well to be plated. Pipette up and down 10 times to mix without introducing any bubbles.
5. Transfer 50 µl directly to the center of a well in the pre-warmed 24-multiwell plate.
6. Carefully return the plate to the cell culture incubator at 37°C for 15 minutes to polymerize the gel.
7. Remove the plate from the incubator. Solidified gel "domes" should be visible.
8. Carefully overlay dome with 500 µl of pre-warmed complete IntestiCult^{TM} Organoid Growth Medium (Stem Cell Technologies).
9. Return plate to the incubator.
10. Replace the medium completely every 2-3 days without disturbing the dome.

Notes:

- Crypts should form spheres within 24 hours. Budding should be visible within 96 hours. Extensive budding should be visible within 144 hours.
- Thaw the Cell Matrix Basement Membrane Gel on ice. For the 5 ml stock tube, thaw overnight at 4°C. For 250 µl aliquots, thaw on ice for ~2 hours. Keep on ice at all times.
- If the Cell Matrix Basement Membrane gels prematurely, pre-chill pipette tips and tubes. Work rapidly.
- Pipette Cell Matrix Basement Membrane Gel directly to the center of the well. Do not allow it to make contact with the well wall.

- If the gel does not solidify after 15 minutes in the incubator, either excessive liquid remained following step 3 or the Cell Matrix Basement Membrane Gel was mishandled.

C. Mouse Small Intestinal Organoid Splitting Protocol

1. Aspirate medium from well.
2. Add 1 ml of ice cold Enzyme Free Dissociation Reagent and incubate at room temperature for 1 minute
3. Using a P1000 tip, repeatedly pipet up and down 10 times within the well to break up the gel dome.
4. Transfer contents of well to a 15 ml conical tube and place on ice.
5. Add an additional 1 ml of ice cold Enzyme Free Dissociation Reagent to the well and transfer to a 15 ml conical tube.
6. Repeat steps 1-5 for all wells to be split.
7. Place conical tube on a rocker at room temperature for 10 minutes.
8. Spin tube at 200 x g for 5 minutes at 4°C.
9. Re-suspend crypts in 10mL ice cold DMEM:F12.
10. Count crypts using a hemocytometer and inverted microscope
11. Proceed from step B.1 above.

Notes:

- Recommended split ratio of intestinal organoids is 1:3 to 1:6.
- Healthy organoids should be split at least every 7 days.

Recommended sources of media and reagents

Non-Enzymatic Cell Dissociation Solution, Cell Matrix Basement Membrane, and DMEM:F12: ATCC
IntestiCult^{TM} Organoid Growth Medium: Stem Cell Technologies www.stemcell.com

8 SOP 8 - IMMUNOFLUORESCENCE ASSAY OF MOUSE INTESTINAL ORGANOID CULTURES

1. Plate 40 µl of organoids suspended in matrigel into one well each of an 8-well chamber-slide (Thermo Fisher cat. # 154532). After letting harden in the incubator for 15 min, add 0.4 mL of growth medium to each well and change every 2-3 days.
2. When ready for fixation, aspirate growth medium from the wells and wash domes three times with 0.4 mL PBS.
3. Aspirate the PBS and add 0.4 ml of 3% paraformaldehyde (PFA) in PBS to each well. Incubate at room temperature for 15 min or overnight at 4°C.
4. Aspirate the fixative and wash the wells twice with PBS.
5. Add 0.4 ml of 0.1% Triton X-100 in PBS to each well and incubate at room temperature for 5 min.
6. Aspirate Triton X-100 and wash twice with PBS. After the last wash, block wells with 0.5 ml of 3% bovine serum albumin (BSA) in PBS for 1 hour at room temperature.
7. Add 150 µL of primary antibody diluted 1:250 to 1:500 in blocking buffer to each well. Incubate at room temperature for 1 hour.
8. Remove the primary antibody solution and wash three times with PBS.
9. Add 150 µL of fluorophore-conjugated secondary antibody diluted 1:2000 in blocking buffer to each well. Incubate at room temperature for of 1 hour in the dark.
10. Remove the secondary antibody solution and wash three times with PBS.
11. Carefully detach the 8-well chambers with a metal spatula. Place 12 µL of mounting medium (Life Technologies ProLong Gold Antifade Reagent with DAPI) on the surface of each sample.
12. Place a cover slip over each sample, avoiding bubbles. Let harden overnight at room temperature in the dark. After imaging, the slide can be stored at 4°C.

