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

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

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

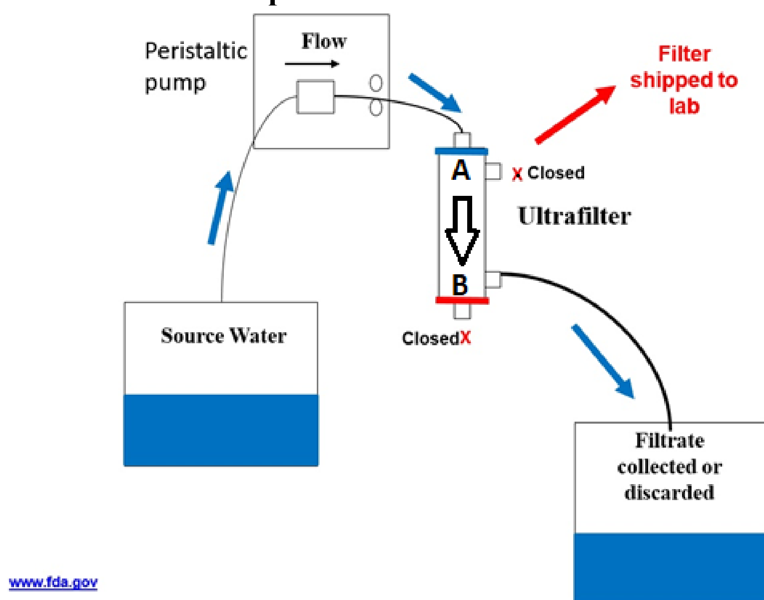


Figure 1.Diagram of the filtration system.

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.

l. 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.

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 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 and 50 mL Conical Tube Adapters, set of 4.
   ii. 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.
   iii. 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.

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 Technologies™ 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 µL 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. PrimeTime^{TM} 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)

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 µL 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 µL 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.

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.

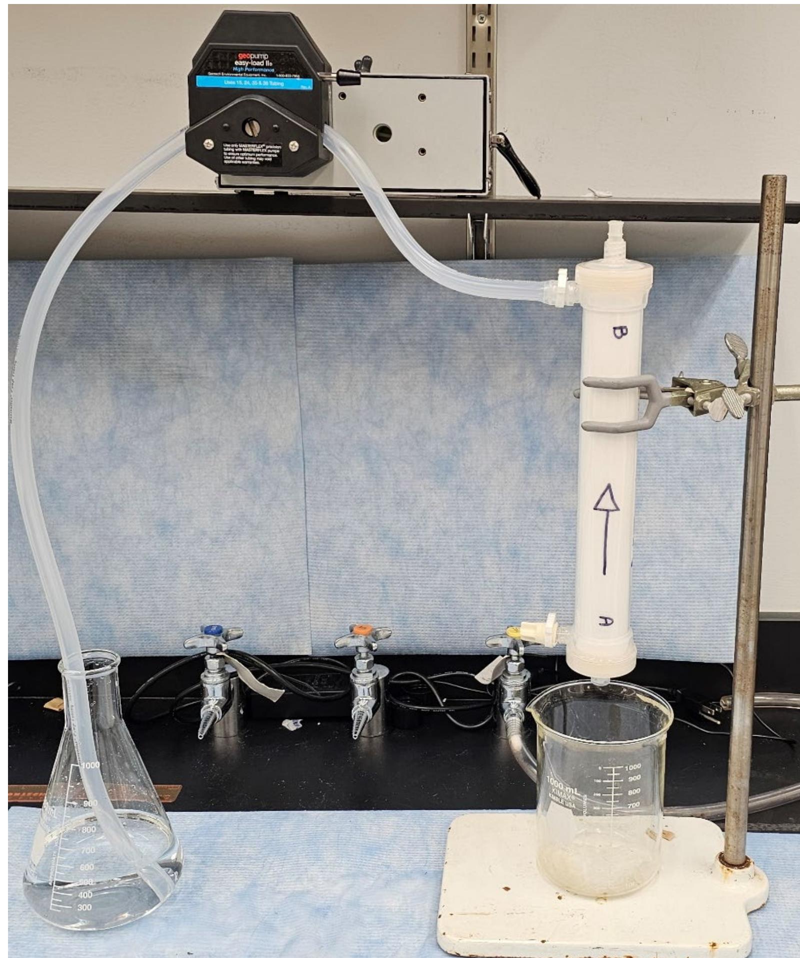


Figure 2. Backflush assembly.

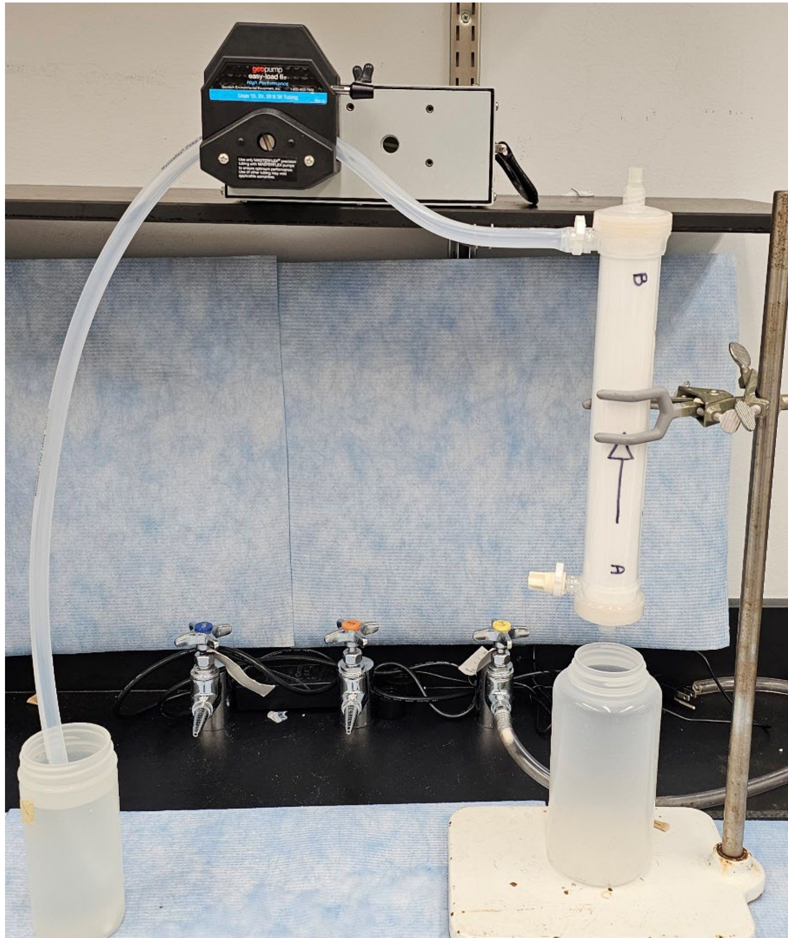


Figure 3. Backflush assembly.

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 4ºC 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 ~1 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

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 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 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 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). *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 µL prepared SEWS-M to each filter. Gently 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).

xx. 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 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. 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 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 s to elute the purified DNA.

viii. Centrifuge the QIAquick column once more for 1 min. Discard the columns and cap the tubes.

