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Produce Washing and DNA Extraction Procedures

This analytical procedure provides steps for isolation of *C. cayetanensis* oocysts from produce by washing and subsequent DNA template preparation from produce washes.

1. Laboratory Facilities

To eliminate the potential for positive results due to contamination, it is necessary to perform the produce sample washing, DNA extraction, and Real-Time PCR steps in areas which are isolated from one another. The following work areas are recommended to complete the produce washing and DNA extraction steps while minimizing the potential for contamination:

- A. A laboratory bench for produce washing.
- B. A hood for DNA extraction procedure.

2. Materials and Equipment

- A. Interscience BagPage®+ 400 mL filter bags, 500/pk, Cat No. EW-36840-56 (Cole-Parmer)
- B. Interscience Bag Clips, 50/pk, Cat No. EW-36850-46 (Cole-Parmer)
- C. Disposable serological pipets, 5 mL and 25 mL or 50 mL
- D. Tray to hold filter bags during washing step (see Figure 1b-c)
- E. Stovall Belly Dancer or comparable orbital mixing platform
- F. Platform rocker
- G. 15 mL and 50 mL conical centrifuge tubes for produce washing
- H. Sorvall Legend RT+ refrigerated centrifuge or equivalent (for centrifugation of 15and 50 mL conical centrifuge tubes)
- I. 2 Liter (or larger) vacuum flask connected to house vacuum
- J. Short glass Pasteur pipets for vacuum aspiration of wash supernatants
- K. Empty 2 mL FastPrep® tubes and caps, Cat nos., 115076400 and 115064002 (MP Biomedicals)
- L. DNase-free 2.0 mL microcentrifuge tubes
- M. FastPrep®-24 Instrument (MP Biomedicals) or comparable homogenizer
- N. 15 mL conical centrifuge tubes for DNA binding step
- O. Benchtop centrifuge capable of spinning 2.0 mL tubes
- P. Micropipettors
- Q. Aerosol resistant micropipette tips
- R. Latex or nitrile gloves
- S. Vortex Mixer

3. Reagents

- A. Powdered Alconox® laboratory glassware detergent, Part no. EW-17775-0 (Cole-Parmer)
 - 1. 1.0% Alconox stock solution (Appendix 1)
 - 2. 0.1% Alconox produce wash solution (Appendix 1)
- B. FastDNA® SPIN Kit for Soil, Part no. 6560-200 (MP Biomedicals)
- C. 100% ethanol for DNA extraction procedure
- D. Sterile nuclease free deionized water for produce wash procedure

4. Wash Procedure for Fresh Produce Samples

The standard wash procedure described below is optimal for leafy greens and herbs or sturdy vegetables. It is important to take note of modifications which are described in the protocol and required for fragile matrices such as raspberries which release larger amounts of debris or pectin if not handled carefully.

Note: Centrifugation of wash solution is performed as described below using a swinging bucket rotor with a brake setting of 6 (on a scale of 0-9) for deceleration.

- A. Weigh produce to be analyzed in a BagPage®+ filter bag (25 grams of fresh produce or 50 grams of fresh berries).
- B. Add 100 mL of 0.1% Alconox to the produce sample in the filter bag. Lay the bottom portion of the bag flat on the bench with the opening edge folded up against a vertical support (Figure 1a). Bags containing leafy greens or sturdy vegetables (but not those containing fragile matrices such as berries) should be massaged gently with fingertips up the length of the bag a few times to remove most of the air. Bags containing berries should be sealed without massaging and without removing air. Seal the bags with the bag clips.
- C. Lay sealed bags containing leafy greens flat in a tray on a rocker platform with the sealed opening edges propped up against the sides of the tray (Figure 1b) to prevent occasional leakage which on rare occasions can occur. The bags are stacked on top of one another in order to accommodate all. Agitate for 30 minutes at 85 rpm (Stovall Belly Dancer set at 7.0 with maximum tilt) at room temperature, inverting the bags after 15 minutes. Bags containing berries are stood upright in the tray (Figure 1c) to achieve better coverage of matrix with wash solution and slowly rocked on a platform rocker for 30 minutes at low speed (e.g., 12 rocks per minute using a Hoefer Red Rocker set at 5.0).
- D. Open bags and transfer the supernatant from the filtrate side of each BagPage®+ filter bag into two labeled 50 mL conical centrifuge tubes using serological pipets.
- E. Isolate wash debris containing oocysts by centrifugation in a swinging bucket rotor for 20 minutes at $2,000 \times g$ with a brake setting of 6 (on a scale of 0-9) for deceleration.
- F. During the centrifugation, add an additional 100 mL of 0.1% Alconox to the produce in each filter bag and tip the bag from side to side three to four times to rinse the food and

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bag surfaces. Lean the bags containing the produce and rinse solution against a vertical surface until needed in **step 4H**.

