

Long-term *in vitro* Culture of *Cryptosporidium parvum*

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[Abstract] Continuous *in vitro* growth of *Cryptosporidium parvum* has proved difficult and conventional *in vitro* culture techniques result in short-term (2-5 days) growth of the parasite resulting in thin-walled oocysts that fail to propagate using *in vitro* cultures, and do not produce an active infection using immunosuppressed or immunodeficient mouse models (Arrowood, 2002). Here we describe the use of hollow fiber bioreactors (HFB) that simulate *in vivo* conditions by providing oxygen and nutrients to host intestinal cells from the basal surface and permit the establishment of a low redox, high nutrient environment on the apical surface. When inoculated with 10⁵ *C. parvum* (Iowa isolate) oocysts the bioreactor produced 10⁸ oocysts per ml (20 ml extra-capillary volume) after 14 days, and was maintained for over 2 years. *In vivo* infectivity studies using a TCR- α -immune deficient mouse model showed that oocysts produced from the bioreactor at 6, 12 and 18 months were indistinguishable from the parent Iowa isolate used to initiate the culture. HFB produced oocysts had similar percent excystation profiles to the parent Iowa isolate.

Keywords: Parasitology, Cell culture, Protozoans, Cryptosporidia, *Cryptosporidium parvum*, Hollow fiber bioreactor

[Background] *Cryptosporidium parvum* is an intracellular obligate parasite of the intestinal tract of man and other mammals resulting in an acute diarrhea. The disease is self-limiting in immunocompetent individuals, however, in immunocompromised adults and young children, the disease can be life-threatening (Kotloff, 2017). It is amongst the top three diagnosed enteric diseases of children in economically low resource countries (Kotloff *et al.*, 2013; Sow *et al.*, 2016), and is estimated to account for 9% of child deaths globally (Bhutta and Black, 2013; Checkley *et al.*, 2015). In countries with a high burden of pediatric diarrheal disease, it has been shown that there is a correspondingly high incidence of malnutrition, stunting, and impaired cognitive functions (Lang and MA-LED Network Investigators, 2015). Despite the significant health risks and global distribution of this disease, there is no consistently effective therapy for the most-at-risk population. Recent advances in manipulating the parasite genome (Vinayak *et al.*, 2015) and chemotherapeutic profiling (Arnold *et al.*, 2017; Love *et al.*, 2017; Manjunatha *et al.*, 2017) are an encouraging sign that this bleak situation will change. Culture of the parasite has concentrated on developing a 3D culture system using adult murine colon cells (Baydoun *et al.*, 2017) or novel bioengineered human intestinal cells (DeCicco RePass *et al.*, 2017) 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* (Morada *et al.*, 2016). 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 (Figure 1). The environment inside the reactor is adjusted to mimic the lumen of the gut hence the apical surface of the intestinal cells are 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 and has the advantage of avoiding the use of harsh chemicals such as K-dichromate, which is used as a long-term storage medium at (4 °C) and has the advantage of sanitizing the oocysts; and chlorine currently used as both a sanitizer and to enhance excystation of oocysts obtained from animal sources (Arrowood, 2008).

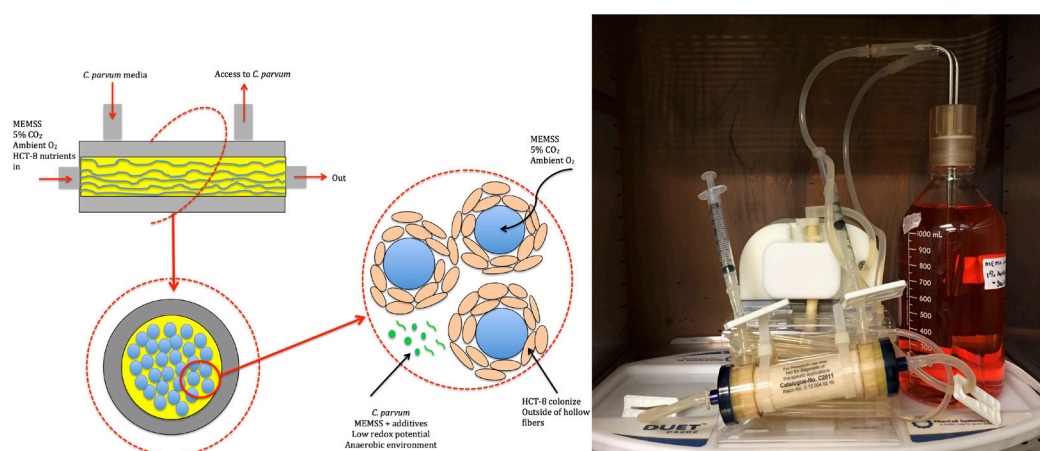


Figure 1. Hollow Fiber Bioreactor. A. Schematic representation of the HFB; B. HFB setup.

Materials and Reagents

1. BrandTech™ BRAND™ reagent reservoirs (BrandTech Scientific, catalog number: 703459)
2. Aluminum foil 18 in x 500 ft (Sigma-Aldrich, catalog number: Z185159-1EA)
3. BD syringe with various tips, 60 ml (BD, catalog number: 309653)
4. BD Diagnostic Systems SYR only Luer-Lok™ 10 ml 200PK RX (Thermo Fisher Scientific, catalog number: B302995)

Manufacturer: BD, catalog number: 302995.