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 µM 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 → choose "Create a custom formulation" → choose "Full product yield, to a specified µMolar concentration" → enter "500" and choose "IDTE 8.0 pH" → 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 *C. cayetanensis* target	Mit1C-f	5'-TCTATTTTCACCATTCTTGCTCAC-3'
Reverse primer for amplification of the mitochondrial *C. cayetanensis* target	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" → choose PrimeTime qPCR Probes → 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 *C. cayetanensis* target	**Probe for detection of IAC target**
Item Name	Mit1P-FAM	dd-IAC-Cy5
Sequence	5'-AGGAGATAGAATGCTGGTGTATGCACC -3'	5'-AGCTAGTCGATGCACTCCAGTCCTCCT-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 µM 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 µM final concentration. Vortex and centrifuge the hydrated probe briefly.

100 µM 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 µM 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)" → 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

TACAGCACCCTAGCTTGGTAGAATCGATCAGCTACGTGAGGTCCTACGACGATC
GCCAAGCATGCCCTAGCTAAGATGCATCGATTGCTCATCACGTACGTTAGGTCG
ACTAGGAGGACTGGAGTGCATCGACTAGCTAAGATGGTTCGATTGCTCATCACG
AAGGTTAGGTCGACTACGAACGAGTCGTATTGCAGGTT

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>TAGAGAAGTTGGTACTACATCAGCTTCTCTGGTTTCATCAATTTGTTTAG
GTGTTATTAGTACTGAGTTACTACTATTTGTTAGCTTCTTCTGGGGTGCATACACC
AGCATTCTATCTCCTAGTTATGTAACAGACTCCACCCTAGTAAGTCCAACTGAGG
GTCTTGTAAGTATCTCTAGTAG

Click "Add to Order" → answer "No" to all questions on the Terms and Disclosure pop up window → type your name in the Signature box → accept the terms and conditions → 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):

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

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-FAM	0.3 µM final in reaction
446 µL TE	
500 µL final volume	

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-synIAC	1E4 copies/µL final in reaction
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_2O (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 µL of sample or appropriate controls to each reaction plate well or tube (see Section C below).

Samples and Controls:

Table 1-8. Sample and Controls volume

NTC	2.0 µL H_2O (DNase-free)
DNA extraction control	2.0 µL
Samples	2.0 µL (1X and 1/10 dilution)
Positive Control	2.0 µL Mit1AA gBlock (5E2 copies/µL)

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 µL of sample to a clean microcentrifuge tube containing 18 µL 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 × 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 ≤ 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 121ºC, 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/µL. Store dilutions and working solution at -20°C.

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

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 µL TE dilution buffer and vortex briefly. Centrifuge again to bring liquid contents to the bottom of the tube.	5E12*
Mix 10 µL of the 5E12 stock + 990 µL TE in a new tube. Centrifuge to bring liquid contents to the bottom of the tube.	5E10
Mix 10 µL of the 5E10 stock + 990 µL TE in a new tube. Centrifuge to bring liquid contents to the bottom of the tube.	5E8
Mix 10 µL of the 5E8 stock + 490 µL TE in a new tube. Centrifuge to bring liquid contents to the bottom of the tube.	1E7

*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 µg) 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 µL TE, the approximate copies is 5E12 copies/µL.

- An optional method for an accurate measurement is to determine the concentration of DNA (above- lyophilized target in 1000 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 1.0E6 copies/µL.

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/µL. 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. 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 µL 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 µL of the 2E9 stock + 990 µL TE in a new tube. Centrifuge to bring liquid contents to the bottom of the tube.	2E7
Mix 10 µL of the 2E7 dilution + 990 µL TE in a new tube. Centrifuge to bring liquid contents to the bottom of the tube.	2E5
Mix 10 µL of the 2E5 dilution + 390 µL TE in a new tube. Centrifuge to bring liquid contents to the bottom of the tube.	5E3
Mix 50 µL of the 5E3 dilution + 450 µL TE in a new tube. Centrifuge to bring liquid contents to the bottom of the tube.	5E2

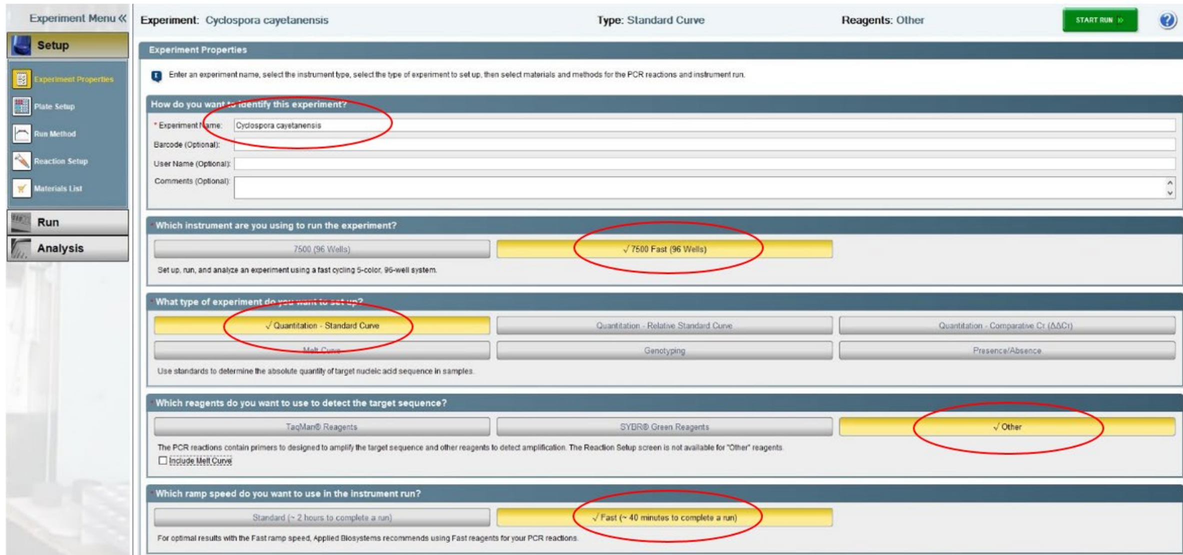
- Note: This value is theoretically derived and is a function of the DNA concentration, Avogadro's number, length of template, and average weight of a basepair. There are freely available online calculators to derive this value.

  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 µL TE, the approximate copies are 2E9 copies/µL.

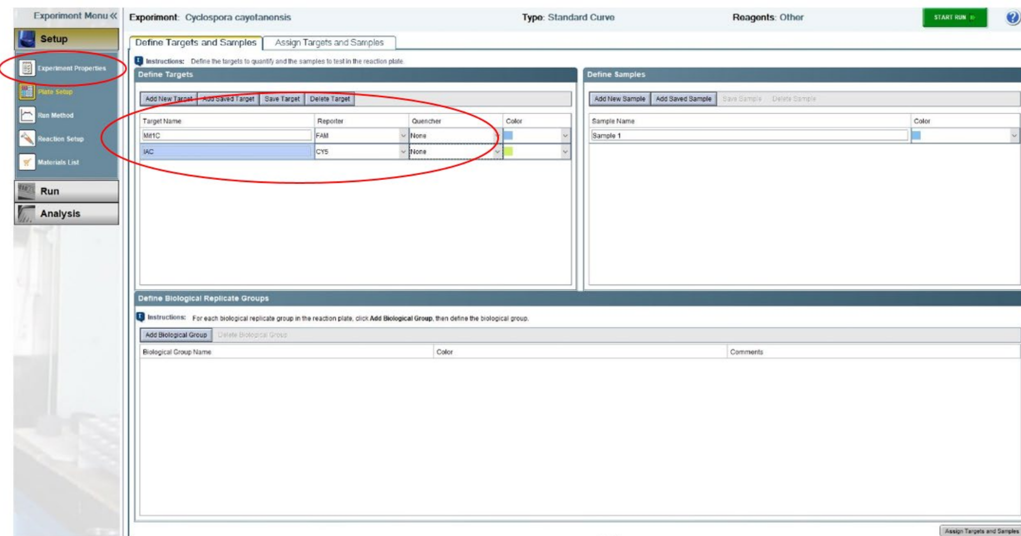
- An optional method for an accurate measurement is to determine the concentration of DNA (above- lyophilized target in 500 µL TE) using preferably a high sensitivity Qubit kit or nanodrop. The concentration (ng/µL) can be plugged into a copy number calculator to derive an accurate copies/µL of the target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

