Recovery and Detection of *Cyclospora cayetanensis* from Agricultural Water

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1. Introduction

Cyclospora cayetanensis is a protozoan parasite that causes human diarrheal disease called cyclosporiasis (1). The symptoms of cyclosporiasis include explosive watery diarrhea, weight loss, cramping, bloating, increasing gas, nausea, fatigue and loss of appetite. Although no fatal cases of this disease have been reported, the infection can lead to hospitalization. Outbreaks and sporadic cases occur annually associated with the consumption of fresh produce such as basil, romaine, cilantro and berries and since 2013, there has been a surge in the number of reported cases of cyclosporiasis. In 2018, 2299 cases of domestically acquired cyclosporiasis were reported to the CDC with approximately one-third of the cases associated with two large multistate outbreaks linked to prepackaged vegetable trays and salads (2). It is not clear how produce becomes contaminated, but it is possible that agricultural water may play a major role in contaminating crops during the irrigation process. Therefore, laboratory methods that can be used to detect the parasite in food and water are essential to identify potential sources of infection and provide critical support for outbreak investigations.

A method for recovering *Cyclospora* and *Cryptosporidium* oocysts from water was published in the FDA BAM, Chapter 19A (11) in 2004. The methodology described in Chapter 19A relies on the use of Envirochek™ sampling capsules to recover *Cyclospora* oocysts from large volume water samples by filtration and is based on a protocol published by the EPA, Method 1623 (3), for isolation of *Cryptosporidium* and *Giardia* from drinking water. In 2013, the US FDA was involved in outbreak investigations which demanded the testing of turbid agricultural water collected from farms that harvested crops epidemiologically linked to cyclosporiasis outbreaks. The Envirochek® filters employed in this method clogged rapidly during the filtration and no effective analysis could be performed on the samples collected. In 2015, a study was initiated to define and compare performance characteristics of dead-end ultrafiltration (DEUF), continuous flow centrifugation (CFC) and the EPA Method 1623 for recovery of *Cyclospora* from agricultural water. It was demonstrated that the hollow fiber filters employed in the DEUF method was less prone to clogging in low quality water such as agricultural water having significantly high amounts of small debris and sediment. The DEUF method is also more practical for field applications and the filters are most cost effective.

In July of 2019, a multi laboratory validation study for a new method based on DEUF for recovery and detection of *Cyclospora* oocysts from agricultural water samples was approved. In the validation study, it was demonstrated that the approach is very sensitive and specific for the detection of *C. cayetanensis*, with a detection limit as low as 6 *C. cayetanensis* oocysts in 10 L of agricultural water samples. The analysis protocol is executed using the following methodology: 1. Recovery of oocysts by DEUF, 2. Disruption of oocysts and extraction of *Cyclospora* DNA, 3. Molecular detection by a real-time PCR assay targeting the *C. cayetanensis* 18S rRNA gene according to BAM Chapter 19B with minor modifications.

2. Supplies and Equipment List

2.1. Laboratory Facilities

A. A laboratory bench for the DEUF backflush 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 positive results due to laboratory contamination.

2.2. Equipment and Supplies

A. Geopump™ Peristaltic Pump Series II Package (includes easy-load II pump head and batteries), 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

C. Laboratory lift stand for pump (optional)

D. Laboratory ring stand and clamps to support filter during backflush procedure

E. 1 L glass beaker for collecting backflushed sample, Fisher, Cat. No. NC9370942

F. 1-L Nalgene bottles, Fisher, Cat. No. 02-893D

G. 500-mL Nalgene bottles, Fisher, Cat No. 02-893C

H. Centrifuge (or equivalent assembly) capable of spinning 15 ml conical tubes and 175 ml centrifuge bottles at 4000 x g:

i. Thermo Scientific™ Sorvall™ Legend™ 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 Conical Tube Adapters, set of 4

ii. 250/175ml bottle adapters for TX-1000 Swinging Bucket Rotor, sold as set of 4, Fisher, Cat. No. 75005392. Not included in above centrifuge package, must be ordered separately.