5. BD Disposable syringes with Luer-Lok™ Tips, 3 ml (BD, catalog number: 309657)
6. Fisherbrand™ sterile alcohol prep pads (Fisher Scientific, catalog number: 22-363-750)
7. BD Vacutainer™ general use syringe needles (BD, catalog number: 305180)
8. Fisherbrand™ borosilicate glass disposable serological pipets with regular tip, standard length, 5 ml (Fisher Scientific, catalog number: 13-678-27E)
9. Fisherbrand™ borosilicate glass disposable serological pipets with regular tip, standard length, 10 ml (Fisher Scientific, catalog number: 13-678-27F)
10. Fisherbrand™ borosilicate glass disposable serological pipets with regular tip, short length, 25 ml (Fisher Scientific, catalog number: 13-678-36D)
11. EMD Millipore™ Millex™-GP sterile syringe filters with PES membrane (Fisher Scientific, catalog number: SLGP033RS)
Manufacturer: Merck, catalog number: SLGP033RS.
12. Corning™ polyethylene terephthalate (PET) centrifuge tubes, 15 ml (Corning, catalog number: 430055)
13. CELLSTAR™ Greiner Bio-One™ TC treated cell culture flasks, 250 ml (Greiner Bio One International, catalog number: 658175)
14. Fisherbrand™ premium microcentrifuge tubes, 1.5 ml (Fisher Scientific, catalog number: 05-408-129)
15. Fisherbrand™ low-retention pipet tips–filtered, 10 µl (Fisher Scientific, catalog number: 02-717-158)
16. Fisherbrand™ low-retention pipet tips–filtered, 20 µl (Fisher Scientific, catalog number: 02-717-161)
17. Fisherbrand™ low-retention pipet tips–filtered, 200 µl (Fisher Scientific, catalog number: 02-717-165)
18. Quibit™ assay tubes (Thermo Fisher Scientific, Invitrogen™, catalog number: Q32856)
19. MicroAmp™ optical 96-well reaction plate with barcode (Thermo Fisher Scientific, Applied Biosystems™, catalog number: 4306737)
20. MicroAmp™ optical adhesive film (Thermo Fisher Scientific, Applied Biosystems™, catalog number: 4311971)
21. Filters, bottle top 1 L (Thermo Fisher Scientific, catalog number: 597-4520)
22. Dynabeads™ MPC™-S magnetic particle concentrator (Thermo Fisher Scientific, catalog number: A13346)
23. Dynabeads™ MPC™-6 magnetic particle concentrator (Thermo Fisher Scientific, catalog number: 12002D)
24. Dynabeads™ L10 tubes (Thermo Fisher Scientific, catalog number: 74003)
25. VWR® 8-425 screw thread vials (VWR, Avantor, catalog number: 66020-950)
26. Cap screw top black, with PTFE/silicone pre-slit septa, 10 mm, packed in a clean environment (PerkinElmer, catalog number: N9306052)
27. *C. parvum* 18S-rRNA primers: Cp18S-995F: 5'-TAGAGATTGGAGGTTCCCT-3' and Cp18S-

- 1206R: 5'-CTCCACCAACTAAGAACGCC-3' (Thermo Fisher Scientific, Waltham, MA, USA)
28. Human 18S-rRNA primers: Hs18S-F1373: 5'-CCGATAACGAACGAGACTCTGG-3' and Hs18S-R1561: 5'-TAGGGTAGGCACACGCTGAGCC-3' (Thermo Fisher Scientific, Waltham, MA, USA)
29. Quant-iT™ Qubit RNA BR assay kit (Thermo Fisher Scientific, Invitrogen™, catalog number: Q10211)
30. RNeasy mini kit, 250 (QIAGEN, catalog number: 74106)
31. RNase-free DNase set, 50 (QIAGEN, catalog number: 79254)
32. iScript™ RT-qPCR sample preparation reagent (Bio-Rad Laboratories, catalog number: 1708898)
33. Luna® Universal one step RT-qPCR kit protocol (New England BioLabs, catalog number: E3005)
34. Sterile phosphate buffered saline (Thermo Fisher Scientific, Gibco™, catalog number: 10010049)
35. Minimum Essential Medium (MEM) with L-glutamine and phenol red, without HEPES (Thermo Fisher Scientific, Gibco™, catalog number: 11095-114)
36. Horse Serum, New Zealand origin (Thermo Fisher Scientific, Gibco™, catalog number: 16050122)
37. UltraPure™ DNase/RNase-free distilled water (Thermo Fisher Scientific, Invitrogen™, catalog number: 10977015)
38. Ethanol, 70% (Sigma-Aldrich, catalog number: 1009744000)
Manufacturer: Merck, catalog number: 100974.
39. Roche Diagnostics™ glucose test strips for Accutrend® Plus meters (Fisher Scientific, catalog number: 22-045-871)
Manufacturer: Roche Diagnostics, catalog number: 11447475160
40. Crypt-a-Glo™ reagent only kit (Waterborne, catalog number: A400FLR-20)
41. Sporo-Glo™ (Waterborne, catalog number: A600FLR-20X)
42. Trypsin-EDTA (0.25%), phenol red (Thermo Fisher-Scientific, Gibco™, catalog number: 25200056)
43. L-glutamine (Sigma-Aldrich, catalog number: G3126)
44. HEPES (Sigma-Aldrich, catalog number: H3375)
45. D-glucose (Sigma-Aldrich, catalog number: G8270)
46. Ascorbic acid (Sigma-Aldrich, catalog number: A0278)
47. P-Aminobenzoic acid (Sigma-Aldrich, catalog number: A9878)
48. Calcium pantothenate (Sigma-Aldrich, catalog number: C8731)
49. Folic acid (Sigma-Aldrich, catalog number: F7876)
50. Heparin (Sigma-Aldrich, catalog number: H3393)
51. Antibiotic/antimycotic 100x (Thermo Fisher Scientific, catalog number: 15240062)
52. Taurodeoxycholate (Sigma-Aldrich, catalog number: T0557)
53. Thioglycolic acid (MP Biomedicals, catalog number: 102933)