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

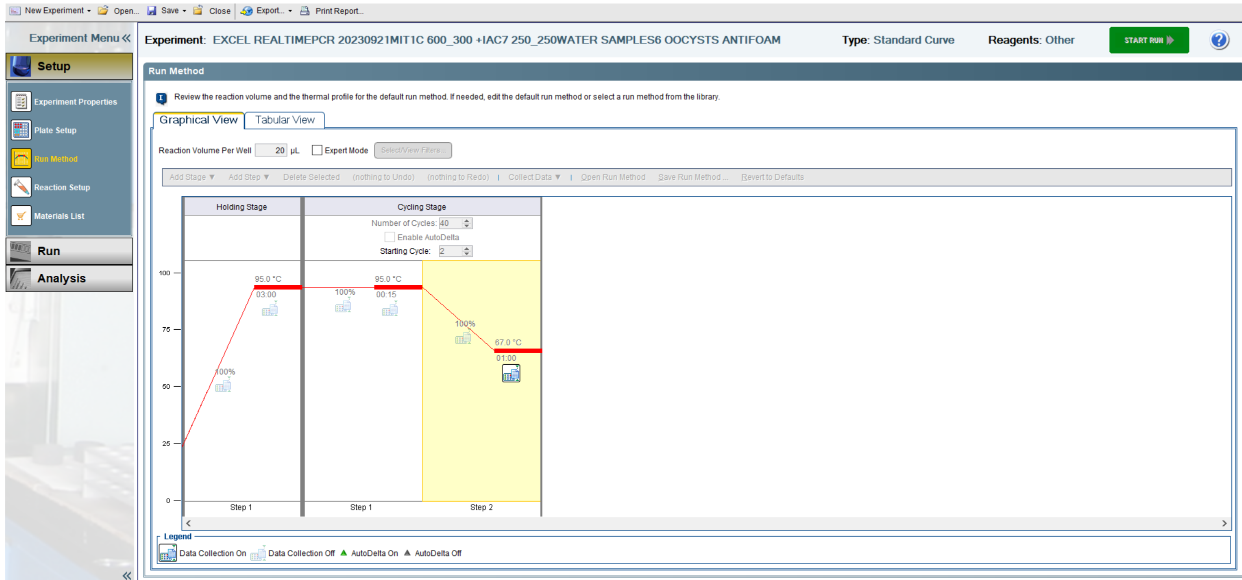
Define a Run Template Using Software v2.0 or 2.3 on the ABI 7500 Fast Instrument:
Turn on the computer and ABI 7500 FAST Real-Time PCR system. Open the 7500 Software v2.0 or 2.3 and click "New Experiment" (Advanced Setup). Define "Experimental Properties" as shown below with "Experiment Name"→ "Cyclospora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



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".



Click “Run Method” on left and define cycling parameters as shown below for a 20 µL 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.

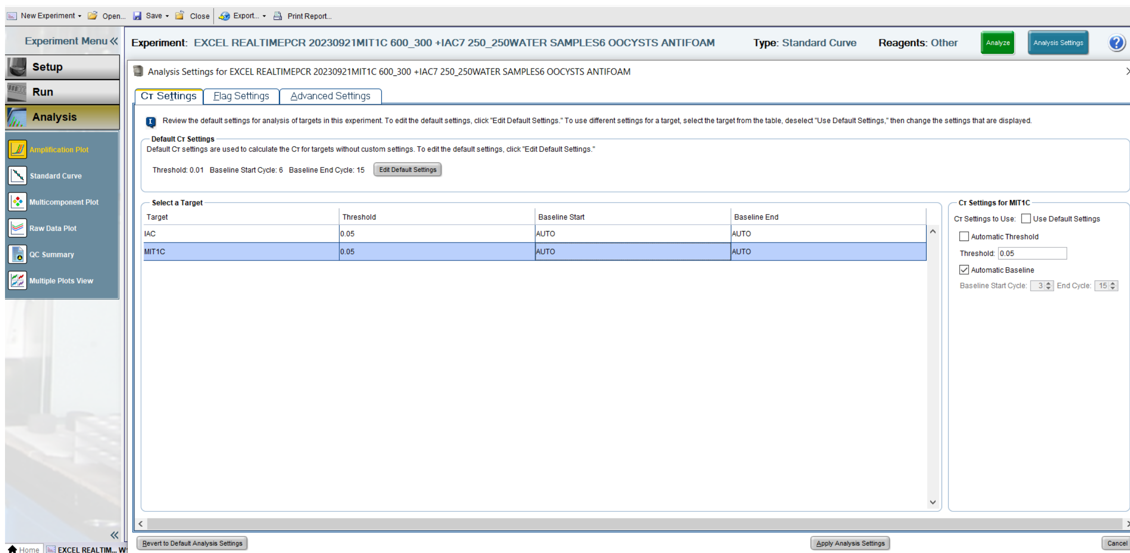


Choose “Analysis” on the left and click “Analysis Settings” in the upper right corner. Define target Ct settings in the pop up window:

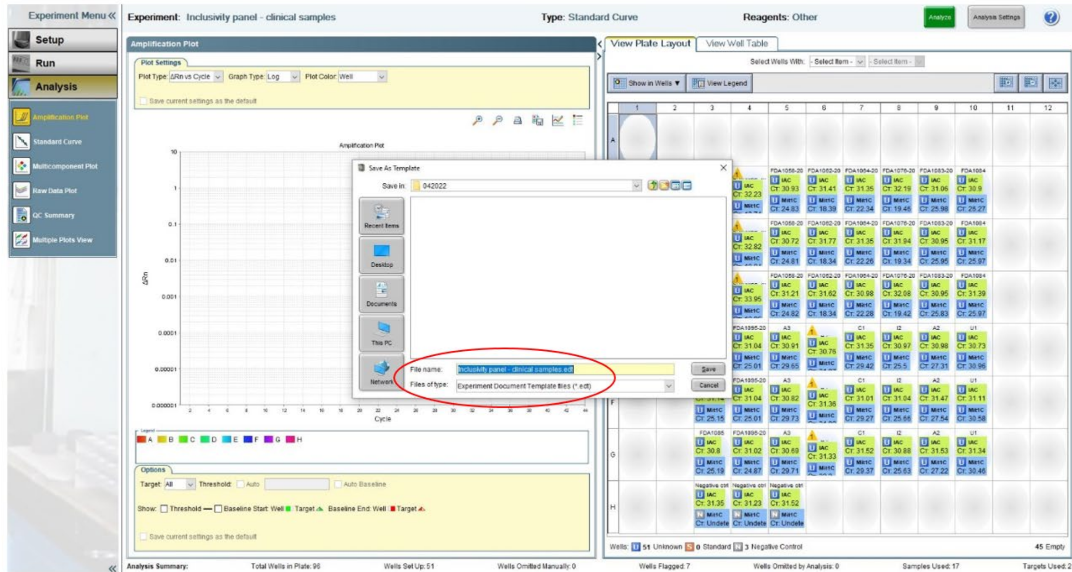
(A) Select the **Mit1C** target: Turn **<u>off:</u> “**Use Default Settings” and “Automatic Threshold”. Set the Threshold to **0.05** and choose “Auto Baseline”**.**

(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”.