- G. After the centrifugation use a short glass Pasteur pipet connected with tubing to a filter flask and house vacuum to aspirate all but approximately 4 mL of the supernatant from each of the 50 mL tubes to waste without disturbing wash debris pellets.
- H. Transfer the rinse from the filtrate side of each BagPage®+ filter bag to the corresponding two 50 mL conical tubes containing the first wash debris pellets from step 4G. Centrifuge for 20 minutes at 2,000 × g to pellet the combined wash and rinse debris. After the centrifugation aspirate all but approximately 4 ml of the supernatant from each of the 50 mL tubes to waste without disturbing the pellets.
- I. Pool each pair of wash debris pellets by resuspending with a 5 mL serological pipet in the residual wash liquid and transferring to a single 15 mL conical centrifuge tube. Rinse the pair of empty 50 mL tubes sequentially with 2 mL of dH₂O and add to the contents of the 15 mL tube. Centrifuge for 20 minutes at 2,000 × g to pellet the debris. After the centrifugation aspirate all but approximately 1 mL of the supernatant from the 15 mL tube. Resuspend the debris pellet in the 15 ml tube in the residual supernatant and transfer to a single empty 2 mL FastPrep lysing tube (without beads). Rinse the empty 15 mL tube with 0.4 mL of dH₂O and add to the contents of the 2 mL tube. *If the total volume of the resuspended pellet and tube rinse exceeds the capacity of the 2 mL tube, centrifuge a portion in the 2 mL FastPrep tube at 14,000 × g for 4 minutes, aspirate the supernatant without disturbing the pellet, and then add the remaining resuspended pellet and tube rinse.*
- J. Centrifuge the 2 mL FastPrep tubes containing wash debris from step 4I at 14,000 × g for 4 minutes. Aspirate all but approximately 100-200 μ L of the supernatant without disturbing the pellet. Note: If a pooled debris pellet sample is greater than approximately 850 μ L, the sample must be split into two 2 mL FastPrep lysing tubes.
- K. Store at 4°C overnight or proceed as described in **Section 5** for isolation of DNA immediately.



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5. Isolation of DNA from Fresh Produce Wash Debris Pellets using the FastDNA® SPIN Kit for Soil

DNA is extracted from produce washes in a laboratory hood using the FastDNA SPIN Kit for Soil following the modified instructions detailed below.

Prepare the following items for the DNA extraction procedure before beginning:

- Add 100 mL of 100% ethanol to SWES-M bottle of wash solution*
- Lysing Matrix E tubes (containing beads)*
- 2 mL microcentrifuge tubes
- o 15 mL Falcon tubes containing 1 mL resuspended Binding Matrix*
- Spin Filters in catch tubes*
- Second set of catch tubes*

*Items provided in the FastDNA® SPIN Kit for Soil.

Modified FastDNA Spin Extraction Protocol

• Assemble the samples to be extracted from wash procedure step 4J and add an empty FastPrep tube as a DNA extraction control.

- Carefully transfer into each tube in **step 5A** the beads from a Lysing Matrix E tube (supplied with the FastDNA Spin Kit).
- \circ Add 122 µL MT buffer (FastDNA protocol step 3).
- Add 978 μL (or less) Sodium Phosphate Buffer (FastDNA protocol step 2) to the maximum fill height; leave at least 1.0 cm of air space at the top of the tube to allow for efficient bead-beating (see Figure 1d). Screw on cap securely.



Figure 1d

- Transfer the samples to a FastPrep-24 bead beater and homogenize at a setting of 6.5 m/s (approximately 4000 rpm) for 60 seconds. Immediately remove the sample holder containing the tubes from the instrument and place on ice for 3 minutes. Return the sample holder to the bead beater and repeat the bead beating and the incubation on ice as above.
- Remove the tubes from the sample holder and centrifuge at $14,000 \times g$ for 15 minutes (FastDNA protocol step 5).
- Transfer the supernatant to a clean 2 mL tube. Add 250 μL PPS and mix by inverting by hand 10 times (FastDNA protocol step 6).
- Centrifuge at 14,000 × g for 5 minutes (FastDNA protocol step 7) then transfer supernatant to a clean 15 mL Falcon tube containing 1.0 mL of resuspended Binding Matrix.
- Place on a rotator or invert by hand for 2 minutes and then allow silica matrix to settle for 3 minutes (FastDNA protocol step 9). Centrifuge the 15 mL tubes briefly at 1000 × g for 1 minute in a swinging bucket rotor.
- $\circ~$ Remove and discard a total of 1.4 mL of supernatant from each tube in two 700 μL aliquots.
- \circ Resuspend the matrix in the remaining supernatant and transfer approximately 700 μL to a SPIN Filter in a catch tube. Centrifuge at 14,000 × g for 1 minute (FastDNA protocol step 11). Empty the catch tube and add any remaining resuspended mixture to the SPIN Filter and spin as before. Empty the catch tube again.
- Add 500 μL prepared SWES-M to each filter. Gently resuspend each by pipetting up and down (FastDNA protocol step 12).