54. Mannitol (Sigma-Aldrich, catalog number: M4125)
55. Glutathione (Sigma-Aldrich, catalog number: G6013)
56. Taurine (Sigma-Aldrich, catalog number: T8691)
57. Betaine hydrochloride (Sigma-Aldrich, catalog number: B3501)
58. Cysteine (Sigma-Aldrich, catalog number: C7352)
59. Oleic acid (Sigma-Aldrich, catalog number: O1257-10MG)
60. Cholesterol (Sigma-Aldrich, catalog number: C4951-30MG)
61. Dynabeads™ anti-*Cryptosporidium* (Thermo Fisher Scientific, catalog number: 73011)
62. Nitrogen Medical Grade 99.998% purity (TW Smith, Brooklyn, NY)
63. Carbon dioxide, Bone Dry, 99.998% (TW Smith, Brooklyn, NY)
64. ECS medium mix (see Recipes)
65. ICS medium mix (see Recipes)

Equipment

1. 1 L Bottles, 33 mm cap (Fisher Scientific, catalog number: 0642114)
Manufacturer: DWK Life Sciences, catalog number: 61111T1000.
2. 125 ml Bottles, 33 mm cap (DWK Life Sciences, catalog number: 219755)
3. Roche Diagnostics™ glucose controls for Accutrend® Plus meters (Fisher Scientific, catalog number: 22-045-733)
Manufacturer: Roche Diagnostics, catalog number: 05213231160.
4. Hausser™ Levy™ hemacytometer chamber set (Hausser Scientific, catalog number: 3520)
5. FiberCell™ Systems Duet Pump (FiberCell Systems, catalog number: P3202)
6. Hollow Fiber Medium Cartridge (FiberCell Systems, catalog number: C2011)
7. UVP UV2 Sterilizing PCR Workstation (VWR, Avantor, Bridgeport, NJ 08014, USA)
8. Class II, Type A, Biohazard Cabinet, model number 10276 (Enviroco Cedar Grove, NJ 07009, USA)
9. Beckman Coulter Microfuge® 16 centrifuge (Beckman Coulter, model: Microfuge® 16)
10. Eppendorf™ centrifuge 5810R (Eppendorf, model: 5810 R)
11. Quant Studio 6 Flex 44 (Applied Biosystems, Life Technology, Thermo Fisher Scientific, Waltham, MA, USA)
12. Thermo Scientific Revco Ultima CO₂ incubator (Thermo Fisher Scientific, Waltham, MA, USA)
13. Dynabeads™ MX mixer base (Thermo Fisher Scientific, catalog number: 15902)
14. Qubit™ 3.0 fluorometer (Thermo Fisher Scientific, catalog number: Q33216)
15. Nikon™ Optiphot fluorescence microscope (Nikon, Westbury, NY)
16. Fisher Scientific Accumet® AR10 pH benchtop meter (Fisher Scientific, catalog number: 13-636-AB150)
17. Fisher Scientific dry bath incubator (Fisher Scientific, catalog number: 11-718-2)
18. Autoclave (LBR Scientific, Rutherford, NJ)

Software

1. Quant Studio RT-PCR software v1.0 (Applied Biosystems, Life Technology, Thermo Fisher Scientific, Waltham, MA, USA)
2. Sigma Plot v2001 (Systat Software, Inc, San Jose, CA, USA)

Procedure

A. Cartridge preparation

1. Sterilization of Reservoir Cap/Bottle Assembly
 - a. Insert a 33 ml reservoir cap into the 125 ml bottle. Fit screw cap loosely.
 - b. Attach fresh reservoir cap tube (non-sterile), included with the cartridge, to the cap.
 - c. Loosely wrap the Luer fittings on the end of the reservoir cap tubing with foil.
 - d. Place entire assembly into an autoclave bag and seal.
 - e. Autoclave at 121 °C, 15 lb/in² for 20 min (High Vacuum).
2. Connection of the reservoir cap to the Flow Path
 - a. Remove Fibercell module from its packaging. Check the Luer fittings between the hollow fiber module and the flow path to ensure that they are finger tight.
 - b. Remove the foil from one of the Luer fittings on the reservoir cap.
 - c. Choose one of the Luer fittings on the flow path. Spray with 70% ethanol and wipe with an alcohol pad.
 - d. Remove the Luer cap from the Luer fitting and attach the Luer fitting to the 33 ml reservoir cap. Apply ½ turn counter rotation to the tubing prior to attachment to prevent kinking of the tubing.
 - e. Repeat steps to attach the other Luer fitting.
 - f. Check fitting and be sure they are tightly attached.

B. Pre-culture

When working with adherent cells, the cartridge should be washed with 2-volumes of 125 ml of sterile PBS each for 24 h prior to filling the cartridge with 125 ml of cell culture medium.