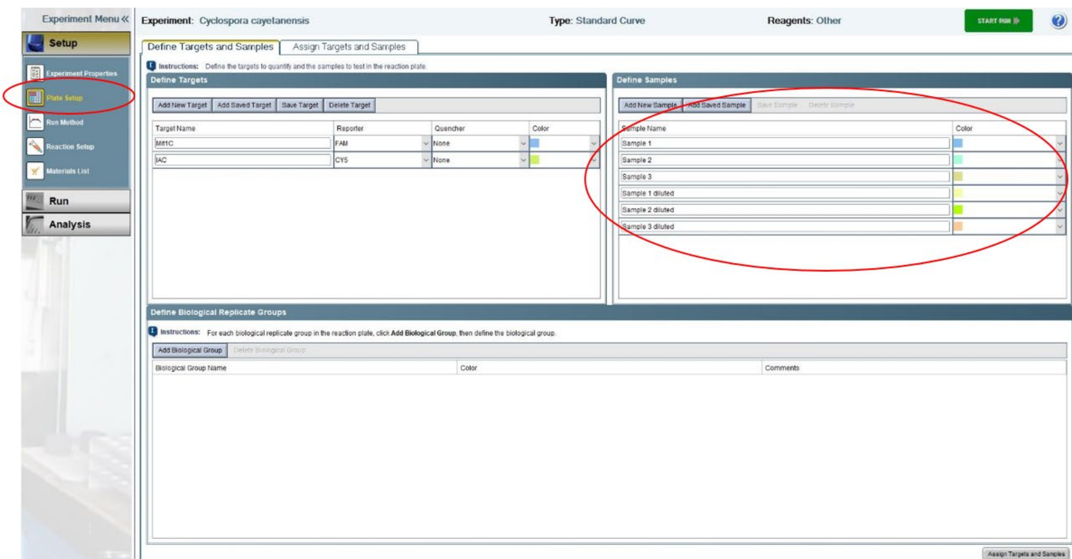


Click “File” → “Save as template…” → “Save”



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

Click “File”→“New Experiment”→”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.



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.

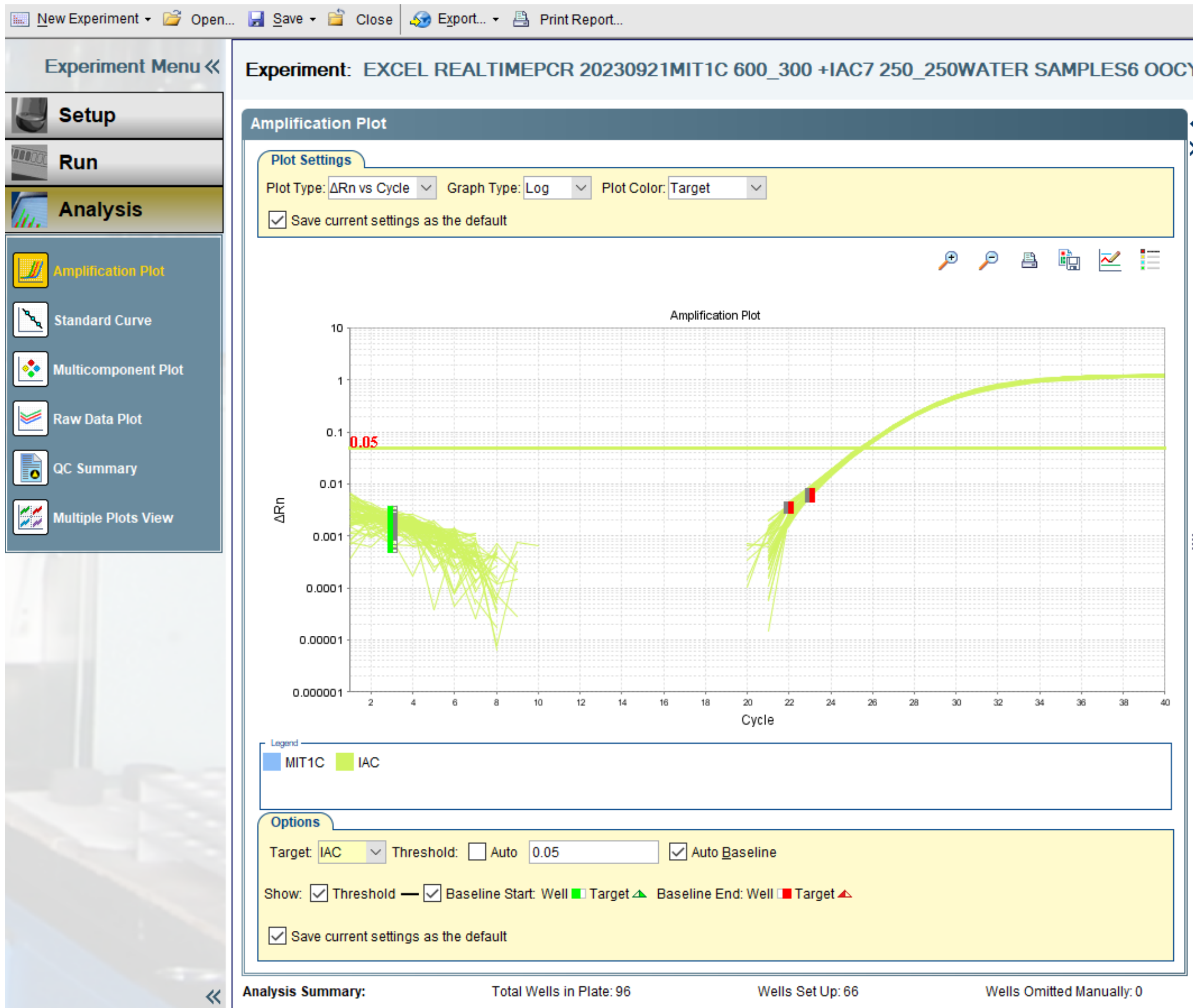
Define all unknown samples or DNA extraction controls one at a time by selecting three wells for each and checking the box next to the sample name in the "Assign sample(s) to the selected wells" panel.

