

August 2024

Table of Contents

| 1. | 1. Standard Operation Procedure – Dead-end Ultrafiltration for the Detection of <i>Cyclospora</i> <i>cayetanensis</i> from Agricultural Water | | | | | |
|----|--|--|-----|--|--|--|
| | 1.1. | Dead-end Ultrafiltration | 2 | | | |
| | 1.2. | Recovery and Detection of Cyclospora cayetanensis from Ultrafilters | 6 | | | |
| | 1.3. | Cyclospora cayetanensis Real-Time PCR Detection Method | .15 | | | |
| 2. | Ap | ppendix 1. Sanitizing Polypropylene (PP) Carboys and Centrifuge mL Bottles | .21 | | | |
| 3. | Ap | ppendix 2. Tris EDTA (TE) pH 7.5 buffer (10mM Tris, 0.1mM EDTA, pH 8.0) | .21 | | | |
| 4. | Ap | opendix 3. Preparation of the Internal Amplification Control (IAC) Target Working Solution | .22 | | | |
| 5. | Ap | ppendix 4. Preparation of the Positive Control Target Working Solution | .23 | | | |
| 6. | Ap | ppendix 5. ABI 7500 Fast v2.0 or 2.3 Method | .24 | | | |
| 7. | Ap | ppendix 6. ABI 7500 Fast v1.4 Method | .33 | | | |

Procedures for Dead-end Ultrafiltration and Detection of *Cyclospora cayetanensis* from Ultrafilter pellets.

This analytical procedure provides steps for isolation of *C. cayetanensis* oocysts from agricultural water samples by dead-end ultrafiltration and subsequent DNA template preparation from ultrafilter pellets. We have divided this protocol in two parts. Part I covers the dead-end ultrafiltration protocol, and Part II covers the recovery and detection of *C. cayetanensis* from the ultrafilters.

1.1. Dead-end Ultrafiltration Equipment and Supplies

- A. Geopump[™] Peristaltic Pump Series II Package (includes easy-load II pump head and a portable battery), Geotech, Cat No. 91352123.
- B. Pump Assembly (or equivalent assembly):
 - i. DIN adapter (for End Port), Molded Products, Cat. No. MPC855 NS.375.
 - ii. Blood port (End) cap, Molded Products, Cat. No. MPC-40.
 - iii. Dialysate Port (Side) cap, Molded Products, Cat. No. MPC-60D.
 - iv. L/S 36 tubing (9.7mm ID), Cole Parmer, Cat. No. EW-96410-36 or EW-06434-36.
 - v. SNP-8 hose clamps (for DIN adapter), Cole Parmer, Cat. No. EW-06832-08.
 - vi. SNP-12 hose clamps (for side port), Cole Parmer, Cat. No. EW-06832-12 (optional).
- C. Hollow fiber ultrafilter Nipro Elisio 25H.
- D. Turbidity Meter (optional) with a resolution of 0.01 NTU, a range up to at least 200 NTU, and compliant with EPA 180.1 method or ISO 7027 standard.
- E. 500 mL plastic bottles for turbidity measurement samples and sodium thiosulfate solution.
- F. Flow meter.
- G. Sterilized scissors (required to cut tubing).
- H. Pliers (to close the hose clamps).
- I. Autoclavable biohazard waste bags.
- J. Appropriate Personal protective equipment (PPE) such as hand sanitizer, gloves, protective eyewear, and lab coats as needed.

Reagents

A. Sodium thiosulfate, Fisher Cat. No S446 (Only needed for chlorinated water), or equivalent.

Ultrafiltration Procedure for the Detection of *Cyclospora cayetanensis* Note: The filtration protocol can be executed in the field.

Ultrafiltration Setup



Figure 1.Diagram of the filtration system.

Ultrafiltration Protocol

Note 1: If the water to be collected contains free chlorine or chlorination is suspected, immediately after the filtration process, treat the filter with a 1% sodium thiosulfate solution following the procedure described in section A.iii.

Note 2: Charge the pump batteries in advance according to the manufacturer's instructions.

- i. Check turbidity of water
 - a. Set aside a sample of approximately 500 mL for turbidity measurements in a 500 mL bottle.
 - b. If a turbidity meter is not available, ship the collected sample together with the filter to the laboratory that will perform the analysis.
 - c. If a turbidity meter is available, allow any debris to settle out of the water for approximately one minute and measure the turbidity according to the manufacturer's instructions. (Do not transfer the settled debris into the turbidimeter).
 - d. If turbidity is higher than 40 Nephelometric Turbidity Units (NTU), or a turbidimeter is not available in the field, monitor the effluent flow rate during the filtration. If the rate decreases abruptly, stop the filtration and record the total volume of

water that was filtered. If the turbidity is less than 40 NTU, the filter should not clog before filtering a 10 L sample.

- ii. Ultrafiltration Procedure
 - a. Select the sampling locations and determine the number of filters to be used. This protocol was optimized for filtration of 10 L water with each filter. The filters cannot be used under freezing conditions.
 - b. Assemble the filtration system with one filter as shown in Figure 1, using the supplies and equipment listed above. Position the peristaltic pump on a flat surface or stand lift platform. Place the filter next to the pump.
 - c. Label one end of the filter "A" and the other end "B".
 - d. Remove the end port cap from the "A" end of the filter.
 - e. Screw in the DIN adapter to the "A" end port.
 - f. Push the influent L/S 36 tubing onto the DIN adapter and secure with a SNP 8 tubing clamp. Use the pliers to secure the clamp in place if needed. Use the scissors to cut sufficient tubing to feed through the pump head and reach the water source.
 - g. Remove the cap from the side port close to the "A" end. Push the Dialysate Port (side) cap onto the side port and secure with a SNP 12 tubing clamp.
 - h. Remove the cap from the **"B"** end port. Screw in the blood port (end) cap to the **"B"** end of the filter opposite to the DIN adapter.
 - i. Feed the influent tubing through the pump head and close the pump head using the lever.
 - j. Remove the cap from the side port close to the "**B**" end. Push effluent L/S 36 tubing onto the open side port as shown above and secure with a SNP 12 tubing clamp.
 - k. If a flow totalizer meter will be used to measure the volume of water filtered, screw a 3/4" GHT adapter onto each end, cut tubing attached to red side port and push tubing onto both sides of the meter (ensure directional flow of the meter is correct), no clamps are needed.
 - 1. Place the influent tubing into the body of water and ensure the end of the tubing will stay below the surface of the water and away from plant material or other large debris which may be present. Be cautious not to disturb the water significantly near the filtration site.
 - m. Plug in the appropriate power cord into the outlet in the back of the pump and the other end of the power cord into the power source. The power source can be any external 12-18 V DC @ 70 watts or 90-260 V AC 47-65 Hz. Place the battery in a place where it will not get wet.
 - n. Determine the desired direction of flow and set the toggle switch for the flow direction. Ensure the speed dial is set to

zero before starting the pump.

- o. Turn the pump "ON" (the black filled circle), record the start time of filtration.
- p. Once pumping has begun, the speed dial can be adjusted to gradually increase the flow to the maximum speed setting.
- q. As soon a volume of 10 L is filtered, reduce the speed to the minimum and turn the pump "OFF". Record the stop time of filtration.
- r. If the water is chlorinated or chlorination is suspected follow the instructions in item iii below to treat the ultrafilter with 1% sodium thiosulfate. If the water is not chlorinated, skip this step.
- s. Remove all tubing from the ultrafilter. Screw a blood port cap into the influent port on the "A" end.
- t. Place a Dialysate Port (side) cap on the side port of the "B" end where the tubing was removed.
- u. Influent tubing can be reused when collecting multiple subsets from the same sample. Influent tubing can be discarded in the biohazard bags after collecting each sample. Effluent tubing can be reused for subsequent samples. All tubing clamps and adapters that can be reused should be sterilized by autoclaving for future use.
- v. **Each filter must be labelled with the following information:** Location, sample name, sample collection date and time, turbidity (if available), the amount of water that was filtered, and contact information from the person collecting the sample. An arrow should be drawn on the filter to indicate the direction of the flow of water (see Figure 1).
- w. Place the filter in a cooler for transport to the laboratory. Use ice packs to keep the cooler cold if necessary for the target/suspected microbes collected.
- iii. Procedure to treat ultrafilters with 1% sodium thiosulfate solution when agricultural water is suspected to have been treated with chlorine.
 - a. Fill a 500 mL bottle with non-chlorinated water and add 5 g sodium thiosulfate, shake to dissolve.
 - b. Place the influent tubing into the 1% sodium thiosulfate solution immediately after the filtration process and pump the entire volume through the filter without pumping air into the ultrafilter.

Dead-end Ultrafiltration for the Detection of Cyclospora cayetanensis from Agricultural Water

1.2. Recovery and Detection of *Cyclospora cayetanensis* from Ultrafilters

Laboratory Facilities

- A. A laboratory bench for the ultrafilter backflushing and concentration procedures.
- B. A laboratory bench or hood for the DNA extraction procedure.
- C. Separate workstations for each step of the qPCR protocol to allow an efficient workflow and eliminate the potential for false positive results due to laboratory contamination.

Equipment and Supplies

- A. Geopump[™] Peristaltic Pump Series II Package (includes easy-load II pump head and batteries), Geotech, Cat No. 91352123.
- B. Turbidity Meter with a resolution of 0.01 NTU, a range up to at least 200 NTU, and compliant with EPA 180.1 method or ISO 7027 standard.
- C. Pump Assembly (or equivalent assembly):
 - i. DIN adapter (for End Port), Molded Products, Cat. No. MPC855 NS.375.
 - ii. Blood port (End) cap, Molded Products, Cat. No. MPC-40.
 - iii. Dialysate Port (Side) cap, Molded Products, Cat. No. MPC-60D.
 - iv. L/S 36 tubing (9.7mm ID), Cole Parmer, Cat. No. EW-96410-36 or EW-06434-36.
 - v. SNP-12 hose clamps (for side port), Cole Parmer, Cat. No. EW-06832-12.
- D. Laboratory lift stand for pump (optional).
- E. Laboratory ring stand and clamps to support filter during backflush procedure
- F. 1 L glass beaker for collecting backflushed sample or 1 L Nalgene bottles, Fisher, Cat. No. 02-893D (or equivalent).
- G. 500-mL Nalgene bottles, Fisher, Cat No. 02-893C (or equivalent).
- H. Centrifuge (or equivalent assembly) capable of spinning 15 mL and 50 mL conical tubes and 500 mL, 225 mL, or 175 mL centrifuge bottles at 4000 x g:
 - i. Thermo ScientificTM SorvallTM LegendTM XTR Centrifuge Package,
 - Fisher, Cat. No.75-217-420. Package must include:
 - a. Thermo Scientific[™] Sorvall[™] Legend[™] TX-1000 high capacity rotor.
 - b. 15 mL and 50 mL Conical Tube Adapters, set of 4.
 - 500 mL bottle adapters for TX-1000 Swinging Bucket Rotor, sold as set of 4, Fisher, Cat. No. 75007302. Not included in above centrifuge package, must be ordered separately.
 - 250/175 mL bottle adapters for TX-1000 Swinging Bucket Rotor, sold as set of 4, Fisher, Cat. No. 75005392 or Cat. No. 75007305 (higher capacity). Not included in above centrifuge package, must be ordered separately.
- I. Vacuum aspiration system (or equivalent assembly):
 - i. Foxx Life Sciences, Vactrap[™], PP (Autoclavable), 4 L, Red Bin, SKU: 305-4001-FLS.
 - ii. Vacuum pump or house vacuum.