1. Ensure the left and right end port side clamps are open. Aseptically fill 100 ml of sterile PBS into 125 ml bottle attach to cartridge using the 33 mm caps (The flow path and cartridge for medium cartridge holds about 30 ml of media).
2. Use thumb and forefinger to manually prime the pump until air is pushed out of the tubing then close the left and right end port side clamps.
3. Fill a sterile 60 ml syringe with 30 ml sterile PBS and attach to the left extra-capillary space (ECS) port and attach an empty 60 ml syringe to the right ECS port.
4. 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.
5. 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.
 6. Connect the Fibercell hollow fiber module to the Fibercell pump unit in a 37 °C 5% CO₂ incubator. Set a medium flow rate of 5, leave for 24 h.
 7. 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.
 8. Aseptically fill a sterile 125 ml reservoir bottle with 100 ml of MEM media. Leave the reservoir cap loose by ½ turn.
 9. Fill a sterile 60 ml syringe with 30 ml sterile MEM and attach to the left ECS port and attach an empty 60 ml syringe to the right ECS port.
 10. 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
 11. 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.
 12. Connect the Fibercell hollow fiber module to the Fibercell pump unit in a 37 °C, 5% CO₂ incubator. Set a medium flow rate of 5 and leave overnight.
 13. Repeat Steps B8-B12 using MEM plus 10% horse serum.

C. Inoculating Cells into the ECS (refer to Video 1)



Video 1. Procedure for loading either host cells or *C. parvum* into the HFB

1. Close the left and right end port slide clamps.
2. HCT-8 monolayers are trypsinized using Trypsin-EDTA (0.25%), phenol red, washed with MEM

and resuspended 1×10^6 in a total volume of 5 ml of ICS mix (see Recipe 2), and placed into a trough.

3. Spray the left side port Luer connection with 70% ethanol and wipe with an alcohol pad. Remove syringes with an alcohol pad.
4. Draw up cells using a 10 ml syringe with a blunt end needle.
5. Following same aseptic procedure attach an empty syringe to the right side port Luer fitting.
6. If closed, open the side port clamps.
7. Gently flush the cell suspension through the ECS back and forth between the 2 syringes 3-5 times. This ensures distribution of the cells.
8. Loosen the reservoir cap $\frac{1}{2}$ turn.
9. Leave the left end port slide clamp closed. Open the right-hand end port slide clamp. This will allow excess medium to flow into the reservoir bottle.
10. Close the right side port slide clamp.
11. Gently depress the plunger of the syringe attached to the left side port until all the medium and cells have been forced into the ECS.
12. Close the left side port slide clamp and open the right side port slide clamp.
13. Gently depress the plunger of the syringe attached to the right side port until all the medium and cells have been forced into the ECS.
14. Close the side port slide clamps. Replace the syringes with new sterile syringes.
15. Tighten the reservoir cap.
16. Open the left end port slide clamp.
17. Place the cartridge into the Fibercell pump inside a 37 °C, 5% CO₂ incubator and set flow rate to 5. After 3 days, increase the flow rate to 10.

D. Addition of *C. parvum* oocysts

Fresh MEM plus 10% HS is replaced daily. The pH and glucose concentration of the 48 h and fresh medium are monitored in the ECS using an Accumet® pH meter and an Accutrend Plus® glucose meter. When the glucose drops by 50% in 24 h, the ECS is seeded with 1×10^5 *C. parvum* oocysts that have been treated with 10% Chlorox®, washed 7x each with 30 ml sterile distilled water and suspended in 10 ml of MEM containing the supplements shown in Recipe 1 (ECS mix). The intra-capillary space (ICS) medium is replaced with that shown in Recipe 2 (ICS mix).

1. Remove the unit to a sterile hood and slide the L and R end port clamps to closed.
2. Spray the left and right ECS ports with 70% alcohol using an alcohol pad and remove the syringe from the left ECS port.
3. Attach the syringe containing the 10 ml of *C. parvum* oocysts suspended in the ECS mix to the left ECS port.
4. Attach a clean sterile 10 ml syringe to the right ECS port.
5. Open the slide clamps to the left and right ECS ports. Push down on the syringe attached to the left port (containing *C. parvum* oocysts), gently pull up on the right syringe.

6. When the left syringe is fully depressed, push down on the right syringe to mix the contents of the ECS.
7. Repeat the procedure (Steps 5 and 6) 3-4 times end up with both the left and right syringe approximately 50% full (5 ml in each).
8. Close the right ECS port clamp, open the right end port slide clamp. Push down on the syringe attached to left ECS port until the contents have been added to the ECS (Take care not to add air to the cartridge). Slide the left ECS port clamp closed. Open the right ECS slide clamp and push down on the syringe until the contents of the syringe attached to this port have been added (Take care not to add air to the cartridge). Slide the right ECS port clamp closed. 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 presumably due to build-up of the host-cell layer and debris from sloughed cells. When using the 3 ml ECS cartridge after a year of continuous culture, the backpressure is significant and it is not recommended for studies beyond this point. The 20 ml ECS cartridge was maintained for over 2 years without encountering this problem and is the cartridge of choice for long-term studies.
9. Slide the left end port clamp to open. Spray the left and right ECS ports with 70% alcohol and use an alcohol pad to remove the syringes and replace with clean, sterile 10 ml syringes.
10. Ensure both left and right end port clamps are closed and replace the reservoir bottle with 125 ml of the complete ICS medium mix (Recipe 2). Open both left and right end ports and return the cartridge to the pump. Set the flow rate to 10.
11. The pH and glucose in the ICS medium are monitored daily, by removing 5 ml directly from the reservoir bottle. When the glucose concentration falls from 360 mg/dl to approx. 200 mg/dl in 24 h, change the reservoir for a 500 ml bottle of the complete ICS medium mix, containing 460 mg/dl of glucose.
12. Continue to monitor the pH and glucose, and when the glucose concentration falls to 50% (200-230 mg/dl) in 24 h, change the reservoir for a 1 L bottle of the complete ICS medium mix, containing 500 mg/dl glucose.
13. The pH and glucose are monitored every 48 h for the duration of the culture as an indicator of the integrity of the host cell layer.