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

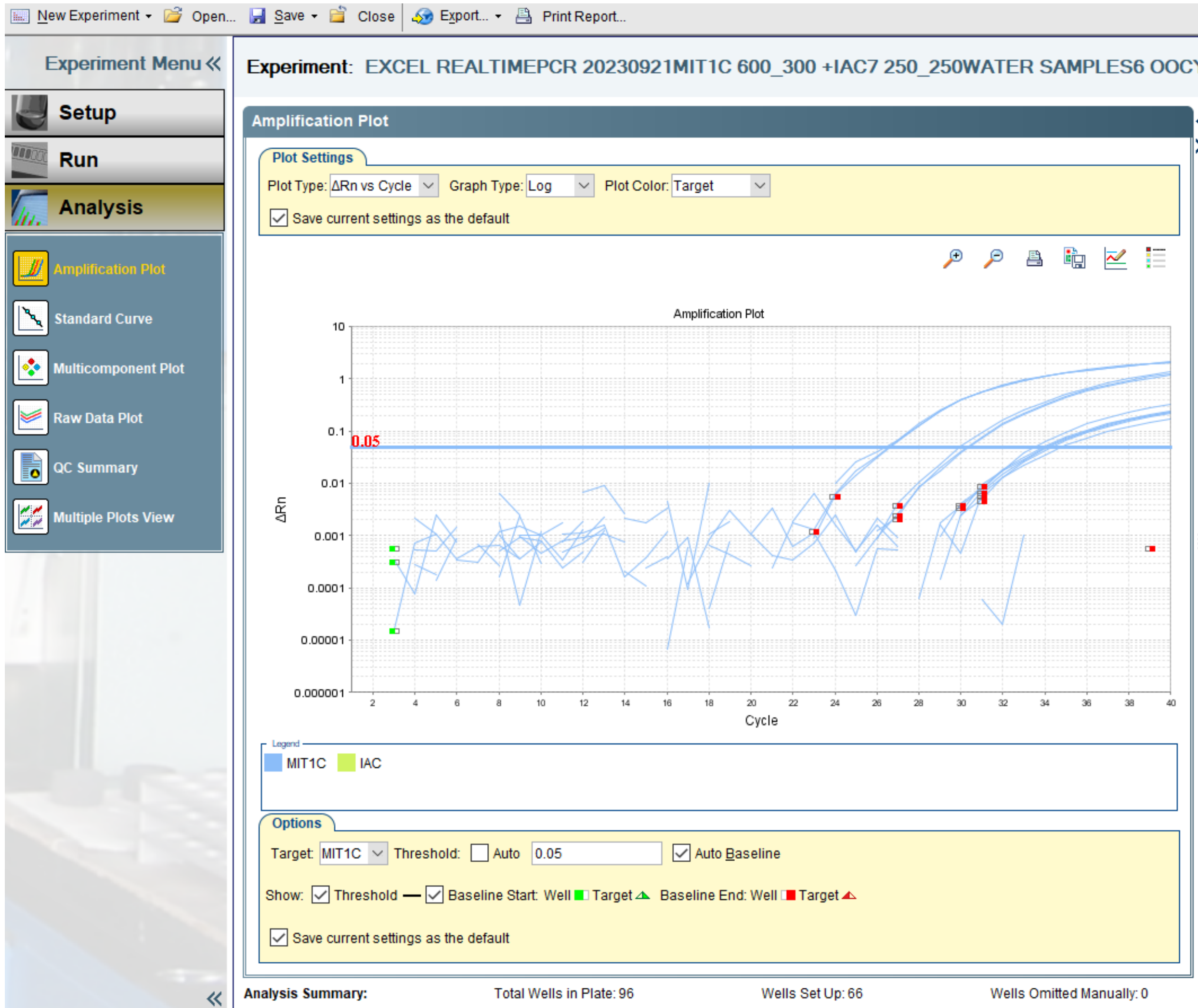
Define the Positive control (Standard) wells by selecting three wells and checking the box next to the Mit1C target choosing "S" as task and "1000" as quantity. "File"→"Save as"→ Experiment Document Single file (*.eds) with a unique name. Insert plate or tube strips and start the run.

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

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.

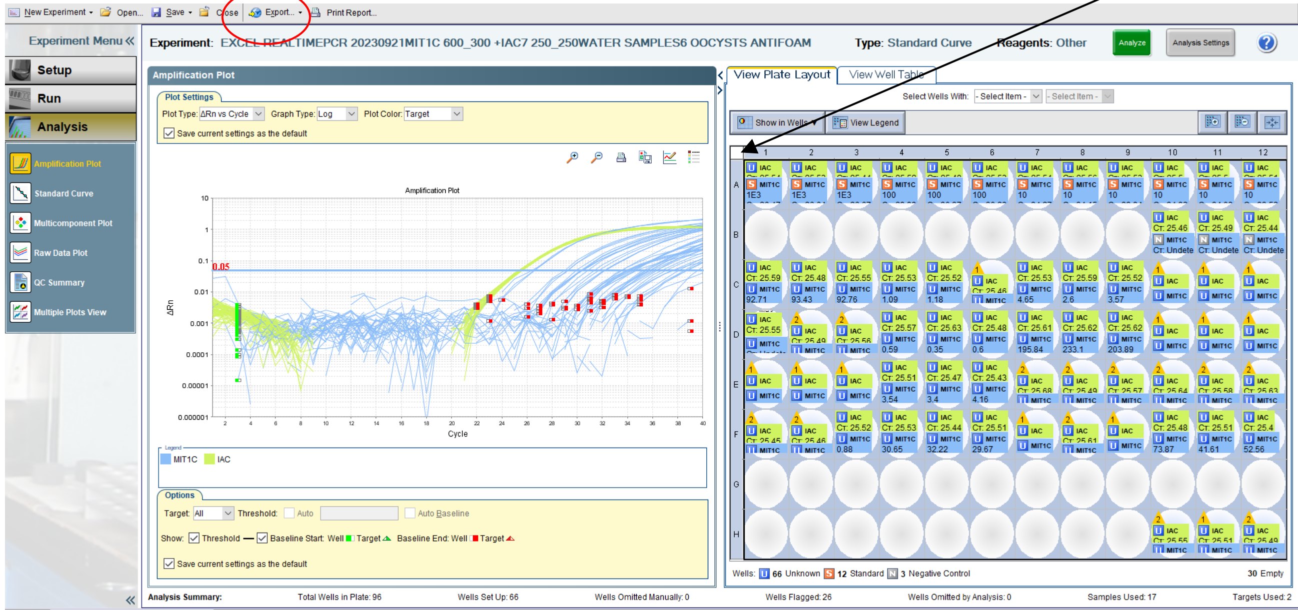


Next, select the “Mit1C” target in the options panel and check that the show threshold and baseline boxes are checked. Verify threshold and baseline settings are accurate as defined in the run template above.



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.



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.

Export Data

Select the type of data to export, select whether to export one file or separate files, then enter export file properties. (Optional) Click "Customize Export" to change the export format and to select fields to export. Click "Start Export" to export your data.

Export Properties | Customize Export

Customize: Results

File Name: Excel Real Ime PCR 20222204 ccayMIT1C+ IAC Perfecta Upper Marlboro_data File Type:

Organize Data

- (•) Down Rows ( ) Across Columns

Select Results Content

- [ ] All Results Fields
- [x] Well
- [x] Sample Name
- [x] Target Name
- [x] Task
- [x] Reporter
- [ ] Quencher
- [x] Cт
- [x] Cт Mean
- [x] Cт SD
- [ ] Quantity
- [ ] Quantity Mean

Field Separator (Delimiter)