9 SOP 9 - INOCULATION AND SCREENING OF MOUSE INTESTINAL ORGANOIDS WITH *CRYPTOSPORIDIUM PARVUM*

Preparation

1. Place 6-well tissue culture plates and 8-well chamber slides in a 37°C humid incubator for at least 15 minutes to warm.
2. Bring complete IntestiCult^{TM} growth medium to room temperature.
3. Thaw required volume of Basement Cell Matrix Basement Membrane on ice.

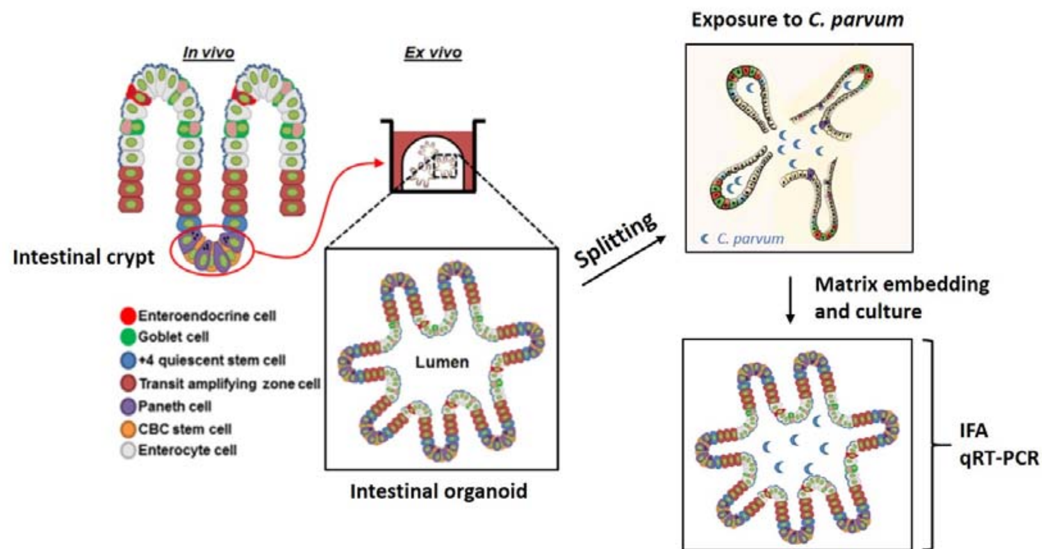
Passaging

4. Aspirate medium completely from a 7-day old mature organoid culture growing in a 6-well plate.
5. Add 2 mL of ice-cold non-enzymatic cell dissociation solution per well.
6. Incubate for 1 minute at room temperature.
7. Use a cell scraper to release organoids from plate.
8. Pre-rinse a P1000 tip with cell dissociation solution and then break up domes by pipetting 10 times.
9. Transfer broken up domes to a 15 mL conical tube.
10. Place on shaker for 15-20 minutes at room temperature.
11. Add ice cold 1% BSA in DMEM:F12 to max volume.
12. Spin at 300xg for 5 minutes at 4°C.
13. Aspirate supernatant.
14. Add ice cold 1% BSA in DMEM:F12 to max volume.