E. Analysis of *C. parvum* growth by changes in *Cp18S-rRNA*

RNA Preparation:

1. Centrifuge a 50 μ l sample, discard the supernatant and add 100 μ l of iScript Buffer (lysis buffer).
2. Freeze/thaw 6x by immersing in liquid nitrogen for 1 min followed by rapid thaw at 70 °C for 1.5 min.
3. Centrifuge the lysate for 5 min at 16,162 \times g (14,800 rpm).
4. Carefully transfer the supernatant using a 200 μ l pipette (DO NOT TOUCH THE PELLETT) to a clean tube.

5. Add 1 volume of 70% ethanol to the lysate, and mix well by pipetting. Do not centrifuge.
6. Transfer up to 700 μ l of the sample, including any precipitate, to an RNeasy Mini spin column placed in a 2 ml collection tube.
7. Centrifuge for 15 sec at 8,000 \times *g* (10,412 rpm). Discard flow through.
8. Add 350 μ l of Buffer RW1 to the column. Centrifuge for 15 sec at 8,000 \times *g* (10,412 rpm).
9. Add 10 μ l DNase I stock solution to 70 μ l buffer RDD. Mix gently by inverting the tube. Centrifuge for 15 sec at 8,000 \times *g* (10,412 rpm).
10. Add DNase I incubation mix (80 μ l) from Step E9 to the column membrane and place on the benchtop for 15 min
11. Add 350 μ l buffer RW1 to the column. Centrifuge for 15 sec at 8,000 \times *g* (10,412 rpm). Discard flow through.
12. Add 500 μ l buffer RPE to the column. Centrifuge for 15 sec at 8,000 \times *g* (10,412 rpm) to wash the membrane. Discard flow through.
13. Add 500 μ l buffer RPE to the column. Centrifuge for 2 min at 8,000 \times *g* (10,412 rpm). Discard flow through
14. Place the column in a new 1.5 ml collection tube. Add 30-50 μ l of RNase free water directly to the column membrane. Centrifuge for 1 min at 8,000 \times *g* (10,412 rpm) to elute the RNA. Label tubes with sample description, date, and amount of water added.
15. Either quantitate RNA immediately or store samples at -80 °C.
16. RNA is quantitated using the broad range Qubit RNA assay kit using a Qubit 3.0 fluorometer according to the manufacturer's instructions.
17. Enumerate *C. parvum* by quantitating *Cp18S*-rRNA in HFB samples and compare it to a standard prepared using 10^6 , 10^7 and 10^8 *C. parvum* oocysts. Carry out the procedure using 5 ng of RNA with Luna Universal One-Step[®] RT-qPCR kit (NEB E3005) employing *C. parvum* 18S-rRNA primers: (*Cp18S*-995F: 5'-TAGAGATTGGAGGTTCCCT-3' and *Cp18S*-1206R: 5'-CTCCACCAACTAAGAACGCC-3') and compare it to human 18S-rRNA primers: (*Hs18S*-F1373: 5'-CCGATAACGAACGAGACACTCTGG-3' and *Hs18S*-R1561: 5'-TAGGGTAGGCACACGCTGAGCC-3'). Perform the procedure according to the manufacturer's instructions using a Quant Studio 6 Flex 44.
18. The HFB reproducibly produces 5×10^7 to 10^8 oocysts per ml after 2 weeks of culture, and this is maintained if not more than 50% of the ECS volume is sampled every 7-10 days. Sampling 90% of the cartridge volume at one time resulted in 4-week recovery time to obtain 10^8 oocysts per ml.
19. Stain oocysts with Crypt-o-Glo[®] according to the manufacturer's instructions and evaluate using a Nikon[™] Optiphot fluorescence microscope.
20. Stain motile stages with Spor-a-Glo[®] according to the manufacturer's instructions and evaluate using a Nikon[™] Optiphot fluorescence microscope.

F. Tips to aid reproducibility

1. Keep the inlet and outlet lines to the reservoir bottle behind the line used to prime the pump, this prevents kinking of the lines and reduces the chance of contamination when handling the cartridge.
2. Use an alcohol pad to wrap around fittings when changing the syringes prevents contamination.
3. If the glucose concentration of the reservoir bottle does not drop significantly after 48 h, replenish the host cells by adding 10^6 HCT-8 cells to the extra-capillary space.
4. To achieve the consistent growth of *C. parvum*, it is important to maintain a low redox balance of the extra-capillary medium.

Note: To achieve this, we prepared the redox mix using sterile distilled water that is boiled and purged with 0.4 μm filtered N_2 gas. When cooled to ambient temperature, thiols are dissolved under an N_2 atmosphere.

5. Removal of 50% (10 ml) of the culture is possible by displacing the extra-capillary medium with an equal volume of fresh ECS medium. When displacing the cells depress the syringe gently (10 sec with a 10 ml syringe) so as not to cause turbulence. It is advised that the culture not be disturbed for 10-14 days post-sampling to enable repopulation of the culture.