- ( ) Tabs ( ) Commas

Results Export

Well	Sample N...	Target Na...	Task	Reporter	Cт	Cт Mean	Cт SD
B1	116	IAC	UNKNOWN	CY5	30.82815	30.860903	0.0654539...
B2	116	IAC	UNKNOWN	CY5	30.81829	30.860903	0.0654539...
B3	116	IAC	UNKNOWN	CY5	30.936268	30.860903	0.0654539...
B4	117	IAC	UNKNOWN	CY5	30.668905	30.761206	0.10648773
B5	117	IAC	UNKNOWN	CY5	30.736996	30.761206	0.10648773
B6	117	IAC	UNKNOWN	CY5	30.877712	30.761206	0.10648773
B7	118	IAC	UNKNOWN	CY5	30.912022	30.697931	0.1854309
B8	118	IAC	UNKNOWN	CY5	30.587885	30.697931	0.1854309
B9	118	IAC	UNKNOWN	CY5	30.593891	30.697931	0.1854309
B10	119	IAC	UNKNOWN	CY5	31.093542	30.831705	0.23527534
B11	119	IAC	UNKNOWN	CY5	30.763517	30.831705	0.23527534
B12	119	IAC	UNKNOWN	CY5	30.638054	30.831705	0.23527534
C1	120	IAC	UNKNOWN	CY5	30.98061	30.977295	0.0035693...
C2	120	IAC	UNKNOWN	CY5	30.977758	30.977295	0.0035693...
C3	120	IAC	UNKNOWN	CY5	30.973516	30.977295	0.0035693...
C4	121	IAC	UNKNOWN	CY5	30.696188	30.950525	0.23369096
C5	121	IAC	UNKNOWN	CY5	30.99962	30.950525	0.23369096
C6	121	IAC	UNKNOWN	CY5	31.15577	30.950525	0.23369096
C7	122	IAC	UNKNOWN	CY5	30.947596	31.0744	0.28495446
C8	122	IAC	UNKNOWN	CY5	31.400745	31.0744	0.28495446
C9	122	IAC	UNKNOWN	CY5	30.874857	31.0744	0.28495446
C10	123	IAC	UNKNOWN	CY5	31.260004	31.083635	0.20481487
C11	123	IAC	UNKNOWN	CY5	30.858997	31.083635	0.20481487
C12	123	IAC	UNKNOWN	CY5	31.131907	31.083635	0.20481487
D1	124	IAC	UNKNOWN	CY5	31.20964	31.11142	0.17755374
D2	124	IAC	UNKNOWN	CY5	31.218159	31.11142	0.17755374

- [ ] Open file(s) when export is complete

- [ ] Save current settings as the default

Start Export | Cancel

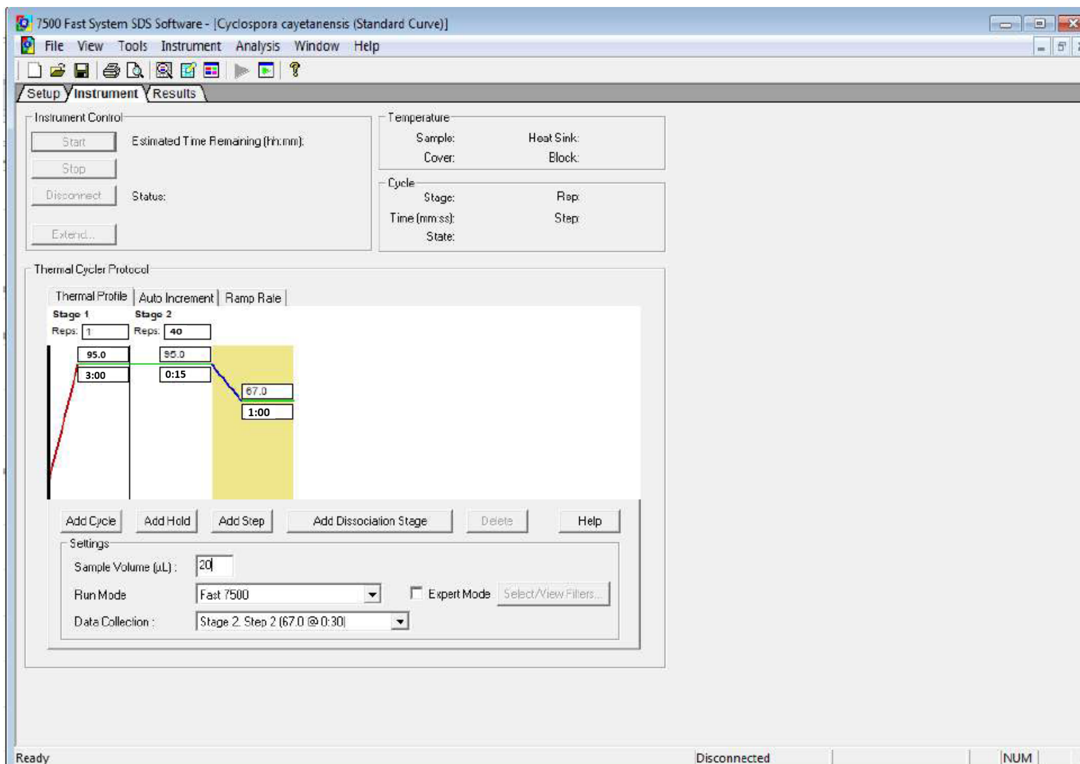
7. Appendix 6. ABI 7500 Fast v1.4 Method.