Inoculation

15. Count the crypts from step 14 using a hemocytometer and divide the suspension into twelve 15 mL conical tubes with ~2 x 10^4 crypts per tube. Bring the volume to 2 mL per tube with 1% BSA in DMEM:F12.
16. Inoculate 6 tubes with 5×10^6 *C. parvum* sporozoites per tube. Leave the remaining 6 tubes uninfected.
17. Incubate uninfected tubes for 0, 15, 30, 60, 120, and 180 min at 37°C.
18. Incubate infected tubes for 0, 15, 30, 60, 120, and 180 min at 37°C.
19. At each time point, spin uninfected and infected tubes at 300xg for 5 minutes at 4°C.
20. Aspirate supernatant.
21. Res-suspend uninfected and infected organoids in 100% Cell Matrix Basement Membrane. Pipet up and down 20 times.
22. Using a P200, dispense approximately six 10-15uL droplets into each well of the pre-warmed 6 well plate. Avoid bubbles. Use three wells for the uninfected sample and three wells for the infected sample.

23. Using a P200, dispense one 10-15uL droplet into each well of the pre-warmed 8 well chamber slide. Avoid bubbles. Use four wells for the uninfected sample and four wells for the infected sample.
24. Invert plate and chamber slides and place in 37°C humid incubator for 15 minutes.
25. Add 2 mL of complete growth medium per well of the six well plate and 0.4 mL of growth medium per well of the 8 well chamber slide.
26. Return plate and slide to incubator.
27. Incubate for 3, 7, and 10 days at 37°C, 5% CO_2. Change medium every 2-3 days.



Schematic diagram of *C. parvum* infection of intestinal organoids. Intestinal stem cells located at the base of the crypts are cultured ex vivo to generate indefinitely propagating organoid cultures. Immediately after splitting, the crypt/villus units are exposed to *C. parvum* sporozoites followed by 3D culture. Infection of the MIOs by the parasite is examined by qRT-PCR and IFA. Images were modified from Zachos, N.C., et al. 2016. JBC. 291:3759-3766 and Mahe, M.M., et al. 2014. Curr. Protoc. Mouse Biol. 3: 217–240.

RNA isolation and qRT-PCR

28. Isolate RNA from uninfected and *C. parvum*-infected cultures incubated in 6-well plates after 3, 7, and 10 days of incubation using the RNeasy Kit (Qiagen).
29. Analyze the purity and concentration of RNA by spectrophotometry using the NanoDrop 1000 system (Thermo Scientific, Pittsburgh, PA).
30. Perform qRT-PCR as described in "SOP 5 - Quantitative reverse transcriptase PCR (qRT-PCR) assay for *Cryptosporidium parvum*-infected Epithelial Cells Growing on Polycarbonate Membrane Supports (Transwells)"

IFA

31. Aspirate the medium from uninfected and *C. parvum*-infected cultures incubated in 8-well chamber slides.
32. Wash each well three times with PBS.
33. Fix samples with 3% paraformaldehyde in PBS and incubate at room temperature for 15 min. Samples may be left at 4°C overnight.
34. Perform IFA as described in “SOP 8 - Immunofluorescence Assay of Mouse Intestinal Organoid Cultures.”