I. Vacuum aspiration system (or equivalent assembly):

i. M-Vac 4L Polypropylene Bottle and Lid with barbed tube fittings (1800mm tubing included), Argos Technologies, Cat. No. EV432

ii. M-Vac Stainless Steel Stand, Argos Technologies, Cat. No. EV445

iii. Vacuum pump or house vacuum

iv. Glass Pasteur pipets for vacuum aspiration of supernatants

J. Autoclavable Polypropylene Centrifuge Tubes, 225 ml, Fisher, Cat. No. 05-538-61

K. Conical Sterile Polypropylene Centrifuge Tubes, 15 ml, Fisher, Cat. No. 339650

L. FastPrep®-24 Instrument (bead beater), 5G or Classic, 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. Eppendorf Tubes® with snap cap, 5.0 mL Biopur, Fisher, Cat. No. 14-282-303
T. Sterile applicator Sticks, Fisher, Cat. No. 22-029-641
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 8-Tube Strip, 0.1 mL and MicroAmp® Optical 8-Cap Strips, Cat Nos. 4358293 and 4323032, (ThermoFisher Scientific) or equivalent, ***OR*** 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 ***OR*** a mini-centrifuge capable of spinning 0.1 mL tube strips
Y. Analytical Balance (0.01g resolution)
Z. Magnetic Stir Plate
AA. Teflon coated stir bar
BB. Digital laboratory timer
CC. Scalpel or scissors
DD. Pliers
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
LL. 20 µL aerosol resistant pipette tips
MM. 200 µL aerosol resistant pipette tips
NN. 1000 µL aerosol resistant pipette tips
OO. Transfer pipettes
PP. 15 ml conical tubes
QQ. Appropriate personal protective equipment (PPE)

2.3. Reagents

A. Sodium polyphosphate (NaPP), Sigma, Cat. No. 305553-25G
B. Tween 80, Fisher, Cat. No. T164-500 ml
C. Antifoam Y-30 emulsion, Sigma, Cat. No. A5758-250 ml
D. Dulbecco's Phosphate Buffered Saline (DPBS), 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
F. QIAquick® PCR Purification Kit (50), 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, (commercially prepared or refer to FDA BAM Chapter 19B, Appendix 2)
J. DNA AWAY Surface Decontaminant, Fisher, Cat. No. 21-236-28 ***OR*** 10% bleach
K. Real-time PCR reagents (refer to FDA BAM Chapter 19B for additional details and ordering instructions)
    i. QuantiFast Multiplex PCR kit (400), Qiagen, Cat. No. 204654
    ii. Negative control (Water, included in the QuantiFast Multiplex PCR Kit above)
    iii. Real-time PCR primers for *Cyclospora* and IAC amplification
    iv. Real-time PCR probes for *Cyclospora* and IAC detection
    v. IAC Target (HMultra130-synIAC)
    vi. Positive control DNA (HMgBlock135m)

3. 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. Up to four additional filters can be processed the next day prior to the DNA extraction step.

3.1. Backflush - Recovery of *Cyclospora cayetanensis* from Dead-End Ultrafilters

A. Preparation of Backflush solution (0.5% Tween 80/0.01% NaPP/0.001% Antifoam Y-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 Y-30 stock solution:
   a. Add 10 ml DI water to a 15 ml conical tube;
   b. Add 1 g NaPP and 100 µL Antifoam Y-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 µL of the 10% NaPP/1% Antifoam Y-30 stock solution to the water;
   c. Add 2.5 ml of Tween 80 with a 10 ml serological pipette;
   d. Stir with a sterile stir bar for at least 5 min to dissolve the Tween 80.

B. Backflush Procedure

i. Select filters to be processed on the first day. **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 1 or 2 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 red end of the filter is on the top.
   c. Remove the side port cap from the red end of the filter.
   d. Push L/S 36 tubing onto the side port and secure with a SNP 12 hose clamp.
   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 blue end port over a 1 L sterile bottle or beaker, and remove the 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 10 seconds.

v. Measure and record backflush volume or weight, if necessary.

vi. Repeat the backflush procedure for the remaining filters of the set.

vii. Proceed to the concentration procedure.