Standard Operation Procedure

Dead-end Ultrafiltration for the Detection of Cyclospora cayetanensis from Agricultural Water

- iii. Glass Pasteur pipettes or equivalent disposable plastic pipettes for vacuum aspiration of supernatants.
- J. Autoclavable Polypropylene Centrifuge Tubes, 500mL, Fisher, Cat. No. 07-200-621 (or equivalent). If using 250/175 mL bottle adapters instead of 500 mL bottle adaptor then purchase Autoclavable Polypropylene Centrifuge Tubes, 225 mL, Fisher, Cat. No. 05-538-61, or Autoclavable Polypropylene Centrifuge Tubes, 175 mLFisher, Cat No. 05-538-62 (or equivalent).
- K. Conical Sterile Polypropylene Centrifuge Tubes, 15 mL or 50 mL (preferred), Fisher, Cat. Nos. 339650 or 14-432-22 (or equivalent).
- L. FastPrep®-24 Instrument (bead beater), 5G (SKU 116005500) or Classic (SKU 116004500), MP Biomedicals.
- M. Benchtop microcentrifuge (24 tube).
- N. Heat block with core for 2.0 mL microcentrifuge tubes.
- O. Benchtop Vortex mixer.
- P. Eppendorf Repeater Repeating Pipette and assorted Combitips (optional).
- Q. Sterile DNase-free polypropylene microcentrifuge tubes, 1.5 mL
- R. Sterile DNase-free polypropylene microcentrifuge tubes, 2.0 mL
- S. 5.0 mL Tubes for Centrifugation (Eppendorf Tubes® with snap cap Biopur, Fisher, Cat. No. 14-282-303 or Argos TechnologiesTM Microcentrifuge tubes, Fisher, Cat. No. 03-391-276).
- T. Sterile applicator Sticks, Fisher, Cat. No. 22-029-641 (optional).
- U. Applied Biosystems 7500 Fast Real-Time PCR System with Software versions 1.4, 2.0, or 2.3 or newer, ThermoFisher Scientific.
- V. VWR Mini Centrifuge (or comparable), VWR, Cat. No. 76269-064.
- W. Applied Biosystems MicroAmp® Fast Optical 96-Well Reaction Plates, 0.1 mL, and MicroAmp® Optical Adhesive Film, Cat Nos. 4346907 and 4311971, (ThermoFisher Scientific) or equivalent.
- X. Benchtop centrifuge capable of spinning 96-Well reaction plates
- Y. Analytical Balance (0.01 g resolution).
- Z. Magnetic Stir Plate.
- AA. Teflon coated stir bar.
- BB. Digital laboratory timer.
- CC. Sterile scalpel or scissors (required to cut tubing).
- DD. Pliers (to close the hose clamps).
- EE. Pipet-Aid
- FF. 1 mL serological pipettes
- GG. 2 mL serological pipettes
- HH. 5 mL serological pipettes
- II. 10 mL serological pipettes
- JJ. Micropipettors
- KK. 2 µL aerosol resistant pipette tips with filter.
- LL. $20 \ \mu L$ aerosol resistant pipette tips with filter.
- MM. 200 µL aerosol resistant pipette tips with filter.
- NN. 1000 µL aerosol resistant pipette tips with filter.
- OO. Transfer pipettes
- PP. Appropriate personal protective equipment (PPE) such as hand sanitizer, protective eyewear, gloves and lab coats as needed.

Reagents

- A. Sodium polyphosphate (NaPP), Sigma, Cat. No. 305553-25G.
- B. Tween 80, Fisher, Cat. No. T164-500 mL
- C. Antifoam L-30 emulsion, Sigma, Cat. No. STS0100
- D. Dulbecco's Phosphate Buffered Saline (PBS), no calcium, no magnesium, Thermo Fisher Scientific, Cat. No. 14190250, (or equivalent)
- E. FastDNA SPIN Kit for Soil, 50 preps, MP Biomedicals, Fisher, Cat. No. MP116560200 or FastDNA SPIN Kit for Soil, 100 preps, MP Biomedicals, Fisher, Cat. No. MP116560300
- F. QIAquick® PCR Purification Kit (50), Qiagen, Cat. No. 28104 or QIAquick® PCR Purification Kit (250), Qiagen, Cat. No. 28106
- G. 100% Ethanol
- H. 3 M Sodium Acetate Solution, pH 5.2, Fisher Scientific, Cat. No. FERR1181
- I. Sterile DNase-free TE buffer, pH 7.5, IDTE Cat. No. 11-01-02-02 (or equivalent such as Appendix 2)
- J. DNA AWAY Surface Decontaminant, Fisher, Cat. No. 21-236-28 **OR** 10% bleach
- K. Real-time PCR reagents
 - i. PrimeTimeTM Gene Expression Master Mix (IDT) (Cat. No 1055770 (1 x1 ml) or Cat No. 1055772 (1 x 5 ml). Separate tubes of reference dye (ROX) are included with the master mix and need to be added to the master mix as a low reference dye for Applied Biosystems 7500 Fast Real-Time PCR instruments following the manufacturer instructions before use
 - ii. Sterile DNase-free TE buffer pH 7.5 (commercially prepared IDTE Cat. No. 11-01-02-02 or see Appendix 2)
- iii. Primers, 500 µM stock solution (See Table 1-2)
- iv. Probes, 100 µM stock solution (See Table 1-3)
- v. IAC Target (HMultra130-synIAC), working concentration 1E7 copies/µL. See Reagent Ordering and Preparation Instructions.
- vi. Positive control (Mit1AA gblock), 5E2 copies/µL. See Reagent Ordering and Preparation Instructions
- vii. Negative control (Water, DNase-free)

Dead-end Ultrafiltration for the Detection of Cyclospora cayetanensis from Agricultural Water

Procedures

Note: The backflush and concentration protocols to prepare samples for DNA extraction can be executed for a set of up to four filters in a single day using one centrifuge and one analyst.

Turbidity Measurement

- A. If an unfiltered water sample is provided for a turbidity measurement, use a turbidimeter to measure the turbidity according to the manufacturer's instructions. The sample should be at room temperature before measuring turbidity. Measure the turbidity at any time before executing the backflush procedure.
 - i. Allow any debris to settle out of the provided unfiltered water sample for approximately one minute and do not transfer the settled debris into the turbidimeter.
 - ii. Record the turbidity of the water sample in Nephelometric Turbidity Units (NTU).

Backflush - Recovery of Cyclospora cayetanensis from ultrafilters

- A. Preparation of Backflush solution (0.5% Tween 80/0.01% NaPP/0.001% Antifoam L-30). Note: the solution should be prepared freshly on the first day and stored at 4 °C for no more than 48 hours.
 - i. Make 10 mL 10% NaPP/1% Antifoam L-30 stock solution:
 - a. Add 10 mL DI water to a 15 mL or 50 mL conical tube.
 - b. Add 1 g NaPP and 100 μ L Antifoam L-30 to the water.
 - c. Vortex to dissolve the NaPP.
 - ii. For each ultrafilter to be processed, make 500 mL backflush solution:
 - a. Add 500 mL DI water to a 500 mL or 1 L bottle.
 - b. Add 500 μ L of the 10% NaPP/1% Antifoam L-30 stock solution to the water.
 - c. Add 2.5 mL of Tween 80 with a serological pipette.
 - d. Stir with a sterile stir bar for at least 5 min to dissolve the Tween 80.

Backflush Procedure

- i. Remove the selected filters from the refrigerator at least one hour prior to executing the backflush procedure.
- ii. Assemble the filtration system with one filter, as shown in Figure 2 or 3 depending on how the pump is supported, using the supplies and equipment listed above:
 - a. Position the peristaltic pump on a level surface or on a lift platform at least 20" above the bench surface.
 - b. Clamp the filter on a ring stand and make certain the **"B"** end of the filter is on the top.
 - c. Remove the side port cap from the **"B"** end of the filter.
 - d. Push L/S 36 tubing onto the side port and secure with a SNP 12 hose clamp.

Standard Operation Procedure

Dead-end Ultrafiltration for the Detection of Cyclospora cayetanensis from Agricultural Water

- e. Feed the tubing through a peristaltic pump and place the end of the tubing into the 500 mL of backflush solution. Close the pump head lever.
- f. Hold the "A" end port over a 1 L sterile bottle or beaker, and remove the end port cap.
- iii. Set the pump dial setting to zero and turn on the pump. Slowly increase the flow rate by turning up the dial to a slow pumping rate. If the pump has a digital display, the flow rate can be set at ~ 650 mL/minute.
- iv. Continue pumping until no backflush solution remains in the container or the tubing and the out flow from the ultrafilter has slowed to a trickle. Do not pump air into the filter for more than approximately 10s.
- v. Measure and record backflush volume or weight, if necessary.
- vi. Repeat the backflush procedure for the remaining filters of the set.
- vii. All DIN adapters, Blood port (End) caps, Dialysate Port (Side) caps and clamps can be autoclaved and reused.
- viii. Proceed to the concentration procedure.



Figure 2. Backflush assembly.

Standard Operation Procedure Dead-end Ultrafiltration for the Detection of *Cyclospora cayetanensis* from Agricultural Water



Figure 3. Backflush assembly.

Concentrating Cyclospora cayetanensis from Backflush Effluent

- A. Each filter yields approximately 600 mL of effluent. Divide the effluent collected from each filter into two 500 mL conical centrifuge bottles.
 Alternatively, four 175/225 mL conical centrifuge bottles can be used.
- B. Balance the conical bottles using sterile PBS and centrifuge at 4,000 x g for 45 min with maximal acceleration and a brake setting of 6 (on a scale of 0-9) for deceleration.
- C. Carefully remove and discard all but ~25 mL of the supernatant from each centrifuge bottle using a glass Pasteur pipette connected to a vacuum aspirator or a 50 mL serological pipette. Using a vacuum aspiration system will be faster and more efficient than using a serological pipette.
- D. Re-suspend each pellet in the remaining supernatant by pipetting up/down and vortexing. Transfer approximately 25 mL aliquots of the re-suspended material from the two 500 mL centrifuge bottles to one 50 mL centrifuge tube (if the pellet is too large, we might split into two or more 50 mL centrifuge tubes). If using 175/225 mL bottles, transfer approximately 30 mL aliquots of the re-suspended material to four 50 mL centrifuge tubes. Alternatively, 13-15 mL aliquots of the re-suspended material from four 175/225 mL centrifuge bottles can be transferred to multiple 15 mL centrifuge tubes. Use a single 10

mL aliquot of PBS to rinse all empty bottles one by one and add the rinse material to the tubes. Keep all centrifuge tubes for the same filter in a single Styrofoam stand or rack. At this point, if needed, samples can be stored at 4°C overnight before continuing the procedure.

- E. Centrifuge the 50 mL (or 15 mL) centrifuge tubes at 4,000 x g for 45 min.
- F. After the centrifugation, aspirate all but $\sim 1 \text{ mL}$ of the supernatant from each of the 50 mL tubes to waste without disturbing the pellets. If using multiple tubes, you need to combine multiples pellets into one 15 mL tube and proceed with an extra centrifugation (4000 x g for 45 min) to obtain a final pellet.
- G. At this point, the content of each ultrafilter is contained in one tube. Label appropriately.
- H. Store the pellets at 4°C for no more than 24 h.
- I. Autoclave the supernatant waste.

DNA Isolation and Purification

i.