Data analysis

A. Growth profile of total parasite stages from the HFB

1. Samples (0.5 ml) were removed from the HFB and RNA isolated and analyzed by qRT-PCR using specific primers for *C. parvum* 18S-rRNA (Cp18S-995F: 5'-TAGAGATTGGAGGTTCCCT-3' and Cp18S-1206R: 5'-CTCCACCAACTAAGAACGCC-3') and compared with HCT-8 18S-rRNA (Hs18S-F1373: 5'-CCGATAACGAACGAGACACTCTGG-3' and Hs18S-R1561: 5'-TAGGGTAGGCACACGCTGAGCC-3') to enumerate parasite number as described by Zhang and Zhu (2015). Parasite numbers increased from $5 \times 10^3/\text{ml}$ on day one to $5 \times 10^8/\text{ml}$ by Day 15 (Figure 2). Using Dynabeads to purify oocysts from the HFB sample we have obtained 10^9 oocysts from 10 ml of culture volume.

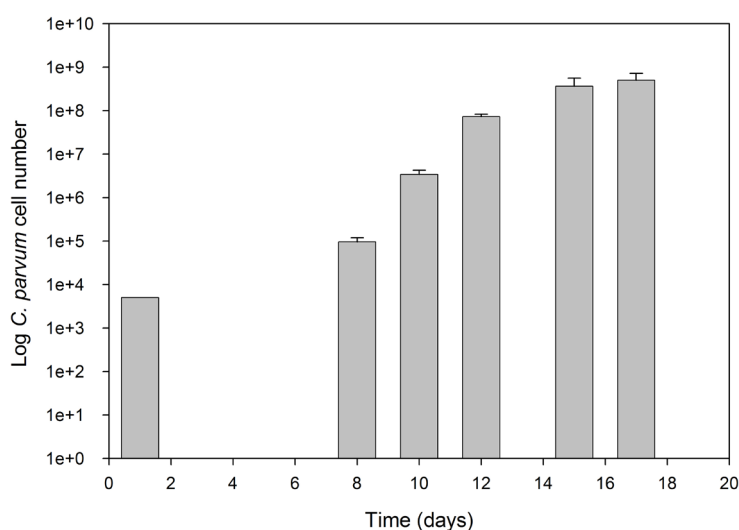


Figure 2. *C. parvum* growth curve in the HFB

- C. parvum* numbers are shown as Log of total parasite stages per ml. Parasite numbers were determined by qRT-PCR using primers for the Cp-18SrRNA as described in the procedure and the ΔC_T values determined from a standard using 10^3 , 10^4 and 10^5 oocysts (Zhang and Zhu, 2015). The culture was inoculated on day 1 with 5×10^3 oocysts/ml (10^5). Results are presented as triplicate counts \pm SEM.
- Oocysts were stained with a monoclonal antibody to the *Cryptosporidium* oocyst wall protein conjugated to a fluorescent probe (Crypt-a-Glo[®]) and examined using a fluorescence microscope with an excitation wavelength of 410-485 nm and an emission wavelength of 515 nm (Figure 3A). Motile extracellular stages were stained using a polyclonal antibody conjugated to a red fluorescent probe (Sporo-Glo[®]) and examined using a fluorescent microscope with an excitation wavelength of 535-550 nm and an emission wavelength of 580 nm (Figure 3B).

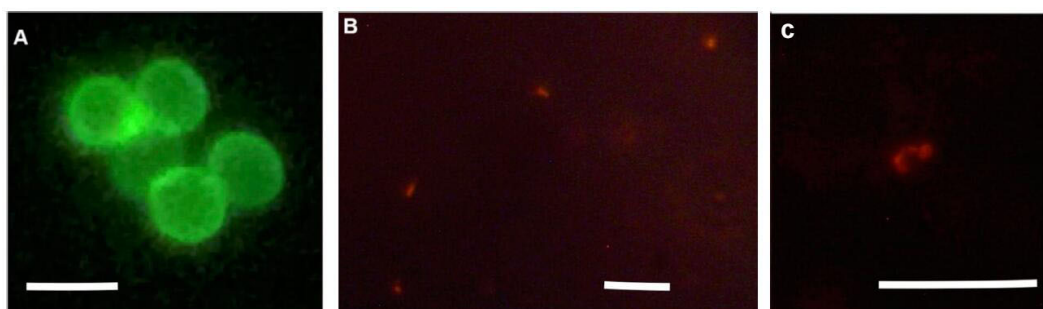


Figure 3. Microscopy of parasites collected from the HFB. A. Oocysts from the HFB stained using Crypt-a-Glo[®]; B and C. Motile stages from the HFB stained using Sporo-Glo[®]. Scale bars = 5 μ m.