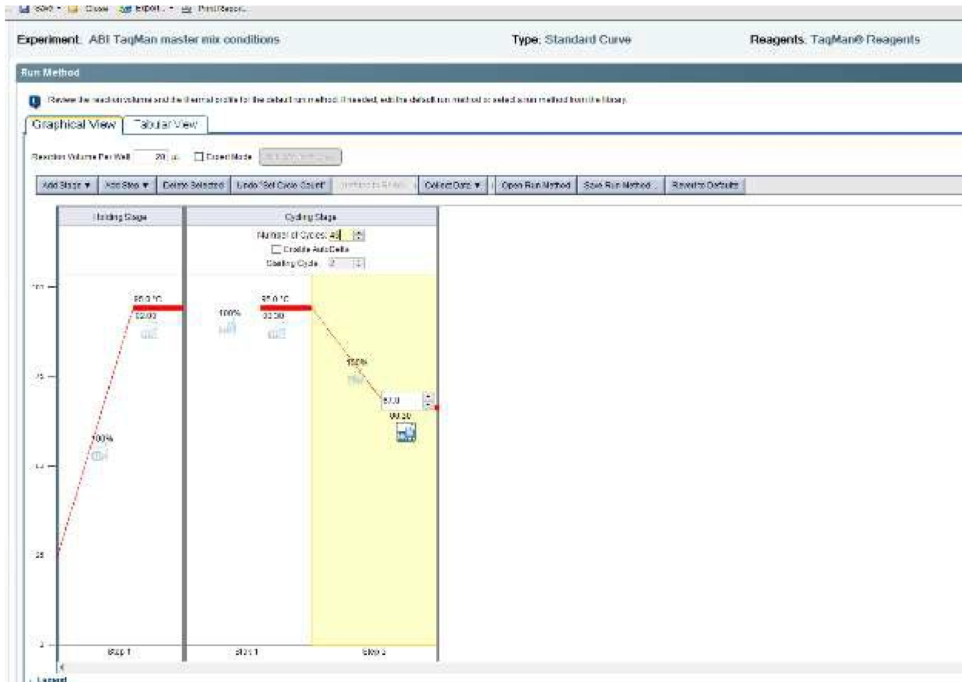
Define Protocol as a Template

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

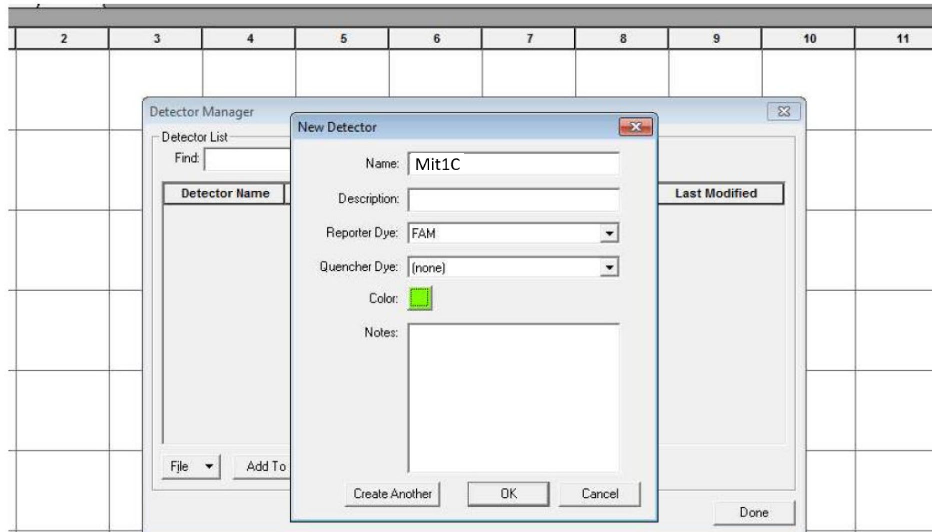
2. Sample Volume (μL): **20**
   Run Mode: Fast **7500**
   Data collection: **Stage 2, step 2 (67 °C @ 1:00)**



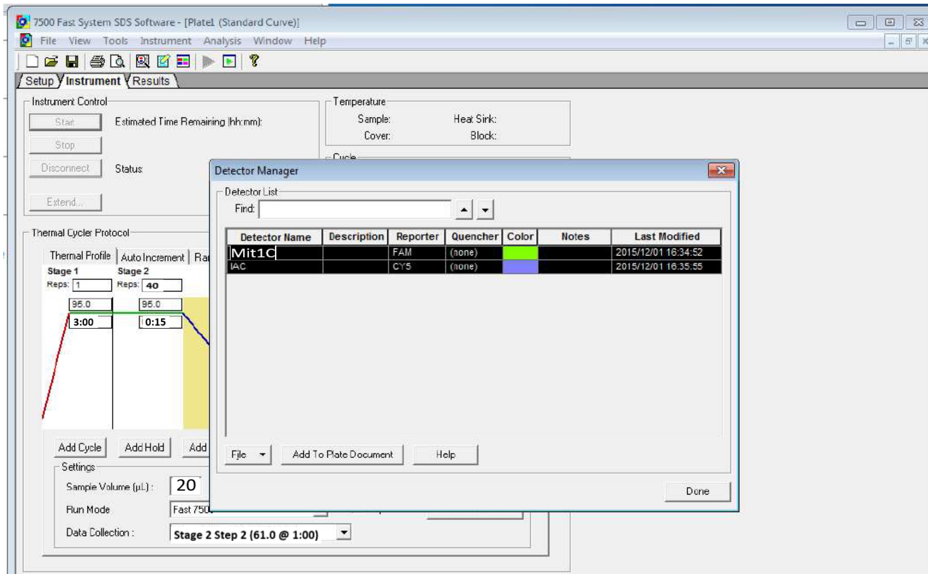


Create Detectors: Mit1C & IAC (internal amplification control)

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



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".



Save Protocol as a SDS Template [*sdt] in Drive D: → Applied Biosystems → 7500 system → templates → 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” → Click “Create New Document” to open “New Document Wizard” and “Define Document”.

Assay: Standard Curve (Absolute Quantitation)

Template: Click on “Browse” → navigate to “templates” folder → Select “Cyclospora cayetanensis.sdt”

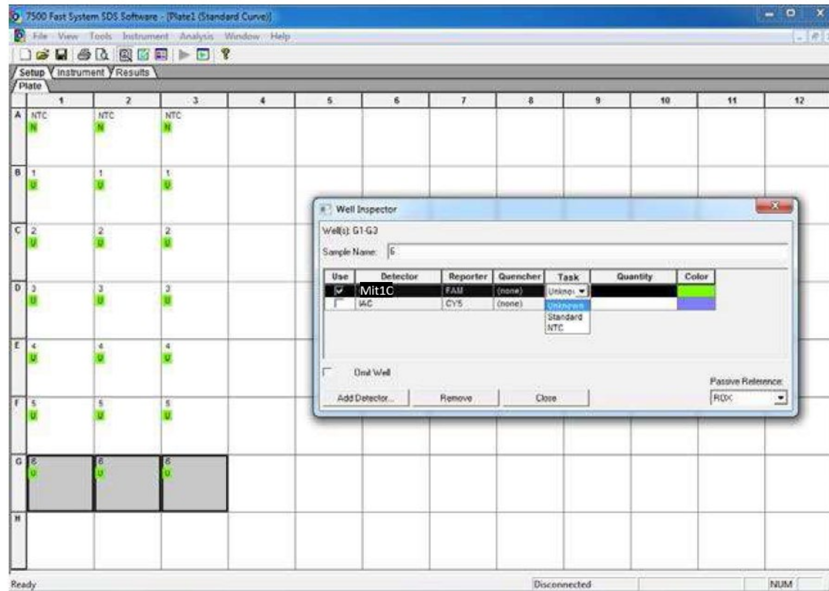
Plate Name: “Cyclospora Test 1” → “Finish”

New Document Wizard
Define Document
Select the assay, container, and template for the document, and enter the operator name and comments.

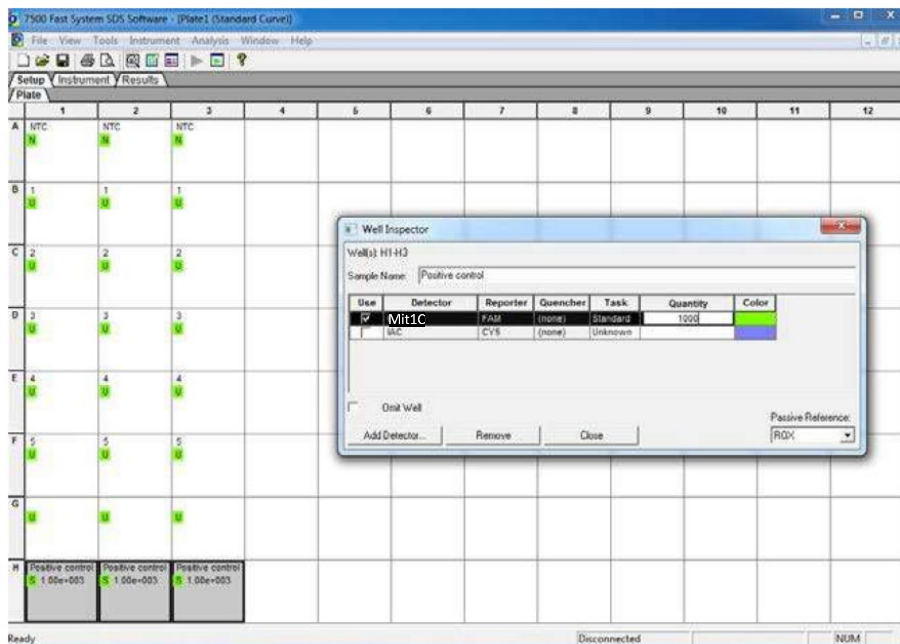
Assay: Standard Curve (Absolute Quantitation)
Container: 96-Well Clear
Template: Cyclospora cayetanensis.sdt Browse...
Run Mode: Fast 7500
Operator: helen.murphy
Comments: SDS v1.5.1
Plate Name: Cyclospora Test 1