Modified DNA Isolation Protocol Using the FastDNA® SPIN Kit for Soil

Material preparation:

- a. Add 100 mL of 100% ethanol to the 12 mL concentrated SEWS-M wash solution when a new kit is opened.
- b. Fill up an ice bucket for cooling the samples after the FastPrep-24 bead beater homogenizing steps (Step <u>vi</u> and <u>vii</u> below).
- c. Set a heat block for 2.0 mL tubes to 55°C.
- d. Label the DNase-free 2.0 mL tubes appropriately. These are the tubes to be used in step <u>ix</u> and are **NOT** provided in the kit.
- e. Program the FastPrep-24 bead beater to a setting of 6.5 m/s (corresponds to approximately 4000 rpm) for 60 s.
- ii. Assemble the sample pellets in the 50 mL or 15 mL tubes for DNA isolation on a workbench cleaned with DNA AWAY or a cleaned Bio-Safety Cabinet (BSC). Include a Lysing Matrix E (LME) tube (supplied with the FastDNA Spin Kit) for the negative DNA extraction control.
- iii. Add 830 μL Sodium Phosphate Buffer (SPB) to the mixture in the 50 mL (or 15 mL) conical tubes (and to the DNA extraction control LME tube). Mix each of the pellets in the 50 mL (or 15 mL) conical tubes with the buffers using a sterile wooden stick or a sterile transfer pipette. Try to minimize generating bubbles.
- iv. Carefully transfer the mixture (pellet, MT and SPB buffers) from each 50 mL (or 15 mL) conical tube into an LME tube using a sterile transfer pipette. Make sure that there is at least 1.0 cm of air space at the top of the tube to allow for efficient bead-beating; do not fill beyond the maximum fill height as shown in Figure 4. If the total volume is too large, split the mixture into extra LME tubes. The sample can be recombined later as described in step xv.
- v. Add 122 µL MT buffer to each of the LME tubes containing pellets. Screw the caps on securely.



Figure 4. Maximum fill height for LME tubes.

vi. Transfer the samples to the FastPrep-24 bead beater, making sure to balance and secure the tubes in the sample holder according to the manufacturer's instructions. Homogenize at a setting of 6.5 m/s for 60 s. Immediately remove the sample holder containing the tubes from the instrument and place on ice for 3 mins.

vii. Return the sample holder to the bead beater and repeat the homogenization and the incubation on ice as above for 3 min.

- viii. Remove the tubes from the sample holder and centrifuge at 14,000 x g for 15 min (FastDNA kit protocol step 5).
- ix. Transfer the supernatant to a clean 2 mL tube (use the 2 mL tubes indicated in step 3.4.A.i.d which are <u>NOT</u> included in the kit). Add 250 μ L PPS solution and mix by inverting by hand 10 times (FastDNA kit protocol step 6).
- x. Centrifuge at 14,000 x g for 5 min (FastDNA kit protocol step 7).
- During the above centrifugation, re-suspend the Binding Matrix provided in the kit by shaking well before use. Transfer 1.0 mL of re-suspended Binding Matrix to a clean 15 mL conical tube for each sample. (For easy pipetting, 5 mL tubes may be used for this step instead of 15 mL tubes).
- xii. Transfer the supernatant from each sample to a tube containing 1.0 mL of re-suspended Binding Matrix.
- xiii. Place the tubes in a rack and mix by inverting the rack for 2 min and then allow the silica matrix to settle for 3 min (FastDNA kit step 9). Centrifuge the tubes briefly at 1000 x g for 1 min in a centrifuge (using a swinging bucket rotor).
- xiv. Remove 1.4 mL (700 μ L x 2) of the supernatant from each tube by pipetting.
- xv. Re-suspend the matrix in the remaining supernatant (with a pipette) and transfer all of the volume to a SPIN Filter in a catch tube provided in the kit. Centrifuge at 14,000 x g for 1 min (FastDNA kit protocol step 11). If a sample was split into two or more tubes at step v above, transfer the matrix from the first tube to one spin filter and perform

the centrifugation. Then, discard the flow through and transfer the matrix from the second tube to the same spin filter and perform another centrifugation.

- xvi. Add 500 μ L prepared SEWS-M to each filter. <u>Gently</u> re-suspend each pellet by stirring (with a P-200 tip) for ~ 10 s. Do not poke the membrane. (FastDNA kit protocol step 12).
- xvii. Centrifuge at 14,000 x g for 1 min. Empty the catch tube and replace the filter back into the same tube. (FastDNA kit protocol step 13). Note: if the sample is too thick and some liquid won't go through, you will need to centrifuge the sample for another 1 or 2 min.
- xviii. Centrifuge at 14,000 x g for 2 min to dry the matrix. Discard the catch tube and replace with a new catch tube provided in the kit (FastDNA kit protocol step 14).
 - xix. Air dry the filter for 5 min at room temperature (FastDNA kit protocol step 15).
 - Add 75 μL DES to the matrix in the spin filter. Re-suspend the Binding Matrix by gently stirring with a P-10 pipette tip. Do not puncture the membrane. Incubate the tubes at 55°C in a heat block for 5 min. (FastDNA kit protocol step 16).
 - xxi. Centrifuge at 14,000 x g for 1 min to recover the eluted DNA and discard the SPIN Filter (FastDNA kit protocol step 17).
- xxii. Proceed to DNA purification.

DNA Purification Using the QIAquick® PCR Purification Kit

- i. Upon opening a new kit:
 - a. Add 96-100% ethanol to Buffer PE wash solution (see bottle label for ethanol volume).
 - b. Add the pH indicator to Buffer PB.
- ii. Add 375 μ L of Buffer PB to the DNA sample and mix. If the color of the mixture is orange or violet, add 10 μ L 3 M sodium acetate, pH 5.2, and mix.
- iii. To bind DNA, apply the DNA sample to the QIAquick column and centrifuge at 14,000 x g for 30–60 s. Discard the flow-through and place the QIAquick column back in the same tube.
- iv. To wash, add 750 μl Buffer PE to the QIAquick column, centrifuge at 14,000 x g for 30–60 s, discard the flow-through, and place the QIAquick column back in the same tube.
- v. Repeat step iv.
- vi. Centrifuge the QIAquick column once more for 1 min to remove residual wash buffer.
- vii. Place each QIAquick column in a clean 1.5 mL microcentrifuge tube. To elute DNA, add 50 μ L Buffer EB (10 mM Tris·Cl, pH 8.5) to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge at 14,000 x g for 30-60 s to elute the purified DNA.
- viii. Centrifuge the QIAquick column once more for 1 min. Discard the columns and cap the tubes.

Standard Operation Procedure

Dead-end Ultrafiltration for the Detection of Cyclospora cayetanensis from Agricultural Water

ix. Proceed to real-time PCR detection, or you may store the DNA samples at 4°C for up to 2 days or at -20 or -80°C for longer term storage.

1.3. *Cyclospora cayetanensis* Real-Time PCR Detection Method Reagent Ordering and Preparation Instructions

All Primers, Probes, and Target control DNAs are commercially synthesized by Integrated DNA Technologies (IDT), Coralville, IA.

Primers: All primers are ordered from IDT normalized to a working concentration of 500 μ M and stored at -20°C.

Primer Ordering Instructions: Choose "Custom DNA Oligos" from the applicable IDT online menu page and select "DNA Oligos". From the "Normalization" drop down menu \rightarrow choose "Create a custom formulation" \rightarrow choose "Full product yield, to a specified µMolar concentration" \rightarrow enter "500" and choose "IDTE 8.0 pH" \rightarrow Name the normalization "500 µM" and Save. Next, on the Oligo Entry page enter the primer options as indicated below for each primer:

Table 1-1. Primer Ordering Instructions.

| Scale: | choose a scale between 25 nmole and 1 µmole |
|----------------|---|
| Normalization: | 500 μM |
| Purification: | Standard Desalting |

Table 1-2. Primer Names and Sequences.

| | Item Name | Sequence |
|--|-----------|--------------------------------|
| Forward primer for amplification of the mitochondrial <i>C</i> . | Mit1C-f | 5'-TCTATTTTCACCATTCTTGCTCAC-3' |
| cayetanensis target | | |
| Reverse primer for amplification of the mitochondrial <i>C</i> . | Mit1C-r | 5'-TGGACTTACTAGGGTGGAGTCT-3' |
| Forward primer for amplification of the IAC target | dd-IAC-f | 5'-CTAACCTTCGTGATGAGCAATCG-3' |
| Reverse primer for amplification of the IAC target | dd-IAC-r | 5'-GATCAGCTACGTGAGGTCCTAC-3' |

Probes: Taqman-style hydrolysis probes are used for detection of the *C*. *cayetanensis* and IAC targets. The *C. cayetanensis* probe is labeled with 5' FAM reporter dye and is double quenched with an internal ZEN quencher and 3' Iowa

Black® FQ (IABkFQ) quencher. The IAC probe is labeled with 5' Cy5 reporter dye and is double quenched with an internal TAO quencher and 3' Iowa Black® RQ-Sp (IAbRQSp) quencher. Probes are ordered from IDT and hydrated to working concentrations as described below and stored at -20°C.

Probe Ordering Instructions: Probes are ordered from the applicable IDT online menu page by choosing "Custom probes" \rightarrow choose PrimeTime qPCR Probes \rightarrow choose 250 nmol or 1 µmol scale. Enter probe nucleotide sequence and choose "5' Dye/3' Quencher" options as indicated for each probe in Table 1-3. (No "Services" options are required.)

Table 1-3. Probe Ordering Information.

| | Probe for detection of | Probe for detection of | |
|------------------------|------------------------|--------------------------|--|
| C. cayetanensis target | | IAC target | |
| Item Name | Mit1P-FAM | dd-IAC-Cy5 | |
| Sequence | 5'- | 5'- | |
| | AGGAGATAGAATGCTG | AGCTAGTCGATGCACTCCAGT | |
| | GTGTATGCACC -3' | CCTCCT-3' | |
| | | | |
| 5' Code | /56-FAM/ | /Cy5/ | |
| 3' Quencher | ZEN-3' Iowa Black® FQ | TAO-3' Iowa Black® RQ-Sp | |
| 3' Code | /3IABkFQ/ | /3IAbRQSp/ | |

Preparation of Probe Working Solutions:

100 μ M Mit1P-FAM: Hydrate the lyophilized probe in sterile DNase-free TE buffer by adding the volume specified on the accompanying IDT probe specification sheet for a 100 μ M final concentration. Vortex and centrifuge the hydrated probe briefly.

100 μ M dd-IAC-Cy5: Hydrate the lyophilized probe in sterile DNase-free TE buffer by adding the volume specified on the accompanying IDT probe specification sheet for a 100 μ M final concentration. Vortex and centrifuge the hydrated probe briefly.

IAC Target: The IAC reaction target (HMultra130-synIAC) is a synthetic 200 bp ultramer DNA sequence based on the internal amplification control developed by Deer et al., (2010).

Ordering Instructions: From the applicable IDT online menu page choose "Ultramer Oligos (up to 200 bases)" \rightarrow On the Oligo Entry page enter or choose the following:

Table 1-4. Ultramer Oligo Description.

| Item Name: | HMultra130-synIAC |
|----------------|---|
| Scale: | 4 nmole Ultramer [™] DNA Oligo |
| Normalization: | None |
| Purification: | Standard Desalting |

Preparation of IAC Target Working Solution: Hydrate the ultramer and prepare dilutions in TE pH 7.5 dilution buffer according to Appendix 3 to obtain the working concentration of 1E7 copies/µL. Store dilutions at -20°C.

Positive Control: The positive control DNA (Mit1AA gblock) is a 245 bp double stranded synthetic gBlocks® Gene Fragment synthesized by IDT. The sequence corresponds to a region (4325 bp - 4569 bp, Genbank: KP231180.1) in the *C. cayetanensis* Mitochondrial gene. In addition, this sequence contains traceable mutations (T4385A and T4386A) within the amplicon generated by the real-time PCR primers used in this protocol.