10 SOP 10 - IMMUNOFLUORESCENCE ASSAY FOR DEVELOPING STAGES OF *CRYPTOSPORIDIUM* ADHERED TO GLASS COVERSLIPS

1. Aspirate growth medium from the culture well of a 24-well plate and wash parasites adhered to the glass coverslip three times with 0.5 mL PBS.
2. Aspirate the PBS and add 0.5 ml of 3% paraformaldehyde (PFA) in PBS. Incubate at room temperature for 15 min or overnight at 4°C.
3. Aspirate the fixative and wash coverslips twice with PBS.
4. Add 0.5 ml of 0.1% Triton X-100 in PBS and incubate at room temperature for 5 min.
5. Aspirate Triton X-100 and wash twice with PBS. After the last wash, block coverslips with 0.5 ml of 3% bovine serum albumin (BSA) in PBS for 1 hour at room temperature.
6. Cover the bottom of a 15 cm Petri dish with wet filter paper and a piece of Parafilm cut to closely fit the bottom of the dish.
7. Add 50 µL of primary antibody diluted diluted 1:500 or 1:1000 in blocking buffer in spots on the Parafilm. Try to maintain the same orientation on the Parafilm as in the 24-well plate.
8. Using a hypodermic needle and forceps, remove coverslips carefully from the wells one at a time.
9. Invert the coverslips individually on top of the spots of primary antibody, ensuring that the entire cell-covered surface is immersed. Cover Petri dish with a lid and incubate at room temperature for of 1 hour.
10. Return the coverslips to the appropriate wells of the 24-well plate, taking care to invert each one so that the cell-covered surface is facing up. Wash three times with PBS.
11. Replace the piece of Parafilm from the Petri dish with a new one. Add 50 µL of fluorophore-conjugated secondary antibody diluted 1:2000 in blocking buffer in spots on the Parafilm. Try to maintain the same orientation on the Parafilm as in the 24-well plate.
12. Using a hypodermic needle and forceps, remove coverslips carefully from the wells one at a time.
13. Invert the coverslips individually on top of the spots of secondary antibody, ensuring that the entire cell-covered surface is immersed. Cover Petri dish with a lid and incubate at room temperature for of 1 hour in the dark.
14. Return the coverslips to the appropriate wells of the 24-well plate, taking care to invert each one so that the cell-covered surface is facing up. Wash three times with PBS.
15. Place 10 µl of mounting medium (Life Technologies ProLong Gold Antifade Reagent with DAPI) on the surface of a microscope slide.
16. Using a hypodermic needle and forceps, remove coverslips carefully from the wells one at a time. Invert the coverslips individually on top of the spots of mounting medium ensuring that the entire cell-covered surface is immersed. Store the slide in the dark at 4°C until imaging.

11 SOP 11 - TRANSMISSION ELECTRON MICROSCOPY FOR AXENIC CULTURES OF *CRYPTOSPORIDIUM*

1. Suspend 1 x 10^8 oocysts in excystation buffer (0.75% sodium taurocholate/0.025% trypsin in PBS) and incubate for 1 h at 37°C. Centrifuge excysted sporozoites at 2,000 x *g*, wash 3X in PBS, and count with a hemacytometer.
2. Inoculate poly-L-lysine coated 6-well culture plates with 1 x 10^7 sporozoites per well using three different media formulations: RPMI, RPMI plus supplements, SFM, and SFM plus supplements.
3. Incubate plates at 37°C under 5% CO_2 for 24h, 48h, and 72h. At each time point, scrape adherent cells from the wells and centrifuge the suspension at 2,000 x *g*.
4. Resuspend the pellet in 0.5 ml of culture medium. Add an equal amount of fixative consisting of 8% paraformaldehyde and 2% glutaraldehyde in 0.12 M Na cacodylate buffer, pH 7.3-7.4.
5. Fix the cells for 30 min at room temperature.
6. Centrifuge the fixed cell suspension at 2,000 x *g*. Cell pellets may be stored at 4°C until ready to process for TEM.
7. Remove fixative solution from the cell pellet. Add a drop of low melting point agarose and gently swirl the pellet into the agarose. Place tube on ice to solidify. Remove agarose and cut into 1 mm cubes.
8. Rinse agarose cubes 3X with 0.12 M Na cacodylate buffer for 10 min each.
9. Post-fix with 1% osmium tetroxide in 0.12 M Na cacodylate for 2 h at room temperature.
10. Wash the samples 3X in 0.1 M Na cacodylate buffer for 10 min each.
11. En bloc stain the samples with 1% aqueous uranyl acetate overnight at room temperature.
12. Wash the samples in distilled water 3X for 10 min each.
13. Dehydrate the samples through a series of dilutions of graded ethyl alcohol:

35% ETOH	3X 15 min
50% ETOH	3X 15 min
70% ETOH	3X 15 min
95% ETOH	3X 15 min
100% ETOH	3X 15 min

14. After the last step, treat samples with 100% propylene oxide (PO) 3X 15 min
15. Perform Epoxy resin infiltration of the samples:
2:1 mix of PO and Embed812 resin 2 h
1:1 mix of PO and Embed812 resin overnight
1:2 mix of PO and Embed812 resin 2 h
100% resin for a minimum of 4 h
16. Embed samples in block molds.
17. Place samples in the 60°C oven for 48 h
18. Cut ultrathin sections of 100 nm and post-stain with uranyl acetate and lead citrate.