< Back Next > Finish Cancel

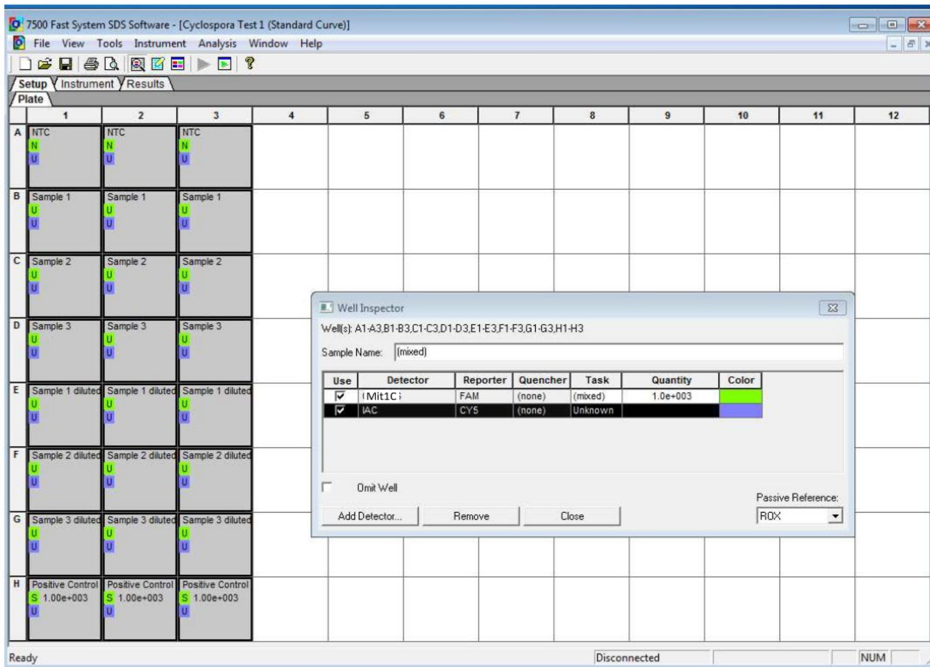
From “View” → Select “Well inspector” to define well assignments. Define the no template control wells by selecting three wells and type “**NTC**” in the “Sample Name” field on the “Well Inspector” window. Check the “Mit1C” detector choosing “NTC” as “Task”. Define all unknown samples and DNA extraction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



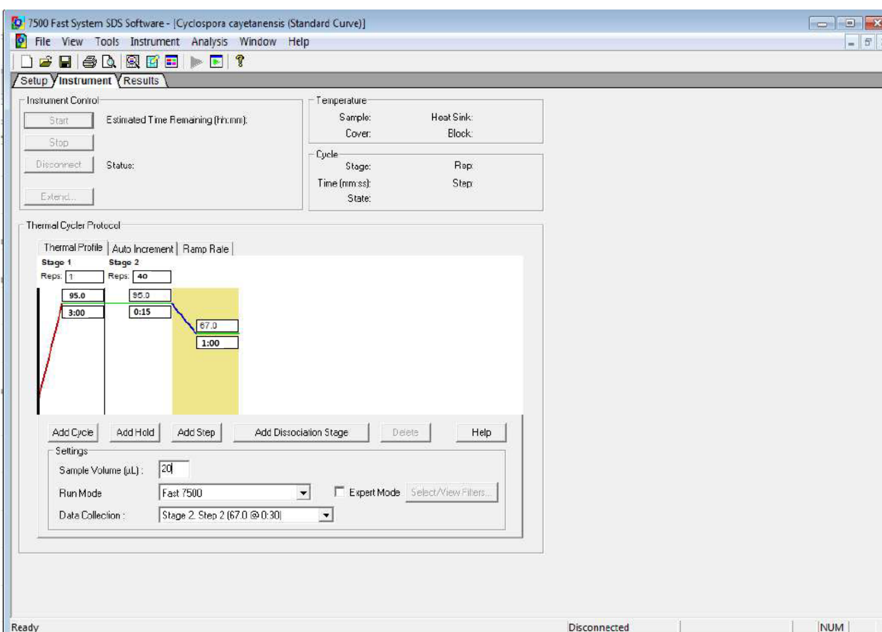
Define the positive control in the same manner but choose “Standard” as “Task” and enter “1000” as “Quantity”.



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 → “Save” to choose a location to save the run file.



Insert the plate to begin the run and click the “Instrument” tab → “Start”.



Results

Click on the “Results” tab → 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:

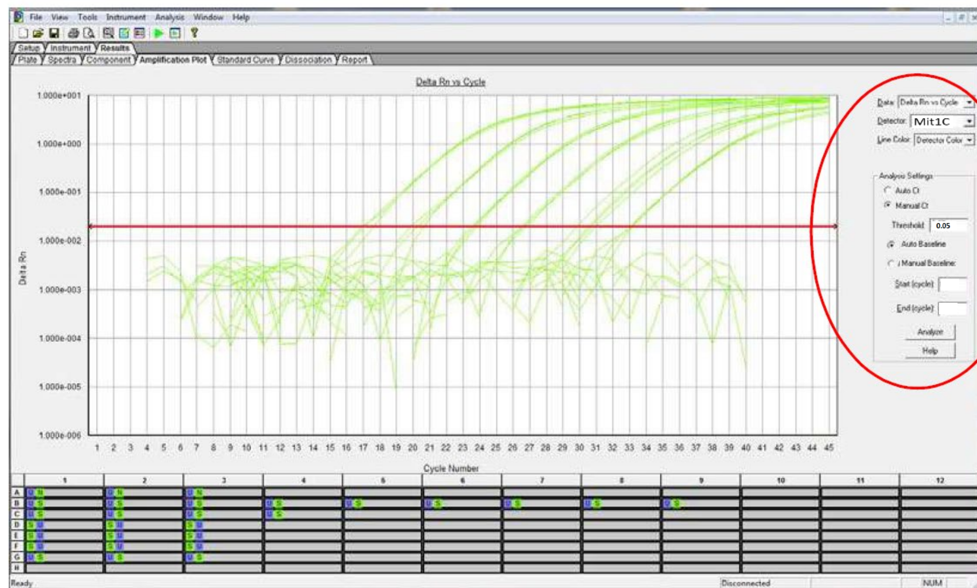
Select Detector “**Mit1C**” and Line Color “**Detector Color**”. In the “Analysis Settings” box select:

> “**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).



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.

Choose Data Columns:
Well
Sample Name
Detector
Task
Ct
StdDev Ct

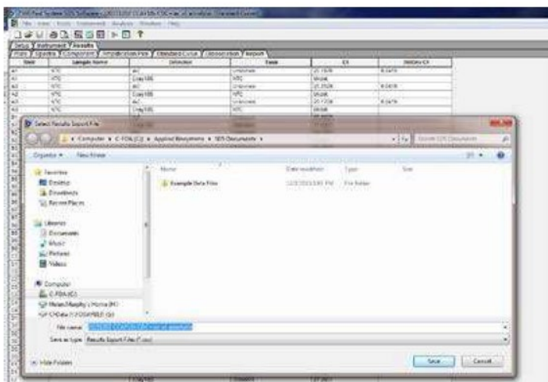
Uncheck all data boxes on the right side of the window.

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 → “Export” → “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 → click “OK”.