Ordering Instructions: From the applicable IDT online menu page choose "Double Stranded DNA Fragments" and select gBlocks Gene Fragments. Enter the following item name and sequence on the gBlocks Gene Fragments Entry page:

Item Name: Mit1AA gblock

Sequence:

ACAGTTGGTTTTCTATTTTCACCATTCTTGCTCACTGTATTAGTATTATTTAATTTT ACT<u>AA</u>TAGAGAAGTTGGTACTACATCAGCTTCTCTGGTTTCATCAATTTGTTAG GTGTTATTAGTACTGAGTTACTACTATTTGTTAGCTTCTTCTGGGGTGCATACACC AGCATTCTATCTCCTAGTTATGTAACAGACTCCACCCTAGTAAGTCCAACTGAGG GTCTTGTAAGTATCTCTAGTAG

Click "Add to Order" \rightarrow answer "No" to all questions on the Terms and Disclosure pop up window \rightarrow type your name in the Signature box \rightarrow accept the terms and conditions \rightarrow click "Add to Cart". The amount delivered will be 250 ng of the gBlock. Hydrate the gBlock and prepare dilutions according to Appendix 4 to obtain the working solution concentration of 5E2 copies/µL. The positive control working solution can be stored at -20 or 4°C. A fresh working solution should be prepared from the frozen 5E3 dilution every 90 days.

Reaction Setup and Execution

A primer/probe mix must be prepared for the *C. cayetanensis* target reaction and for the IAC target reaction. Briefly mix and centrifuge all reagents to resuspend and bring down contents before assembling mixes.

Primer/Probe Mixes (store at -20°C in dark):

| Volume | Final concentration |
|------------------------|---------------------------------|
| 12.0 µL 500 µM Mit1C-f | 0.6 µM final in reaction |
| 12.0 µL 500 µM Mit1C-r | 0.6 µM final in reaction |
| 30.0 µL 100 µM Mit1P- | $0.3 \ \mu M$ final in reaction |
| FAM | |
| 446 μL ΤΕ | |
| 500 μL final volume | |

Table 1-5. 20X Mit1C Pr/Pro (12 μM each primer, 6 μM probe).

Table 1-6. 20X synIAC Pr/Pro (5 µM each primer, 5 µM probe, 2E5 copies synIAC target).

| Volume | Final Concentration |
|---------------------------------|---------------------------------|
| 5.0 μL 500 μM dd-IAC-f | 0.25 µM final in reaction |
| 5.0 μL 500 μM dd-IAC-r | 0.25 µM final in reaction |
| 25.0 μL 100 μM dd-IAC-Cy5 | 0.25 µM final in reaction |
| 10 μL 1E7 copies/μL HMultra130- | 1E4 copies/µL final in reaction |
| synIAC | |
| 455 μL TE | |
| 500 μL final volume | |

Real-Time PCR reaction mix for 20 µL volume reactions:

All samples and all controls are always run in triplicate.

Briefly mix and centrifuge all reagents to resuspend and bring down contents before assembling reaction mix. The master mix formula below is sufficient to run one (1) replicate of one sample. For each qPCR experimental run, prepare sufficient reaction mix to run the no template control (NTC), the positive control, and samples all in triplicate. Calculate the total number of replicates being run (N) in one experiment and prepare a volume of master mix between N+1 and N+3 to assure sufficient reagents for all replicates.

Table 1-7. Reaction mix for 20 µL volume reactions.

| Master mix components | 10.0 | μL 2X PrimeTimeTM Gene Expression Master Mix low ROX (IDT) |
|-----------------------|------|---|
| Master mix components | 1.0 | μL 20X Mit1C Pr/Pro Mix |
| Master mix components | 1.0 | μL 20X synIAC Pr/Pro Mix |
| Master mix components | 6.0 | μL H ₂ O (DNase-free) |
| Sample | 2.0 | μL sample or control |
| Total Volume | 20.0 | μL total volume |

Aliquot 18 µL of reaction mix to each reaction well or tube.

Add 2.0 μ L of sample or appropriate controls to each reaction plate well or tube (see Section C below).

| NTC | 2.0 µL H ₂ O (DNase-free) |
|------------------|--------------------------------------|
| DNA extraction | 2.0 μL |
| control | |
| Samples | 2.0 µL (1X and 1/10 dilution) |
| Positive Control | 2.0 µL Mit1AA gBlock (5E2 |
| | copies/µL) |

Samples and Controls: Table 1-8. Sample and Controls volume

Always briefly vortex and centrifuge controls and samples before adding to reaction wells or tubes.

ALL SAMPLES ARE TO BE ANALYZED AT 1X AND A 1/10 DILUTION IN THE SAME INITIAL EXPERIMENTAL RUN (Controls are not tested at a 1/10 dilution). Prepare diluted samples following instructions below.

1/10 Sample Dilution Protocol:

Transfer 2 μ L of sample to a clean microcentrifuge tube containing 18 μ L of TE. Mix well and centrifuge briefly.

After addition of samples and controls to reaction wells or tubes, seal the plate with the adhesive film or seal the tube strips with cap strips and centrifuge at $400 \times g$ for 30 s.

Run the plate or tube strips in the ABI 7500 Fast Real-Time PCR Instrument using a pre-defined protocol template and run method as described below.

Real-Time PCR Cycling Protocol Templates for the ABI 7500 Fast Instrument:

Prior to initiating a run each laboratory should define a protocol template as described in Appendix 5 for ABI Fast instruments running v2.0 or 2.3 software or Appendix 6 for instruments running v1.4 software.

Run Methods on the ABI 7500 Fast Instrument:

Cycling Parameters.

Follow the run method detailed in Appendix 5 for ABI Fast instruments running v2.0 or 2.3 software or Appendix 6 for instruments running v1.4 (or any v1.x) software:

• Set-up the program in the instrument with an initial step of 95 °C for 3 min followed by 40 cycles of [95 °C for 15 sec + 67 °C for 60 sec]. Data collection should be on during the 67°C step.

The following software analysis settings are applied to data when instructions for protocol templates and run methods in Appendices 5 or 6 are followed:

- Manual Threshold = 0.05 (*C. cayetanensis* target; Mit1C)
- Manual Threshold = 0.05 (IAC target)
- Auto Baseline

Document the experimental run by saving the run file and an exported results data file according to the instructions in Appendix 5 or 6. Print the exported results data file and include with analytical worksheet packet.

Interpretation of Results

Positive Samples:

- Samples are only considered positive for the presence of *C. cayetanensis* if, on initial testing or re-testing, one (1) or more sample replicates produces a smooth exponential/sigmoidal amplification signal with $Ct \le 38.0$ for the *C. cayetanensis* mitochondrial target (Mit1C) reaction and the IAC target reaction is either Negative or Positive.
- ONLY ONE REPLICATE OF AN UNKNOWN SAMPLE OR 1/10 DILUTION OF THAT SAMPLE NEEDS TO BE POSITIVE FOR THE *C. cayetanensis* Mit1C TARGET IN ORDER TO CONSIDER A SAMPLE POSITIVE.

Samples for Further Analysis:

Any sample producing a smooth exponential/sigmoidal amplification signal in one (1) or more replicates for the *C. cayetanensis* Mit1C target reaction crossing the threshold with Ct(s) > 38.0 and the IAC target reaction is either Negative or Positive: Consult CFSAN SME (depending on the SME advice the sample might need to be re-tested one time (in triplicate) at both 1X and 1/10 dilution).

Negative Samples:

• If a sample *C. cayetanensis* Mit1C target reaction produces all replicates with undetermined Ct **and** sample IAC target reaction produces an average Ct value that is not more than 3 cycles higher compared to the NTC (no template control): **Sample is NEGATIVE**, **no further action**.

Invalid Results:

- If one (1) or more replicates of the NTC sample or the DNA extraction control sample Mit1C target reactions produces a positive result crossing the threshold, the experimental run is **invalid and must be repeated**.
- If after repeat of an invalid experimental run, the DNA extraction control repeatedly produces a positive result and the NTC sample is negative, the DNA extraction procedure was likely contaminated. The DNA extraction procedure must be repeated for the entire set of samples if available.
- If one (1) or more replicates of the positive control sample for *C. cayetanensis* Mit1C is undetermined, the experimental run is **invalid and must be repeated**.

Inconclusive Results:

• If on the initial test (or after re-test if required), a sample produces no replicate with Ct ≤ 38.0 (for *C. cayetanensis* mitochondrial Mit1C target), and sample IAC target is undetermined or produces an average Ct value more than 3 cycles higher compared to the NTC: Sample is INCONCLUSIVE → consult CFSAN SME

2. Appendix 1. Sanitizing Polypropylene (PP) Carboys and Centrifuge mL Bottles

Procedure:

- A. Spray a liberal amount of a 0.65% bleach solution into the carboy/bottle. Add water to create approximately a 1:1 by volume bleach/water mixture at the bottom of the carboy/bottle.
- B. Screw the cap of the carboy/bottle back on and gently shake the carboy/bottle, ensuring that the bleach/water mixture rinses the entire inside of the carboy/bottle and cap.
- C. If you are cleaning a centrifuge bottle, brush the bottle with a tube brush to remove any residual pellet material.
- D. Dispose of the bleach/water mixture into a waste container carefully. Leave the carboy/bottle to air dry for approximately two hours. The carboy/bottle does not need to be completely dry for the next step.
- E. Rinse the carboy/bottle with DI water once to remove residual bleach. Discard the liquid into the waste container.
- F. Autoclave the carboy/bottle at 121°C, 15 psi for approximately 20 min.

3. Appendix 2. Tris EDTA (TE) pH 7.5 buffer (10mM Tris, 0.1mM EDTA, pH 8.0).

Table 3-1. Tris EDTA components

| Reagent | Volume |
|------------------------------------|---------|
| 1 M Tris pH 7.5 | 100 µL |
| 0.05M EDTA | 20 µL |
| PCR-grade water (Dnase/Rnase free) | 9.88 mL |

4. Appendix 3. Preparation of the Internal Amplification Control (IAC) Target Working Solution.

Hydrate the IAC Target ultramer (HMultra130-synIAC) in TE pH 7.5 dilution buffer according to instructions below to obtain the working concentration of 1E7 copies/ μ L. Store dilutions and working solution at -20°C.

| HMultra130-synIAC IAC Target: Hydration and Dilution Procedure | Concentration (copies/µL) |
|--|------------------------------|
| Centrifuge the lyophilized ultramer (4 nmoles) prior to opening to ensure the contents are in the bottom of the tube. Hydrate in original tube with 1000 μ L TE dilution buffer and vortex briefly. Centrifuge again to bring liquid contents to the bottom of the tube. | 5E12* |
| Mix 10 μ L of the 5E12 stock + 990 μ L TE in a new tube. Centrifuge to bring liquid contents to the bottom of the tube. | 5E10 |
| Mix 10 μ L of the 5E10 stock + 990 μ L TE in a new tube. Centrifuge to bring liquid contents to the bottom of the tube. | 5E8 |
| Mix 10 μ L of the 5E8 stock + 490 μ L TE in a new tube. Centrifuge to bring liquid contents to the bottom of the tube. | 1E7 |

Table 4.1. Internal Amplification Control (IAC) Target Working Solution

*Note: This value is theoretically derived and is a function of the DNA concentration, Avogadro's number, length of template, and average weight of a basepair. There are freely available online calculators to derive this value.

Step 1: Convert 4 nmol to μg DNA using a molar quantity to weight calculator for nucleic acids. The derived weight is 519.2 μg .

Step 2: Use the calculated DNA weight (519.2 μ g) and the molecular weight of the sequence (61882) to compute the copy number. This will be approximately 5.138E15 copies (using 650 Da as the mass of a DNA base pair). Since the lyophilized target is dissolved in 1000 μ L TE, the approximate copies is 5E12 copies/ μ L.

• An optional method for an accurate measurement is to determine the concentration of DNA (above- lyophilized target in 1000 μ L TE) using preferably a high sensitivity Qubit kit or nanodrop. The concentration (ng/ μ L) can be plugged into a copy number calculator to derive an accurate copies/ μ L of the target which can then be diluted to achieve 1.0E6 copies/ μ L.