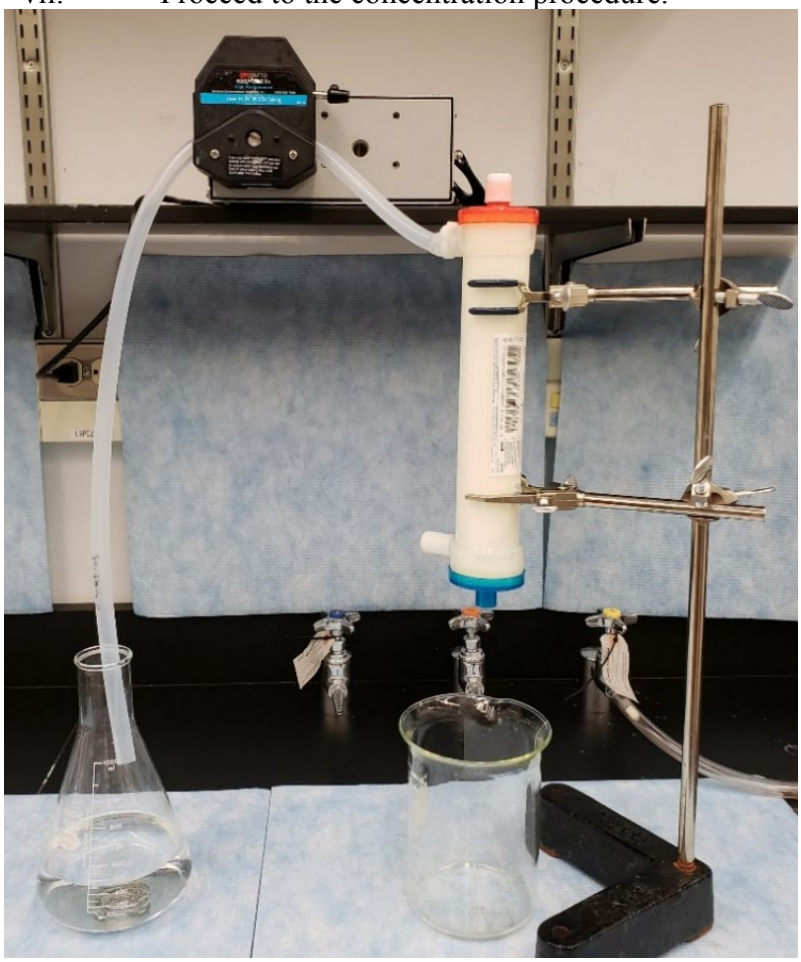

Figure 1 Backflush assembly

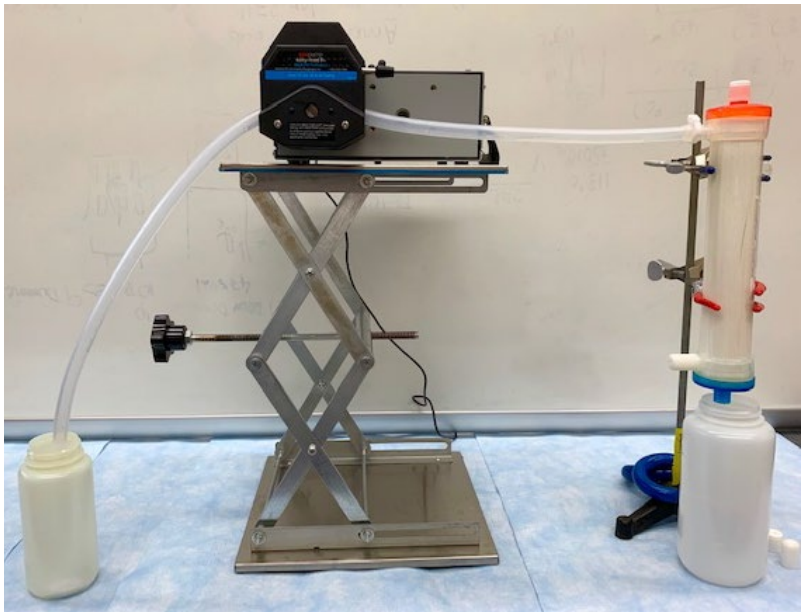

Figure 2 Backflush Assembly

3.2. Concentrating *Cyclospora cayetanensis* from Backflush Effluent

A. Each filter yields approximately 600 ml of effluent. Divide the effluent collected from each filter into four 225ml conical centrifuge bottles. You will have a total of four 225 ml conical bottles for each filter.