19. Image the grids in a FEI TALOS transmission electron microscope at 80 KV on a 4K x 4K CETA camera.

NOTES:

- Use polypropylene tubes for mixing the resin.
- Use disposable pipettes for solution exchanges and proper waste containers for all of the chemicals.
- Use the "hard" resin recipe for the EmBed812 epoxy resin.
- Use agitation/mixing/shaking through all the steps for better solution exchange in the sample.

Recommended source for all TEM reagents: Electron Microscopy Sciences
www.emsdiasum.com/

12 SOP 12 - TRANSMISSION ELECTRON MICROSCOPY FOR *CRYPTOSPORIDIUM PARVUM*-INFECTED INTESTINAL EPITHELIAL CELLS GROWING ON POLYCARBONATE MEMBRANE SUPPORTS (TRANSWELLS)

1. Remove the transwell with sterile forceps, aspirate the culture medium, and transfer to a new 6-well plate.
2. Add 0.5 ml of fresh growth medium to the top chamber and 1.5 ml of growth medium to the bottom chamber of the transwell.
3. Add 0.5 ml of fixative to the upper chamber and 1.5 ml of fixative to the bottom chamber of the transwell. The fixative consists of 8% paraformaldehyde and 2% glutaraldehyde in 0.12 M Na cacodylate buffer, pH 7.3-7.4.
4. Fix the cells for 30 min at room temperature.
5. Carefully scrape the cells from the polycarbonate membrane and transfer the contents from both chambers to a 15 ml polypropylene centrifuge tube.
6. Centrifuge the fixed cell suspension at 2,000 x *g*. Cell pellets may be stored at 4°C until ready to process for TEM.
7. Remove fixative solution from the cell pellet. Add a drop of low melting point agarose and gently swirl the pellet into the agarose. Place tube on ice to solidify. Remove agarose and cut into 1 mm cubes.
8. Rinse agarose cubes 3X with 0.12 M Na cacodylate buffer for 10 min each.
9. Post-fix with 1% osmium tetroxide in 0.12 M Na cacodylate for 2 h at room temperature.
10. Wash the samples 3X in 0.1 M Na cacodylate buffer for 10 min each.
11. En bloc stain the samples with 1% aqueous uranyl acetate overnight at room temperature.
12. Wash the samples in distilled water 3X for 10 min each.
13. Dehydrate the samples through a series of dilutions of graded ethyl alcohol:
35% ETOH 3X 15 min
50% ETOH 3X 15 min
70% ETOH 3X 15 min
95% ETOH 3X 15 min
100% ETOH 3X 15 min
14. After the last step, treat samples with 100% propylene oxide (PO) 3X 15 min
15. Perform Epoxy resin infiltration of the samples:
2:1 mix of PO and Embed812 resin 2 h
1:1 mix of PO and Embed812 resin overnight
1:2 mix of PO and Embed812 resin 2 h
100% resin for a minimum of 4 h
16. Embed samples in block molds.
17. Place samples in the 60°C oven for 48 h
18. Cut ultrathin sections of 100 nm and post-stain with uranyl acetate and lead citrate.

19. Image the grids in a FEI TALOS transmission electron microscope at 80 KV on a 4K x 4K CETA camera.

NOTES:

- Use polypropylene tubes for mixing the resin.
- Use disposable pipettes for solution exchanges and proper waste containers for all of the chemicals.
- Use the “hard” resin recipe for the EmBed812 epoxy resin.
- Use agitation/mixing/shaking through all the steps for better solution exchange in the sample.

Recommended source for all TEM reagents: Electron Microscopy Sciences
www.emsdiasum.com/