5. Appendix 4. Preparation of the Positive Control Target Working Solution.

Hydrate and dilute the Positive Control gBlock (Mit1AA) in TE pH 7.5 dilution buffer according to instructions below to obtain the working concentration of 5E2 copies/ μ L. Store dilutions at -20°C. The working solution can be stored at -20 or 4°C. A fresh working solution should be prepared from the frozen 5E3 dilution every 90 days.

| Table 5.1. Toshive Control Target Working Solution | Table 5.1. | Positive | Control | Target | Working | Solution |
|--|------------|----------|---------|--------|---------|----------|
|--|------------|----------|---------|--------|---------|----------|

| Mit1AA Positive Control: Hydration and Dilution Procedure | Concentration (copies/µL) |
|---|------------------------------|
| Centrifuge the lyophilized gBlock (250 ngrams) prior to opening. Hydrate in original tube with 500 μ L TE dilution buffer. Vortex briefly and incubate at 50°C for 20 minutes. Vortex briefly and centrifuge again to bring liquid contents to the bottom of the tube. | 2E9 |
| Mix 10 μ L of the 2E9 stock + 990 μ L TE in a new tube. Centrifuge to bring liquid contents to the bottom of the tube. | 2E7 |
| Mix 10 μ L of the 2E7 dilution + 990 μ L TE in a new tube. Centrifuge to bring liquid contents to the bottom of the tube. | 2E5 |
| Mix 10 μ L of the 2E5 dilution + 390 μ L TE in a new tube. Centrifuge to bring liquid contents to the bottom of the tube. | 5E3 |
| Mix 50 μ L of the 5E3 dilution + 450 μ L TE in a new tube. Centrifuge to bring liquid contents to the bottom of the tube. | 5E2 |

• Note: This value is theoretically derived and is a function of the DNA concentration, Avogadro's number, length of template, and average weight of a basepair. There are freely available online calculators to derive this value.

Step 1: Use the DNA amount (250 ng) and the molecular weight of the sequence (151227.2) to compute the copy number. This will be approximately E12 copies (using 650 Da as the mass of a DNA base pair). Since the lyophilized target is dissolved in 500 μ L TE, the approximate copies are 2E9 copies/ μ L.

• An optional method for an accurate measurement is to determine the concentration of DNA (above- lyophilized target in 500 µL TE) using preferably a high sensitivity Qubit kit or nanodrop. The concentration (ng/µL) can be plugged into a copy number calculator to derive an accurate copies/µL of the target which can then be diluted to achieve 5E2 copies/µL.

6. Appendix 5. ABI 7500 Fast v2.0 or 2.3 Method

Define a Run Template Using Software v2.0 or 2.3 on the ABI 7500 Fast Instrument: Turn on the computer and ABI 7500 FAST Real-Time PCR system. Open the 7500 Software v2.0 or 2.3 and click "New Experiment" (Advanced Setup). Define "Experimental Properties" as shown below with "Experiment Name"→ "Cyclospora cayetanensis"

| Experiment Menu « | Experiment: Cyclospora cayetanensis | Type: Standard Curve | Reagents: Other | START RUN 🐎 | ? |
|-----------------------|--|---|-------------------------------|-------------|----|
| Setup | Experiment Properties | | | | |
| Experiment Properties | Enter an experiment name, select the instrument type, select the type of experiment to set up, the | en select materials and methods for the PCR reactions and instrument run. | | | |
| Plate Setup | How do you want to identify this experiment? | | | | |
| Run Method | * Experiment Fame: Cyclospora cayetanensis | | | | |
| Reaction Setup | Barcode (Uptional): | | | | |
| 🛒 Materials List | Comments (Optional) | | | | \$ |
| Run | · Which instrument are you using to run the experiment? | | | | |
| Analysis | 7500 (96 Wells) | √ 7500 Fast (96 Wells) | | | |
| | Set up, run, and analyze an experiment using a fast cycling 5-color, 95-well system. | | | | |
| | What type of experiment do you want to set up? | | | | |
| | √ Quantitation - Standard Curve | Quantitation - Relative Standard Curve | Quantitation - Comparative Cr | (ΔΔC1) | |
| | Melt Cune | Genotyping | Presence/Absence | | |
| | Use standards to determine the absolute quantity of target nucleic acid sequence in samples. | | | | |
| | • Which reagents do you want to use to detect the target sequence? | | | | |
| | TagMan® Reagents | SYBR® Green Reagents | √ Other | | |
| | The PCR readions contain primers to designed to amplify the target sequence and other reagents t firstade MetiCouve | to detect amplification. The Reaction Setup screen is not available for "Other" reagents. | | | |
| | "Which ramp speed do you want to use in the instrument run? | | | | |
| | Standard (~ 2 hours to complete a run) | √ Fast (~ 40 minutes to complete a run) | | | |
| | For optimal results with the Fast ramp speed, Applied Blosystems recommends using Fast reagent | As for your PCR reactions. | | | |

Click "Plate Setup" on left. Add targets to the "Define Targets and Samples" tab as shown below. Define the targets Mit1C as "FAM" and IAC as "CY5" with quencher set as "None".

| Exporiment Menu « | Experiment: Cyclospora cayeta | nonsis | Type: Standard Curve | Reagents: Other | START RUN | ? |
|-----------------------|---|---|---------------------------------|--|--|---------|
| Setup | Define Targets and Samples | Assign Targets and Samples | | | | |
| | Instructions; Define the targets to gua | initiv and the samples to test in the reaction plate. | | | | |
| Experiment Properties | Define Targets | and any second se | Define Samples | And Personal Property and Personal Property in which the Person of the P | The second s | |
| Plate Seep | Add New Toront | Stup Target Delate Target | LAST Mary Dormals | dd Onuad Damala - Dan Gamela - Dalais Causala | | |
| Eve Mathed | North Internet | | Southern Derivite | Care Carlos Correction Correction | | |
| | Target Name | Reporter Quencher | Color Sample Name | | Color | _ |
| Reaction Setup | MrtC | FAM V None | Sample 1 | | | ~ |
| Waterials List | WC | CT5 Picne | × × | | | |
| | | | | | | |
| Run | | | | | | |
| Analysis | | | | | | |
| 907 | | | | | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |
| | Define Biological Replicate Group | ps | | | | |
| | Instructions: For each biological repl | icate group in the reaction plate, click Add Biological Group, the | in define the biological group. | | | |
| | Add Biological Group Delete Biologic | | | | | |
| | Biological Group Name | c | alor | Comments | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |
| | | | | | | - 11 |
| | | | | | | - 11 |
| | | | | | | - 11 |
| | | | | | | |
| | | | | | | - 11 |
| | | | | | | |
| | | | | | | |
| 12.04 | | | | | Assign Targets and | Samples |
| | | | 1/ | | | |

Click "Run Method" on left and define cycling parameters as shown below for a 20 μ L reaction. Define the program with an initial step of 95°C for 3 min followed by 40 cycles of [95°C for 15 sec + 67°C for 1 min]. Data collection should be on during the 67°C hold.

| 🔝 New Experiment 👻 Open | . 🛃 Save 🔹 🖆 Close 🛷 Export 🔹 | Print Report | | | | | | |
|-------------------------|--|---------------------------------------|--|--|----------------------|-----------------|-------------|----|
| Experiment Menu « | Experiment: EXCEL REALTIN | EPCR 20230921MIT1C | 600_300 +IAC7 250_2 | 50WATER SAMPLES6 OOCYSTS ANTIFOAM | Type: Standard Curve | Reagents: Other | START RUN 🅪 | () |
| Setup | Run Method | | | | | | | |
| Experiment Properties | Review the reaction volume and the | thermal profile for the default run m | ethod. If needed, edit the default r | run method or select a run method from the library. | | | | |
| Plate Setup | Graphical View Tabular V | iew | | | | | | |
| Run Method | Reaction Volume Per Well 20 µL | Expert Mode Scient/view F | itera | | | | | |
| Reaction Setup | Add Stage V Add Step V Dele | te Selected (nothing to Undo) | (nothing to Redo) Collect D: | ata 🔻 🛛 Open Run Method 🛛 Save Run Method Revert to Defaults | | | | |
| Materials List | Holding Stage | Cycling Number of Cyc | Stage les: 40 🗢 | | | | | |
| Run | | Starting Cycl | e: 2 \$ | | | | | |
| M. Analysis | 100 96.0 °C 75 - 00 - 35 - | 950 °C 100% 00.15 000 000 | 1005 87.8 °C 97.00 000 000 000 000 | | | | | |
| | Step 1 | Step 1 | Step 2 | | | | | > |
| « | Data Collection On | lection Off 🔺 AutoDelta On 🔺 Au | toDelta Off | | | | | |

Choose "Analysis" on the left and click "Analysis Settings" in the upper right corner. Define target Ct settings in the pop up window:

- (A) Select the Mit1C target: Turn <u>off:</u> "Use Default Settings" and "Automatic Threshold". Set the Threshold to **0.05** and choose "Auto Baseline".
- (B) Select the IAC target: Turn <u>off:</u> "Use Default Settings" and "Automatic Threshold". Set the Threshold to 0.05 and choose "Auto Baseline".
- (C) Click "Apply Analysis Settings".

| 📃 New Experiment 🔹 🎯 Open | 😡 Save 🔹 🖆 Close 🧑 Export + 📇 Print Report | | | | | | |
|---------------------------|---|---|--|---|------------------------------|----------------------------|--------|
| Experiment Menu « | Experiment: EXCEL REALTIMEPCR 202 | 30921MIT1C 600_300 +IAC7 250_250WAT | ER SAMPLES6 OOCYSTS ANTIFOAM | Type: Standard Curve Reagents | s: Other Analyz | e Analysis Settings | 0 |
| Setup | Analysis Settings for EXCEL REALTIMEPCR 202 | 30921MIT1C 600_300 +IAC7 250_250WATER SAMPL | ES6 OOCYSTS ANTIFOAM | | | | × |
| Run | Cr Settings Eag Settings Advance | d Settings | | | | | |
| Analysis | Review the default settings for analysis of targets in | this experiment. To edit the default settings, click "Edit Default | Settings." To use different settings for a target, select the targ | et from the table, deselect "Use Default Settings," then chan | nge the settings that are di | splayed. | |
| Amplification Plot | Default CT Settings Default CT settings are used to calculate the CT for targe | ts without custom settings. To edit the default settings, click "Er | dit Default Settings." | | | | |
| Standard Curve | Threshold: 0.01 Baseline Start Cycle: 6 Baseline Er | d Cycle: 15 Edit Default Settings | | | | | |
| Multicomponent Plot | Select a Target | | | | CT Settings for | MIT1C | |
| Dave Data Dist | Target | Threshold | Baseline Start | Baseline End | CT Settings to U | se: 🔲 Use Default Setting: | |
| Kaw Data Plot | IAC | 0.05 | AUTO | AUTO | Automatic | Threshold | |
| QC Summary | MIT1C | 0.05 | AUTO | AUTO | Threshold: 0. | 05 | |
| Multiple Distr Monu | | | | | Automatic | Baseline | |
| | | | | | Baseline Start | Cycle: 3 Cycle: | 15 💠 |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | ~] | | |
| | < | | | | | | > |
| | Bevert to Default Analysis Settings | | | Apply Analysis Settings | | | Cancel |

Click "File" \rightarrow "Save as template..." \rightarrow "Save"



Run Method Using Software v2.0 or 2.3 on the ABI 7500 Fast Instrument

Click "File" \rightarrow "New Experiment" \rightarrow "From Template". Choose the "Cyclospora cayetanensis.edt" template file created according to Appendix 2 instructions above. Under "Setup" on the left click "Plate Setup" and define all unknown samples or DNA extraction controls on the plate on the "Define Targets and Samples" tab by clicking "Add New Sample" until all samples are defined.