B. Balance the four conical bottles with 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 ~25-30 ml of the supernatant from each centrifuge bottle using a glass Pasteur pipet connected to a vacuum aspirator or a 50 ml serological pipette. A vacuum aspiration system will save time.

D. Re-suspend each pellet in the remaining supernatant by pipetting up/down and vortexing. Transfer approximately 13-15 ml aliquots of the re-suspended material from the four 225 ml centrifuge bottles to multiple 15 ml centrifuge tubes. Use a single 10 ml aliquot of PBS to rinse all four empty bottles one by one and add the rinse material to the 15 ml tubes. Between eight to ten 15 ml centrifuge tubes will be required to accommodate the re-suspended material and the rinse from the four 225 ml centrifuge bottles for each filter. 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^{o}C overnight before continuing the procedure.

E. Centrifuge the 15 ml centrifuge tubes at 4,000 x g for 45 min.

F. After the centrifugation, aspirate all but ~1.5 ml of the supernatant from each of the 15 ml tubes to waste without disturbing the pellets.

G. Re-suspend the pellets in the remaining supernatant and pool the pellets for each filter by transferring the re-suspended material into a single 15 ml tube. Use a single 2 ml aliquot of PBS to rinse all of the eight to ten tubes one by one and add the rinse material to the single 15 ml tube. Centrifuge at 4,000 x g for 45 min.

H. Carefully aspirate all but approximately 300 µl of the supernatant above the pellet in each of the 15 ml tubes to waste without disturbing the pellets. The sample in each 15 ml tube represents the sample from each ultrafilter.

I. Store the pellets at 4°C for no more than 24 hours.

J. Autoclave the supernatant waste.

3.3. DNA Isolation and Purification

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

i. 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 vi and vii 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 ix 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 sec.

ii. Assemble the sample pellets in the 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 122 µl MT buffer to each of the pellets in the 15 ml conical tubes (and to the DNA extraction control LME tube). (FastDNA kit protocol step 3).

iv. Add 830 µl Sodium Phosphate Buffer (SPB) to the mixture in the 15 ml conical tubes (and to the DNA extraction control LME tube). Mix each of the pellets in the 15 ml conical tubes with the buffers using a sterile wooden stick or a sterile transfer pipet. Try to minimize generating many bubbles.

v. Carefully transfer the mixture (pellet, MT and SPB buffers) from each 15 ml conical tube into a LME tube using a sterile transfer pipette. Screw the caps on securely. The LME tubes have limited capacity. 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 the Figure 3. If the total volume is too large, split the mixture in two LME tubes. The sample can be recombined later as described in step xv

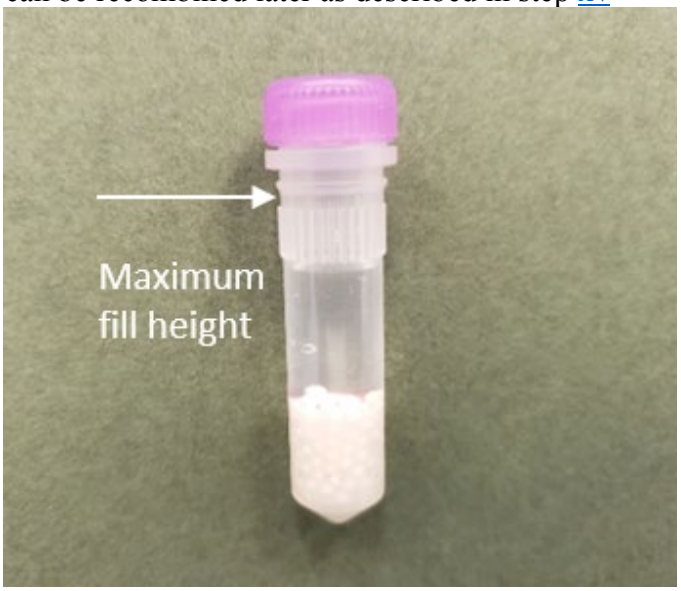

Figure 3 Maximum fill height for LME tubes

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 sec. Immediately remove the sample holder containing the tubes from the instrument and place on ice for 3 minutes.