B. *In vivo* Infectivity of HFB produced *C. parvum*

Oocysts collected from the HFB culture were used to infect TCR- α -deficient mice to demonstrate

the maintenance of virulence factors. Parasites cultured in 2D culture flasks for 48 h fail to cause an infection in either immunosuppressed or immune-deficient mouse models. Oocysts were collected from the HFB and purified using Dyna beads[®] according to the manufacturer's instructions. Groups of three TCR- α -deficient mice on a C57BL/6 background (Jackson Laboratories, Bar Harbor, ME, USA) were orally dosed with 10^6 *C. parvum* oocysts (0.1 ml in PBS) from the HFB, and compared with 10^6 oocysts of the parent Iowa isolate (positive control) and mice dosed with 0.1 ml PBS (negative control). There is a statistical difference in weight gain of the *C. parvum* infected mice from 10 days onward compared to negative controls dosed with PBS (Figure 4A), indicative of an active *C. parvum* infection. Fecal pellets were collected and softened in PBS prior to smearing on microscope slides and staining by the modified cold Kinyoun acid-fast technique and enumerated microscopically at 400x using a confocal microscope (Nikon, Westbury, NY, USA). Oocysts were detected after 7-10 days and shedding increased daily up to 25 days (Figure 4B).

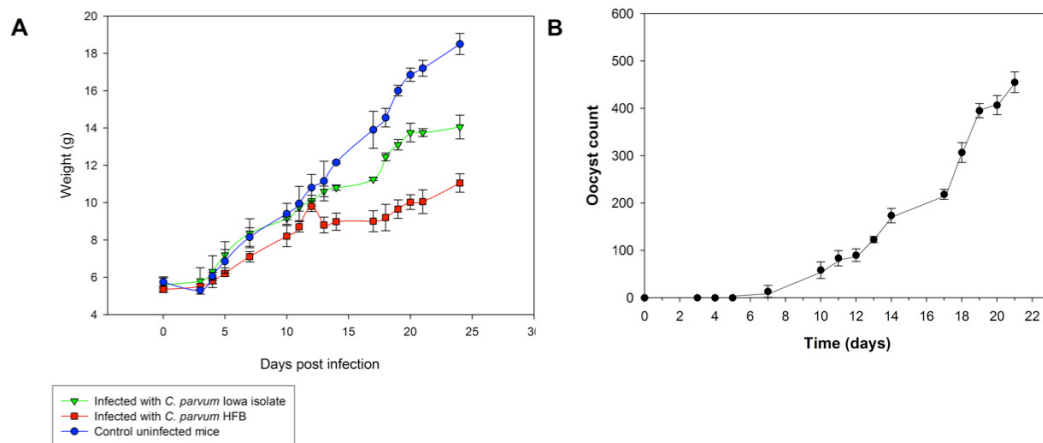


Figure 4. In vivo analysis of *C. parvum* oocysts from the HFB. A. Mouse model *C. parvum* infection. Triplicate groups of mice were infected with 0.1 ml by oral gavage containing 10^6 *C. parvum* oocysts suspended in PBS collected from the HFB ■, Six-week old *C. parvum* Iowa isolate (same source as used to initiate the culture) ▼, and compared to controls gavaged with 0.1 ml PBS ●. Mice were weighed daily. B. Oocyst shedding by HFB cultured mouse model infection. Mouse feces were softened with 0.1 ml distilled water and smeared onto a glass slide, stained using a modified cold Kinyoun acid-fast technique and oocysts counted microscopically at 400x magnification.

C. Excystation of oocysts collected from the HFB

The excystation rate of *C. parvum* oocysts from the HFB was compared to control Iowa isolate oocysts as an index of oocyst viability. *C. parvum* oocysts were incubated in PBS, pH 7.4 containing 150 mg of Na taurocholate and 50 mg of trypsin at 37 °C for 30 min and dual stained using Crypt-a-Glo[®] and Sporo-Glo[®]. The percent of excysted oocysts was determined by counting oocysts and sporozoites using a hemacytometer using a fluorescent microscope (Nikon Optiphot) at 400x magnification. Crypt-a-Glo[®] stained oocysts were visualized using an excitation wavelength of 410-

485 nm and an emission wavelength of 515 nm; Sporo-Glo[®] stained sporozoites (spz) were visualized using an excitation wavelength of 535-550 nm and an emission wavelength of 580 nm. The results show that approx. 80% of the oocysts collected from the HFB culture system excyst in 30 min, which is similar to the data obtained with the *C. parvum* parent Iowa isolate. Oocysts from both the HFB culture and the Iowa isolate stored at 4 °C demonstrated a gradual decrease in percent excystation with time of storage, falling to approx. 40% after 6 months (Figure 5).

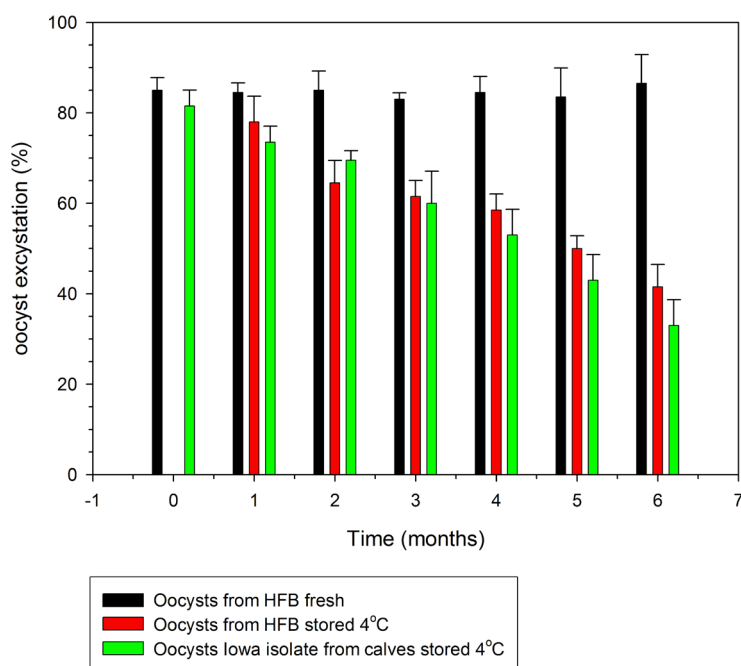


Figure 5. Excystation of *C. parvum* oocysts. Oocysts were excysted in PBS, pH 7.4 containing 150 mg of Na taurocholate and 50 mg of trypsin at 37 °C for 30 min. The percentage of excysted oocysts determined by dual staining with Crypt-a-Glo[®] and Sporo-Glo[®] and counting using an Improved Neubauer Hemacytometer. The percent excystation is calculated from the following formula: $\text{spz count}/4 \times \text{oocyst count} \times 100\%$.