| Experiment Menu « | Experiment: Cyclospora cayeta | nensis | | Type: Standard Curve | Reagents: Other | START FAM ()) |
|-----------------------|---|---|------------------------------|----------------------|---|----------------------------|
| Setup | Define Targets and Samples | Assign Targets and Samples | | | | |
| | Instructions: Dates the targets to gut | offer and the exemples to bed in the reaction rel | te | | | |
| Experiment Properties | Define Targets | ning and the participes to worth and reaction pr | | Define Samples | | |
| Plate Setup | | | | | | |
| | Add New Target Add Saved Target | Save Target Delete Target | | Add New Sample | Add Baved Sample Slove Somple. Deleter Gample | |
| Kun Mothod | Target Name | Reporter | Quencher | Color Semple Name | | Color |
| Reaction Setup | MrtC | FAM | ~ None | Sample 1 | | |
| Materials List | LAC . | CYS | V None | Sample 2 | | |
| | | | | Sample 3 | | |
| Run | | | | Sample 1 dluted | | ¥ |
| 1 Analysis | | | | Sample 2 diuted | | |
| Millin, Analysis | | | | Sample 3 diuted | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |
| | Define Distanted Deplicate Oraus | | | | | |
| | Denne Biological Replicate Group | | | | | |
| | Instructions: For each biological repli | cate group in the reaction plate, click Add Blok | ogical Group, then define th | e biological group. | | |
| | Add Biological Group Delete Biologic | a Group | Tourses | | | |
| | Biological Group Name | | Color | | Comments | |
| | | | | | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |
| | L | | | | | |
| 1 | | | | | | Assign Targets and Samples |

Click the "Assign Targets and Samples" tab to define well assignments. Define the NTC wells by selecting three wells and checking the box next to the Mit1C target choosing "N" as task. Also, assign the internal amplification control by selecting all reaction wells and checking the box next to the IAC target choosing "U" as task. Confirm that ROX is selected as a passive reference dye.

| Experiment Menu « | Experiment: Cyclospora cayetanensis | Type: Standard Curve | Reagents: Other | START RUN ()) |
|-----------------------|--|---|-----------------------------------|---------------|
| Setup | Define Targets and Samples Assign Targets and Samples | | | |
| Experiment Properties | To set up standards: Click "Define and Set Up Standards." To set up unknowns: Select wells, assign targe(s), select "U" (Unknown) as the task for ea To set up unknowns: Select wells, assign targe(s), hen select "N" (Negative Combo | ch target assignment, then assign a sample. I) as the task for each target assignment. | | |
| Plate Setup | Assign target(s) to the selected wells. | it View Well Table | 2000 F 2000 | |
| Run Method | Assign Target Task Obenity | Select Wells | s With: - Select Item Select Item | |
| Reaction Setup | MATC Show in Wells V | View Legend | | |
| 🛒 Materials List | | 2 4 5 | 6 7 8 9 10 | 11 12 |
| | | | | |
| Run | | | | |
| Analysis | Mixed 🗓 Unknown 🔯 Standard 🔝 Negative Control B | | | |
| | 14 Define and Set Up Standards | | | |
| | Assign sample(s) to the selected wells. | | | |
| | Assign Sample | | | |
| | Sample 1 | | | |
| | Sample 2 | | | |
| | Sample 3 | | | |
| | V 6 | | | |
| | Assign sample(s) of selected well(s) to biological group. | | | |
| | Assign Biological Group F | | | |
| | | | | |
| | 0 | | | |
| | | | | |
| | Select the dye to use as the passive reference. | | | |
| | ROX | | | |
| | Vielis: 🚺 3 Unknown 📓 | 0 Standard 🔝 3 Negative Control | | 93 Empty |

Define all unknown samples or DNA extraction controls one at a time by selecting three wells for each and checking the box next to the sample name in the "Assign sample(s) to the selected wells" panel.

| Experiment Menu « | Experiment: Cyclospora cayetanensis | | Type: Standard Curve | Reagents: Other | START RUN ()) |
|-----------------------|---|--|---|---|---------------|
| Setup | Define Targets and Samples Assign Targets and Sample | es | | 10 10 | |
| Experiment Properties | To set up standards: Click' Define and Set Up Standards." To set up uninnowns: Select wells, assign target(s), select 'U' (Ur To set up negative controls: Select wells, assign target(s), then s | known) as the task for each target assignment, t elect "N" (Negative Control) as the task for each to | hen assign a sample. Irget assignment. | | |
| Plate Setup | Assign target(s) to the selected wells. | View Plate Layout View Well Ta | ble | | |
| Run Method | Assign Target Task Quantity | | Select We | els With: -Select item - 🗸 -Select item - 🗸 | |
| Reaction Setup | | 9 Show in Wells View Legend | | | 胞胞 |
| Matagala List | | 1 2 3 | 4 5 | 6 7 8 9 | 10 11 12 |
| | | | | | |
| Run | | N METC N METC | | | |
| Analysis | Mitted 1 Unknown S Standard Negative Control | B Sample 1 Sample 1 Samp | | | 10.00 |
| | Assign sample(s) to the selected wells. Assign Dample | C Sample 2 Sample 2 Samp | 4e 2 | | |
| | Sample 1 Sample 2 Sample 3 | D Sample 3 Sample 3 Samp | se 3 | | |
| | Sample 1 dikted Sample 2 dikted Sample 2 dikted Sample 2 dikted | E Sample 1 divided Sample 1 divided Sample 1 | diuled | | |
| | Assign sample(s) of selected well(s) to biological group. | F Semple 2 diluted Semple 2 diluted Semple 2 | t difuted | | |
| | Assign Biological Croup | 0 Sample 3 diuted Sample 3 diuted Sample 3 | diures | | |
| | Select the dye to use as the passive reference. | н | | | |
| " | | Wells: 🔟 3 Unknown 🔯 0 Standard 💟 3 Ne | gative Control | | 93 Empty |

Then select all unknown samples or DNA extraction controls and check the box next to Mit1C and IAC targets choosing "U" as task.

| Experiment Menu « | Experiment: Cy | clospora cayetanensis | | | Туре: | Standard C | urve | | Reag | ents: Other | | | START RU | • • |
|-----------------------|------------------|---|---|---|---|---------------------------|--------------|--------------------|----------------|-------------|---|----|----------|----------|
| Setup | Define Targets | and Samples Assign Targets and S | Samples | | | | | | | | | | | |
| Experiment Properties | instructions: To | o set up standards: Click "Define and Set Up Standards 5 set up unknowns: Select wells, assign target(s), selec 5 set up negative controls. Select wells, assign target(s | s." ct "U" (Unknown) as the t i), then select "V" (Negati | ask for each target a re Control) as the tas | ssignment, then ass sk for each target ass | ion a sample. ignment. | | | | | | | | |
| Plate Setup | Assign target(s | i) to the selected wells. | View Plate | Layout Vie | w Well Table | | | | | | | | | |
| Run Method | Assign Ta | get Task Quantity | ار | | | | Select Wells | With: - Select ite | m - v - Select | item - 🗸 | | | | |
| Reaction Setup | Mit Mit | | 9 Show in V | Vells View | v Legend | | | | | | | | B | |
| Materials List | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| | | | | I IAC | U MC | | | | | | | | | |
| Run | | | | MittC | Milling | | | | | | | | | |
| Analysis | | | Sample | Sample 1 | Sample 1 | | | | | | | | | |
| | Mixed | Unknown Standard Negative Control | | U MILLO | U MIRIC | | | | | | | | | |
| | A Define and | Set Up Standards | | | | | | | | | | - | | |
| | Assign sample | (s) to the selected wells. | | Lac IAC | Lac Sample 2 | | | | | | | | | |
| | Assign | Sample | 11 MRIC | 11 MittC | II MIEIC | | | | | | | | | |
| | | Sample 1 | Sample : | Sample 3 | Sample 3 | | | | | | | | | |
| | | Sample 2 | D Marc | Miesc | MittC | | | | | | | | | |
| | | Sample 3 | | 4 | | | | | | | | | | |
| | | Sample 1 diluted | E II IAC | Inc Sample 1 dilute | I IAC | | | | | | | | | |
| | | Sample 2 diluted | U Marc | U Masc | 1 Mitto | | | | | | | | | |
| | | Sample 3 diluted | Sample 2 dil | ited Sample 2 dilute | d Sample 2 dilutes | | | | | | | | | |
| | | | F HAC | II IAC | I IAC | | | | | | | | | |
| | Assign sample | s) of selected well(s) to biological group. | | | | | | | | | | | | |
| | Assign | Biological Group | G LIAC | Sample 3 difute | id Sample silved | | | | | | | | | |
| | Select the dye | to use as the passive reference. | H | \smile | | | | | | | | | | |
| | R0X v | | | | | | | | | | | | | |
| | | | Wells: 1 21 U | nknown 🔝 0 Stand | tard 🔝 3 Negative (| Control | | | | | | | | 75 Empty |

Define the Positive control (Standard) wells by selecting three wells and checking the box next to the Mit1C target choosing "S" as task and "1000" as quantity. "File" \rightarrow "Save as" \rightarrow Experiment Document Single file (*.eds) with a unique name. Insert plate or tube strips and start the run.

| Experiment Menu « | Experiment: Cyclos | spora cayetanensis | | | Type: : | Standard C | urve | | Reag | jents: Other | | C | START RU | • * 🕐 |
|-----------------------|--|--|--|---|---|-------------------------|--------------|--------------------|----------------|--------------|---|----|----------|----------|
| Setup | Define Targets an | d Samples Assign Targets and Sam | mples | | | | | | | | | | | |
| Experiment Properties | Instructions: To set To set To set | up standards: Click "Define and Set Up Standards." up unknowns: Select wells, assign target(s), select "U" up negative controls: Select wells, assign target(s), the | " (Unknown) as the task in select "N" (Negative C | for each target ass iontrol) as the task | ignment, then assig for each target assi | in a sample. gnment. | | | | | | | | |
| Plate Setup | Assign target(s) to | the selected wells. | View Plate La | ayout View | Well Table | | | | | | | | | |
| Run Method | Assign Target | Task Quarmy | > | | | | Select Wells | With: - Select ite | m - v - Select | Item - 🐱 | | | | |
| Reaction Setup | Mit1C | 1,000 | Show in Well | s 🔻 📳 View I | .egend | | | | | | | | | • |
| | M IAC | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| Materiais List | | | | | | | | | | | | | | |
| Run | | | | Land research | Las contra | | | | | | | | | |
| Analysis | Mixed 🛄 U | Jnimown 💽 Standard 🔝 Negative Control | B IAC MetC | Sample 1 IAC I MintC | Sample 1 LAC MatC | | | | | | | | | |
| | Assign sample(s) to | o the selected wells. | C Sample 2 | Sample 2 | Sample 2 | | | | | | | | | |
| 7.20 | Assign | Sample | | In manual | C. marce | | | | | | | | | |
| | | Sample 1 | Sample 3 | Sample 3 | Sample 3 | | | | | | | | | |
| | | Sample 3 | U MittC | U MietC | U Mitto | | | | | | | | | |
| | | Sample 1 diluted | Sample 1 diluted | Sample 1 diluted | Sample 1 diluted | | | | | | | | | |
| | | Sample 2 diluted | E U MRIC | U Miesc | U MetC | | | | | | | | | |
| | | Sample 3 diluted | Sample 2 diluted | Sample 2 diluted | Sample 2 diluted | | | | | | | | | |
| | Assign sample(s) o | of selected well(s) to biological group. | U MittC | U Miesc | U MittC | | | | | | | | | |
| | Assign | Biological Group | 0 Sample 3 diluted | Sample 3 diluted | Sample 3 diluted | | | | | | | | | |
| | Select the dye to u | ise as the passive reference. | H MAC H MAC 1E3 | Matc 1E3 | Matc 1E3 |) | | | | | | | | |
| | | | Wells: U 24 Unkn | own <u>S</u> 3 Standa | rd 🔣 3 Negative C | ontrol | | | | | | | | 72 Empty |

Analysis Using Software v2.0 or 2.3 on the ABI 7500 FastInstrument

When the run is complete select "Amplification Plot" under "Analysis" on the left. Ensure that all wells are selected on the "View Plate Layout" tab to the right of the amplification curves. In the options panel below the amplification curves, select the "IAC" target and check that the show threshold and baseline boxes are both checked. Verify threshold and baseline settings are accurate as defined in the run template above.