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

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 3.3.A.i.d. They are NOT included in the kit). Add 250 µl 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).

xi. 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 Eppendorf 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 µl 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). Empty the catch tube. *If a sample was split into two 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 µl prepared SEWS-M to each filter. Gently re-suspend each by stirring (with a P-200 tip) for ~ 10 sec. 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, some liquid won't go through and 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 (kit protocol step 15).

xx. Add 75 µl DES to the matrix in the spin filter. Re-suspend the Binding Matrix by gently stirring with a P-10 pipet tip. Do not poke 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.

B. 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 µl of Buffer PB to the DNA sample and mix. If the color of the mixture is orange or violet, add 10 µl 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. Centrifuge the QIAquick column once more for 1 min to remove residual wash buffer.

vi. Place each QIAquick column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 30 µl 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 seconds to elute the purified DNA. Discard the columns and cap the tubes.

vii. 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.

3.4. Detection of *Cyclospora cayetanensis* by Real-time PCR Targeting the 18S rRNA Gene

A. Real-time PCR

i. For real-time PCR detection of *C. cayetanensis* in DNA extracts, follow the protocol described in BAM Chapter 19B, "*Cyclospora cayetanensis* Real-Time PCR Detection Method", sections 1-5 (https://www.fda.gov/food/foodscienceresearch/laboratorymethods/ucm553445.htm) for reagent ordering and preparation, reaction setup, and execution with **three modifications** to the method described in Chapter 19B:
   a. The real-time PCR reactions are run for **40 cycles** instead of 45.
   b. Each DNA sample is analyzed in triplicate both undiluted and at a **1/10 dilution instead of a ¼ dilution**.

      1/10 Sample Dilution Protocol: Transfer 2.0 µL of sample to a clean microfuge tube containing 18.0 µL of TE. Mix well and centrifuge briefly.

   c. The threshold for the *C. cayetanensis* target is manually set at **0.03** instead of 0.02.

B. Interpretation of Results

Positive Samples:

i. Samples are only considered Positive for the presence of *C. cayetanensis* if one (1) or more sample replicates produces a positive result crossing the threshold for the Ccay18S target reaction and the IAC target reaction is either Negative or Positive.

ii. ONLY ONE REPLICATE OF AN UNKNOWN SAMPLE OR 1/10 DILUTION OF THAT SAMPLE NEEDS BE POSITIVE FOR THE Ccay18S TARGET IN ORDER TO CONSIDER A SAMPLE POSITIVE.

Negative Samples:

iii. If a sample Ccay18S target reaction produces all replicates with undetermined Ct **and** at least one replicate of the IAC target reaction produces a positive result crossing the threshold: **Sample is NEGATIVE, no further action.**

Invalid Results:

iv. If one (1) or more replicates of the NTC sample or the DNA extraction control sample Ccay18S target reactions produces a positive result crossing the threshold, the experimental run is **invalid and must be repeated.**

v. 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, and the results must be considered invalid for the entire set of samples.

vi. If one (1) or more replicates of the positive control sample Ccay18S target reaction is undetermined, the experimental run is **invalid and must be repeated.**

Inconclusive Results:

vii. If a sample Ccay18S target produces all replicates with undetermined Ct and sample IAC target is undetermined: **Sample is INCONCLUSIVE → consult CFSAN SME.**

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5. Appendix

5.1. Sanitizing Polypropylene (PP) Carboys and 225 mL Bottles

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 225mL 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^{o}C, 15 psi for approximately 20 minutes.