D. Changes in glucose concentrations of HFB

The glucose concentration of the intra-capillary and extra-capillary space was measured as an indicator of host cell growth (Figure 6). When the medium glucose concentration falls to 50% of its starting concentration, the new medium must be replenished to prevent loss of host cells. The extra-capillary glucose concentration remains constant at approximately 275 mg/dl (Figure 6, dashed line).

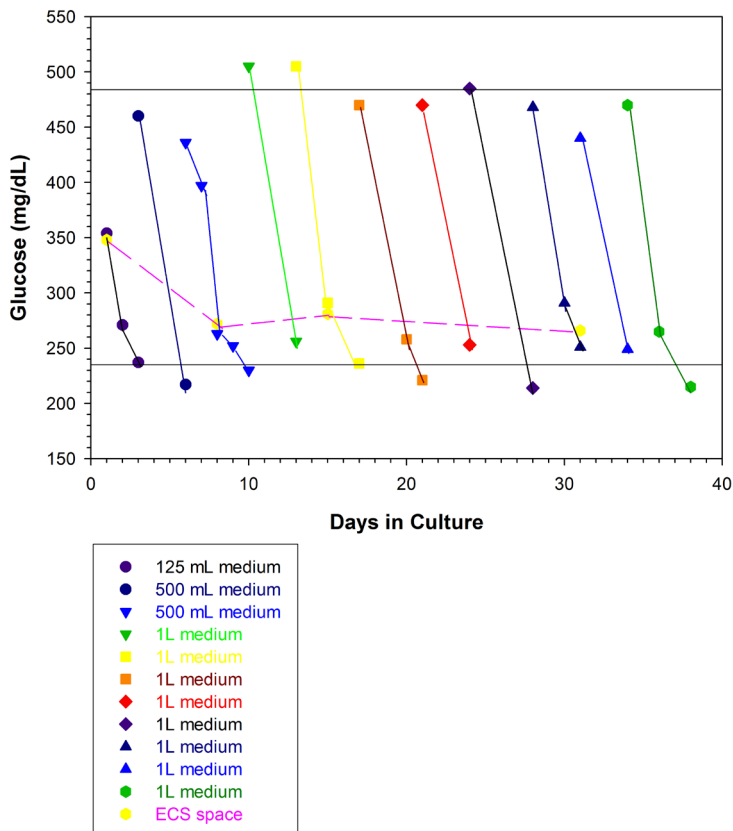


Figure 6. Glucose concentration of intra-capillary space medium. The glucose concentration of the medium providing nutrients to the host cells is monitored daily. The starting concentration is 350 mg/dl in a 125 ml flask which is changed to 425 mg/dl/500 ml flask on day 4. When the glucose concentration drops to 210-225 mg/dl in 48 h, (typically by Day 10) the glucose is increased to 475-500 mg/dl in a 1 L flask. The glucose concentration typically falls to 50% in 48 h, hence the medium is replenished on a Monday, Wednesday, Friday schedule thereafter. The glucose in the extra-capillary space, which contains the parasites, remains constant at 275 mg/dl.

Recipes

1. ECS medium mix

Supplement	Description	Stock (mg/10 ml)	Add to 10 ml MEM (ml)	Final conc. in ECS (mg)
conditioning mix (dissolved in MEM, pH 7.8)	TDC	150	9.0 total with all three supplements	135
	Thioglycolic Acid	5		4.5
	Mannitol	55		49.5
thiol mix* (aqueous)	Glutathione	200	0.15	3.0
	Taurine	200	0.15	3.0
	Betaine hydrochloride	200	0.15	3.0
	Cysteine	200	0.15	3.0
lipid mix# (aqueous)	Oleic Acid	67	0.2	1.34
	Cholesterol	180	0.2	3.6
			Total 10 ml	

Components are added and the medium is filter sterilized.

*Prepared using nitrogen degassed water and stored in 1 ml aliquots under a nitrogen gas phase at -20 °C.

#Stock kept at 4 °C

2. ICS medium mix

Supplement	Description	Final conc. in MEM (g/L)
medium base	MEM	
conditioning	heparin	0.058
	2 mM L-glutamine	0.29
buffer	100 mM HEPES pH 7.8 (5 M NaOH)	23.8
nutrient	50 mM D-glucose	4.5
vitamin mix	35 µg/ml ascorbic acid	0.035
	4 µg/ml PABA	0.04
	2 µg/ml Ca pantothenate	0.02
	1 µg/ml folic acid	0.001
antibiotic/antimycotic	10,000 units/ml penicillin	100,000 units
	10,000 µg/ml streptomycin	100,000 µg
	25 µg/ml amphotericin B	250 µg
serum	horse serum	

Components are added and the medium is filter sterilized.

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