Next, select the "Mit1C" target in the options panel and check that the show threshold and baseline boxes are checked. Verify threshold and baseline settings are accurate as defined in the run template above.



Review the amplification plots and Ct's for each target. Verify that all criteria for a valid experimental run are met as defined in the "Interpretation of Results" section of the protocol.

Then, assure that all reaction wells on the plate are selected by clicking the upper left corner of the plate layout. Click "Export" to open the Export Tool window.

Standard Operation Procedure Dead-end Ultrafiltration for the Detection of *Cyclospora cayetanensis* from Agricultural Water



On the "Export Properties" tab, select the following:

- 1. Select "Results" only.
- 2. Choose "One File"
- *3.* Name: *use experiment name*. Location: *define a location of your choice*. File type: *choose ".xls"*

Click the "Customize Export" tab and select the following results content: Well, Sample Name, Target Name, Task, Reporter, Ct, Ct Mean, Ct SD.

Click on the "Target Name" column header to sort the table by target name. Click "Start Export". Close the export tool.

| sults Export ell Sample 116 116 116 | I N. Target Na ^{r.1} IAC IAC | Jask UNKNOWN | Reporter CY5 | Ст | Ct Mean | File Name: Excel | Real Ime PCR 20222204 ccayMIT1C+ IAC Perfecta Upper Marlboro_data File | e Type: 휨 |
|---|---|---|---|---|---|---|---|--|
| sults Export ell Sample 116 116 116 | IAC IAC |) ask UNKNOWN | Reporter CY5 | Ст | Ct Mean | CT SD | | |
| ell Sample 116 116 116 | N. Target Na ^r IAC IAC | Jask UNKNOWN | Reporter CY5 | Ст | Ct Mean | CT SD | | |
| 116 116 116 | IAC IAC | UNKNOWN | CY5 | | | | | |
| 116 | IAC | | 0.000 | 30.82815 | 30.860903 | 0.0654539 | | |
| 116 | 11.00 | UNKNOWN | CY5 | 30.81829 | 30.860903 | 0.0654539 | | |
| 447 | IAC | UNKNOWN | CYS | 30.936268 | 30.860903 | 0.0654539 | | |
| 117 | IAC | LINKNOWN | CYS | 30.008905 | 30.761206 | 0.10648773 | | |
| 117 | IAC | UNKNOWN | CY5 | 30,877712 | 30 761206 | 0 10648773 | | |
| 118 | IAC | UNKNOWN | CY5 | 30,912022 | 30 697931 | 0 1854309 | | |
| 118 | IAC | UNKNOWN | CY5 | 30.587885 | 30.697931 | 0.1854309 | | |
| 118 | IAC | UNKNOWN | CY5 | 30.593891 | 30.697931 | 0.1854309 | | |
| 0 119 | IAC | UNKNOWN | CY5 | 31.093542 | 30.831705 | 0.23527534 | | |
| 1 119 | IAC | UNKNOWN | CY5 | 30.763517 | 30.831705 | 0.23527534 | | |
| 2 119 | IAC | UNKNOWN | CY5 | 30.638054 | 30.831705 | 0.23527534 | | |
| 120 | IAC | UNKNOWN | CY5 | 30.98061 | 30.977295 | 0.0035693 | | |
| 120 | IAC | UNKNOWN | CY5 | 30.977758 | 30.977295 | 0.0035693 | | |
| 120 | IAC | UNKNOWN | CY5 | 30.973516 | 30.977295 | 0.0035693 | | |
| 121 | IAC | UNKNOWN | CY5 | 30.696188 | 30.950525 | 0.23369096 | | |
| 121 | IAC | UNKNOWN | CTS | 30.99962 | 30.950525 | 0.23369096 | | |
| 121 | IAC | UNKNOWN | CTS | 31.155// | 30.950525 | 0.23369096 | | |
| 122 | IAC | LINKNOWN | CVE | 21 400745 | 31.0744 | 0.20495446 | | |
| 122 | IAC | LINKNOWN | CY5 | 30 874857 | 31 0744 | 0 28495446 | | |
| 0 123 | IAC | UNKNOWN | CY5 | 31 260004 | 31 083635 | 0 20481487 | | |
| 1 123 | IAC | UNKNOWN | CY5 | 30.858997 | 31.083635 | 0.20481487 | | |
| 2 123 | IAC | UNKNOWN | CY5 | 31.131907 | 31.083635 | 0.20481487 | | |
| 124 | IAC | UNKNOWN | CY5 | 31.20964 | 31.11142 | 0.17755374 | | |
| 124 | IAC | UNKNOWN | CY5 | 31.218159 | 31.11142 | 0.17755374 | | |
| | 117 118 118 118 0 119 1 119 2 119 120 120 120 121 121 121 122 122 122 122 | 117 IAC 118 IAC 118 IAC 118 IAC 118 IAC 119 IAC 119 IAC 120 IAC 120 IAC 120 IAC 121 IAC 122 IAC 121 IAC 122 IAC 122 IAC 122 IAC 123 IAC 124 IAC 125 IAC 122 IAC 123 IAC 124 IAC 123 IAC 124 IAC 124 IAC | 1177 IAC UHKNOWN 118 IAC UHKNOWN 118 IAC UHKNOWN 118 IAC UHKNOWN 118 IAC UHKNOWN 119 IAC UHKNOWN 119 IAC UHKNOWN 120 IAC UHKNOWN 120 IAC UHKNOWN 120 IAC UHKNOWN 121 IAC UHKNOWN 121 IAC UHKNOWN 121 IAC UHKNOWN 122 IAC UHKNOWN 121 IAC UHKNOWN 122 IAC UHKNOWN 122 IAC UHKNOWN 122 IAC UHKNOWN 122 IAC UHKNOWN 123 IAC UHKNOWN 123 IAC UHKNOWN 123 IAC UHKNOWN 124 IAC UHKNOWN 124 IAC UH | 117 IAC UBRNOWN CY5 118 IAC UBRNOWN CY5 118 IAC UBRNOWN CY5 118 IAC UBRNOWN CY5 118 IAC UBRNOWN CY5 119 IAC UBRNOWN CY5 120 IAC UBRNOWN CY5 120 IAC UBRNOWN CY5 120 IAC UBRNOWN CY5 120 IAC UBRNOWN CY5 121 IAC UBRNOWN CY5 121 IAC UBRNOWN CY5 121 IAC UBRNOWN CY5 122 IAC UBRNOWN CY5 122 IAC UBRNOWN CY5 122 IAC UBRNOWN CY5 123 IAC UBRNOWN CY5 123 IAC UBRNOWN CY5 124 IAC UBRNOWN CY5 | 117 M.C. UNRNOWN CYS 30.877712 118 M.C. UNNNOWN CYS 30.877712 118 M.C. UNNNOWN CYS 30.912022 118 M.C. UNNNOWN CYS 30.987818 118 M.C. UNNNOWN CYS 30.987818 119 M.C. UNNNOWN CYS 31.083542 1 119 M.C. UNNNOWN CYS 30.93061 120 M.C. UNNNOWN CYS 30.93051 120 M.C. UNNNOWN CYS 30.977518 121 M.C. UNNNOWN CYS 30.99851 121 M.C. UNNNOWN CYS 30.997516 121 M.C. UNNNOWN CYS 30.997551 122 M.C. UNNNOWN CYS 30.997551 122 M.C. UNNNOWN CYS 31.400745 122 M.C. UNNNOWN CYS 31.400745 | 117 IAC UNKNOWN CY5 30.877712 30.78720 118 IAC UNKNOWN CY5 30.28720 30.879711 118 IAC UNKNOWN CY5 30.587885 30.697931 118 IAC UNKNOWN CY5 30.587885 30.697931 119 IAC UNKNOWN CY5 30.58381 30.697931 1 119 IAC UNKNOWN CY5 30.53814 30.83705 120 IAC UNKNOWN CY5 30.538064 30.83705 120 IAC UNKNOWN CY5 30.537156 30.977295 120 IAC UNKNOWN CY5 30.597255 30.977295 121 IAC UNKNOWN CY5 30.590525 121 IAC UNKNOWN CY5 30.590525 121 IAC UNKNOWN CY5 31.577 30.590525 122 IAC UNKNOWN CY5 31.54759 31.0744 | 117 IAC UNKNOWN CY5 30.877712 30.781208 0.104.8473 118 IAC UNKNOWN CY5 30.987712 30.781208 1084.873 118 IAC UNKNOWN CY5 30.987818 30.897931 11854309 118 IAC UNKNOWN CY5 30.98391 30.87931 11854309 119 IAC UNKNOWN CY5 31.093542 20.831705 2.2527534 1 119 IAC UNKNOWN CY5 30.638054 30.831705 2.2527534 120 IAC UNKNOWN CY5 30.638054 30.831705 2.32527534 120 IAC UNKNOWN CY5 30.9377516 30.977285 0.0035693. 120 IAC UNKNOWN CY5 30.937516 30.977285 0.035693. 121 IAC UNKNOWN CY5 31.97796 3.0350525 2.2380096 121 IAC UNKNOWN CY5 3.1407744 2.24495446< | 117 IAC UNKNOWN CYS 30.877712 30.78120 0.1048073 118 IAC UNKNOWN CYS 30.587865 30.697931 0.185409 118 IAC UNKNOWN CYS 30.587865 30.697931 0.185409 118 IAC UNKNOWN CYS 30.587865 30.697931 0.185409 0 119 IAC UNKNOWN CYS 30.53751 0.3831705 0.23527534 1 119 IAC UNKNOWN CYS 30.638054 30.831705 0.23527534 1 100 IAC UNKNOWN CYS 30.638054 30.637725 0.035693. 120 IAC UNKNOWN CYS 30.937756 30.977255 0.035693. 121 IAC UNKNOWN CYS 30.936725 0.2339006 121 IAC UNKNOWN CYS 30.95652 0.2339006 122 IAC UNKNOWN CYS 30.966759 31.30744 0.2 |

7. Appendix 6. ABI 7500 Fast v1.4 Method.

Define Protocol as a Template

Turn on the ABI 7500 FAST Real-Time PCR instrument and computer. Open 7500 Fast System Software v1.4. It shows "Quick start" \rightarrow click "cancel". From "File" \rightarrow New \rightarrow New Document Wizard (Define Document) \rightarrow "Next: \rightarrow "Select Detectors" \rightarrow "Next" \rightarrow "Set Sample Plate" \rightarrow "Finish" \rightarrow Click on the "Instrument" tab.

1. Key in the following parameters: **Stage 1: Reps:1** 95 °C; 3:00 **Stage 2: Reps: 40** 95 °C; 0:15 67 °C; 1:00

2. Sample Volume (μL): 20 Run Mode: Fast 7500 Data collection: Stage 2, step 2 (67 °C @ 1:00)

| 7500 Fast System SDS Software - (Cyclospora cayetanens | is (Standard Curve)] | | |
|---|---------------------------------|-----------|-----|
| File view loois instrument Analysis Window | нер | | - 5 |
| Setup Vinstrument VResults | | | |
| Instrument Control | Temperature | 7 | |
| Start Estimated Time Remaining (hh:nm); | Sample: Heat Sink: | | |
| Stop | Cover: Block: | | |
| | Cycle | | |
| Statue: | Stage: Rep Time (musc) | | |
| Extend | State: | | |
| | | | |
| Thermal Cycler Protocol | | | |
| Thermal Profile Auto Increment Ramp Rate | | | |
| Stage 1 Stage 2 | | | |
| | | | |
| 3:00 0:15 | | | |
| 67.0 | | | |
| 1:00 | | | |
| | | | |
| | | | |
| | | | |
| the second se | | | |
| Add Cycle Add Hold Add Step Add D | Dissociation Stage Delete Help | | |
| Settings | | | |
| Sample Volume (µL) : 24 | | | |
| Run Mode Fast 7500 | Expert Mode Select/View Filters | | |
| Data Collection : Stage 2. Step 2 (67.0 @ 0.30 | 1 - | | |
| | | | |
| | | | |
| | | | |
| | | | |
| | | | |
| | | Discourse | |

Standard Operation Procedure Dead-end Ultrafiltration for the Detection of *Cyclospora cayetanensis* from Agricultural Water



Create Detectors: Mit1C & IAC (internal amplification control)

Select "Tools" \rightarrow "Detector Manager" \rightarrow File \rightarrow "New" \rightarrow "Name": MIt1C "Reporter Dye; FAM. "Quencher Dye": None, "Color": Green \rightarrow OK. Click "Create Another" \rightarrow File \rightarrow New \rightarrow "Name": IAC, Reporter Dye: Cy5, "Quencher Dye": None, "Color": Purple \rightarrow "OK". Click "OK" on "New detector" window.

| 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | |
|---|------------|-----------------|---------------|--------|----|----------|---------------|----|---|
| | Detector M | Manager List | New Detector | | | | | 8 | |
| | Find: | | Name: | Mit1C | | | | _ | |
| | Dete | ector Name | Description: | | | | Last Modified | | _ |
| | | | Reporter Dye: | FAM | | - | | | |
| | | | Quencher Dye: | (none) | | <u> </u> | | | |
| | | | Notes: | | | | | | |
| | | | | | | | | - | - |
| | | | | | | | | | |
| | | | | | | | | | |
| | File | Add T | 0 | | | 1 | | | |
| | | | Create A | nother | UK | Cancel | | 1 | |

Highlight both the Mit1C and IAC detectors. Click "Add to Plate document". Click "OK" after each detector is added to the plate. Click "Done" on "Detector Manager Window".

| 🗋 🖬 🖨 🖪 🖾 🖬 🕨 | | 9 | | | | | |
|---|------------------|----------------------------------|----------------------|-------|-----------------------------|-----|--|
| Setup VInstrument VResults | | | | | | | |
| Instrument Control Stat Stop | ng (hh:nm): | Temperature Sample: Cover: | Heat Sink: Block: | | | | |
| Disconnect Status | Detector Manager | . Luca | | 8 | F | × | |
| | Detector List | | | | | | |
| Extend | Find: | | | | | | |
| Thermal Cycler Protocol | Detector Name | Description Reporter | Quencher Color | Notes | Last Modified | 7 I | |
| There all Parents in the second second | Mit1C | FAM | (none) | | 2015/12/01 16:34:52 | | |
| Triemal Frone Auto Increment Rai | invite 2 C | | | | | | |
| Stage 1 Stage 2 | IAC | CY5 | (none) | | 2015/12/01 16:35:55 | | |
| Stage 1 Stage 2 Reps: 1 Reps: 40 | IAC | CY5 | (none) | | 2015/12/01 16:35:55 | | |
| Stage 1 Stage 2 Reps: 1 Reps: 40 | IAC | CYS | (hone) | | 2015/12/01 16:35:55 | | |
| Inemial Flow Auto Increment Hai Stage 1 Stage 2 Reps: 1 Reps: 40 35.0 95.0 35.0 95.0 32.00 10.15 15 10.15 <td< td=""><td>IAC</td><td>CY5</td><td>(none)</td><td></td><td>2015/12/01 16:35:55</td><td></td><td></td></td<> | IAC | CY5 | (none) | | 2015/12/01 16:35:55 | | |
| Stage 1 Stage 2 Reps: 1 Reps: 40 95.0 95.0 3:00 0:15 | IAC | CYS | (none) | | 2015/12/01 16:35:55 | | |
| Bige 1 Stage 2 Reps: 1 95.0 95.0 3:00 0:15 | IAC | CY5 | (none) | | 2015/12/01 16:35:55 | | |
| Stage 1 Stage 2 Stage 2 <t< td=""><td>AC</td><td>CY5</td><td>(10000)</td><td></td><td>2015/12/01 16:35:55</td><td></td><td></td></t<> | AC | CY5 | (10000) | | 2015/12/01 16:35:55 | | |
| Stage 1 Stage 2 Rep: The stage 2 The stage 2 <thte 2<="" stage="" th=""> <thte 2<="" stage="" th=""> <t< td=""><td>AC</td><td>CYS</td><td>(10000)</td><td></td><td>2015/12/01 16:35:55</td><td></td><td></td></t<></thte></thte> | AC | CYS | (10000) | | 2015/12/01 16:35:55 | | |
| Bage 1 Reps 40 Bage 2 Reps 40 Bage 3 Reps 40 Bage 3 Reps 40 | AC | CYS | (1000e) | | 2015/12/01 16:35:55 | | |
| Stage 1 Sage 2 Rept 1 Rept 40 920 950 93:00 90:15 | | CYS | (none) | | 2015/12/01 16:35:55 | | |
| Bage 1 Rep: [10:05mm], Ha Stage 1 Rep: [40] 950 950 33:00 [0:15] | iac | CY3 | (aone) | | 2019/12/01 18:35:55 | | |
| International Folds Autoincement, Hai Stage 1 Sage 2 Mass 1 Autoincement, Hai Sage 3 Sage 2 Mass 1 Autoincement, Hai Sage 3 Sage 2 Mass 1 Autoincement, Hai Sage 3 Sage 2 Mass 2 Sage 2 Autoin 1 Autoin 2 | | CYS | (aone) | | 2015/12/01 18:35:55 | | |
| Add Cycle Add Hold Add | Fjie - Add T | CYS Plato Document | (aone) | | 2019/12/01 18:35:55 | | |
| Add Cycle Add Hold Add | Fije - Add T | CTS | (aone) | | 2015/12/01 18 35 55 | | |
| Add Cycle Add Hold Add Cycle Sample College | File - Add T | CYS | (terre) | | 2015/12/01 16 35:55 Done | | |
| Add Cycle Add Hold Add Add Cycle Add Hold Add Sample Add Hold Add Sample Add Hold Add Petring: Sample Valume (µ1): [20] | File - Add T | CTS | (cone) | | 2015/12/01 16 32 55 | | |
| Add Cycle Add Hold Add Add Cycle Add Hold Add Sample Sample Zor | File - Add T | Plate Document | (cone) | | 2015/12/01 16 32 55 | | |

Save Protocol as a SDS Template [*sdt] in Drive D: \rightarrow Applied Biosystems \rightarrow 7500 system \rightarrow templates \rightarrow File Name: "Cyclospora cayetanensis".

Start a new Run

Turn on the ABI 7500 FAST Real-Time PCR instrument and computer. Open 7500 Fast System Software v1.4

From "Quick Startup" \rightarrow Click "Create New Document" to open "New Document Wizard" and "Define Document".

Assay: Standard Curve (Absolute Quantitation)

Template: Click on "Browse" \rightarrow navigate to "templates" folder \rightarrow Select "Cyclospora cayetanensis.sdt"

Plate Name: "Cyclospora Test 1" \rightarrow "Finish"

| Container [| | | | |
|--------------|-----------------------------|---|--------|---|
| container. J | 96-Well Clear | | | |
| Template: | Cyclospora cayetanensis.sdt | • | Browse | |
| lun Mode: | Fast 7500 | * | | |
| Operator: | helen.murphy | | | |
| omments: | SDS v1.5.1 | | | * |
| | | | | Ŧ |

From "View" \rightarrow Select "Well inspector" to define well assignments. Define the no template control wells by selecting three wells and type "NTC" in the "Sample Name" field on the "Well Inspector" window. Check the "Mit1C" detector choosing "NTC" as "Task". Define all unknown samples and DNA extraction control samples in the same manner but choose "Unknown" as "Task".



Define the positive control in the same manner but choose "Standard" as "Task" and enter "1000" as "Quantity".



Assign the IAC detector to all wells by highlighting all reaction wells" Check the "IAC" detector in the "Well Inspector" window and choose "unknown" as "Task". Close the "Well Inspector" window. From the "File" menu \rightarrow "Save" to choose a location to save the run file.



Insert the plate to begin the run and click the "Instrument" tab \rightarrow "Start".

| 7500 Fast System SDS Software - [Cyclospora cayetanensis | (Standard Curve)] | | |
|---|-------------------------------------|--------------|----------|
| File View Tools Instrument Analysis Window | Help | | = 5 % |
| | | | |
| / Setup y Instrument (Results) | | | |
| -Instrument Control | Temperature Council Units Circle | | |
| Start Estimated Time Remaining (hn:nm): | Cover: Block: | | |
| Stop | - fucle | 1 | |
| Disconnect Statue: | Stage: Rep | | |
| The second se | Tine (nm:ss): Step | | |
| L XICTIC | State: | | |
| Thermal Cycler Protocol | | | |
| Thermal Profile Auto Increment Ramp Rate | | | |
| Stage 1 Stage 2 | | | |
| Reps: 1 Reps: 40 | | | |
| 95.0 95.0 | | | |
| 67.0 | | | |
| 1:00 | | | |
| | | | |
| | | | |
| | | | |
| Contract Contract Contract Contract | | | |
| Add Lyce Add Hold Add Step Add Dis | Esociation Stage Delete Help | | |
| Settings | | | |
| Sample Volume (LL): | | | |
| Run Mode Fast 7500 | Expert Mode Select/View Filters | | |
| Data Collection : Stage 2. Step 2 (67.0 @ 0:30) | | | |
| | | | |
| | | 1 | |
| | | | |
| | | | |
| | | | l have l |
| Keady | | Disconnected | NUM // |

Results

Click on the "Results" tab \rightarrow Select the "Amplification Plot" tab. Select all wells in the lower portion of the window. On the "Data" drop down menu on the right selection choose "**Delta Rn vs. Cycle**", and then define Analysis Settings for each target:

Select Detector "Mit1C" and Line Color "Detector Color". In the "Analysis Settings" box select:

"Manual Ct" Threshold = 0.05 "Auto Baseline"

Select Detector "IAC" and Line Color "Detector Color". In the Analysis Settings" box select:

"Manual Ct" Threshold = 0.05 "Auto Baseline"

Click "Analyze". Save the run analysis results as an SDS Document (*SDS).



Standard Operation Procedure

Dead-end Ultrafiltration for the Detection of Cyclospora cayetanensis from Agricultural Water

Click on the "Report" tab. Assure that all wells are still detected in the lower portion of the window.

Click on the "Report" tab. Assure that all wells are still detected in the lower portion of the window.



Open a window to define the report settings by clicking the "Report Settings" icon.

Click OK on the report settings window.

Review the Amplification Plots and Ct's for each target. Verify that all criteria for a valid experimental run are met as defined in the "Interpretation of Results" section of the protocol.

Save an **Experimental Results Report** by clicking the "File" drop down menu \rightarrow "Export" \rightarrow "Results". On the "Select Results Export File" window choose a location to save the results file.

File name: *use experiment run name*. Save as type: *Results Export File (*.cvs)*.



Click "Save". Check both options on the "Export Settings" window that appears \rightarrow click "OK".