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- Centrifuge at 14,000 × g for 1 minute. Empty catch tube and replace (FastDNA protocol step 13).
- \circ Centrifuge at 14,000 × g for 2 minutes to dry the matrix. Discard the catch tube and replace with a new catch tube (FastDNA protocol step 14).
- Air dry the filter for 5 minutes at room temperature (FastDNA protocol step 15).
- Add 75 µL DES to the matrix in the spin filter. Resuspend the Binding Matrix by gently stirring with a small pipet tip. Incubate for 5 minutes in a heat block at 55°C. (FastDNA protocol step 16).
- \circ Centrifuge at 14,000 × g for 1 minute to recover the eluted DNA and then discard the SPIN Filter (FastDNA protocol step 17).
- Store the DNA samples at 4 degrees C for up to 2 days or at -20 or -80 degrees C for longer term prior to performing the Real-Time PCR detection step described below.

Cyclospora cayetanensis Real-Time PCR Detection Method

This analytical procedure provides a real-time PCR method for molecular detection of *C. cayetanensis* which replaces the real-time PCR methods found in the FDA BAM Chapter 19A and 19B. The real-time PCR protocol described below offers several advantages including increased specificity. In addition, the use of a real-time PCR method minimizes laboratory environment contamination by amplicons commonly associated with conventional nested PCR. The method was developed for the Applied Biosystems 7500 Fast Real-Time PCR System for detection of *C. cayetanensis* in food samples and is based on a new target on the mitochondria of *C. cayetanensis*. The multi-copy nature of mitochondrial genomes in cells provides higher target sequence concentration than nuclear sequences for molecular methods, such as PCR and NGS. The real-time PCR assay is a duplex reaction which targets the *C. cayetanensis* multicopy mitochondrial gene and uses an internal amplification control to monitor for potential matrix derived inhibition of the reaction. The method also provides a synthetic positive control, allowing sequence verification to identify false positives stemming from inadvertent laboratory contamination.

1. Equipment and Supplies

- A. Applied Biosystems 7500 Fast Real-Time PCR System with Software versions 1.4, 2.0, or 2.3 or newer
- B. 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
- C. Benchtop centrifuge capable of spinning 96-Well reaction plates or a mini-centrifuge capable of spinning 0.1 mL tube strips
- D. Benchtop centrifuge capable of spinning 1.5-2.0 mL tubes
- E. Micropipetors
- F. Aerosol resistant micropipette tips
- G. Latex or nitrile gloves
- H. Vortex Mixer
- I. DNase-free microcentrifuge tubes, 1.5 mL, low retention

2. Reagents

- A. PrimeTime[™] 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.
- B. Sterile DNase-free TE buffer pH 7.5 (commercially prepared or see Appendix 2).
- C. Primers, 500 µM stock solution (See Table 1-1 and Table 1-2).
- D. Probes, 100 µM stock solution (See Table 1-3).
- E. IAC Target (HMultra130-synIAC), working concentration 1E7 copies/μL. See Reagent Ordering and Preparation Instructions, Section 3.
- F. Positive control (Mit1AA gblock), 5E2 copies/µL. See Reagent Ordering and Preparation Instructions, Section 3.
- G. Negative control (Water, DNase-free).

3. Reagent Ordering and Preparation Instructions

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

A. Primers

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

Primer Ordering Instructions: Choose "Custom DNA Oligos" from the IDT online Order Menu page. 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

	Item Name	Sequence
Forward primers for	Mit1C-f	5'-
amplification of		TCTATTTTCACCATTCTTGCTCA
the mitochondrial C.		C-3'
cayetanensis target		
Reverse primers for	Mit1C-r	5'-
amplification of		TGGACTTACTAGGGTGGAGTCT-
the mitochondrial C.		3'
cayetanensis target		
Forward primers for	dd-IAC-f	5'-
amplification of		CTAACCTTCGTGATGAGCAATC
the IAC target		G-3'
Reverse primers for	dd-IAC-r	5'-
amplification of		GATCAGCTACGTGAGGTCCTAC-
the IAC target		3'

Table 1-2. Primer Names and Sequences.

B. 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 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 IDT online order menu page by choosing "Custom qPCR 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/
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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.

C. 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 IDT online order 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/ μ L. Store dilutions at -20° C.

D. 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 IDT online order menu page choose "gBlocks Gene Fragments". Enter the following item name and sequence on the gBlocks® Gene Fragments

Entry page:

Item Name: Mit1AA gblock

Sequence:

ACAGTTGGTTTTCTATTTTCACCATTCTTGCTCACTGTATTAGTATTATTTAATTTTAC T<u>AA</u>TAGAGAAGTTGGTACTACATCAGCTTCTCTGGTTTCATCAATTTGTTTAGGTGTT ATTAGTACTGAGTTACTACTATTTGTTAGCTTCTTCTGGGGGTGCATACACCAGCATTC TATCTCCTAGTTATGTAACAGACTCCACCCTAGTAAGTCCAACTGAGGGTCTTGTAA GTATCTCTAGTAG

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 ngrams 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 degrees C. A fresh working solution should be prepared from the frozen 5E3 dilution every 90 days.

4. 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.

A. 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-	$0.3 \mu\text{M}$ 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

B. 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 microliters volume reactions.

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

C. Samples and Controls:

Table 1-8. Sample and Controls volume.

NTC	2.0 µL H ₂ O (DNase-free)
DNA extraction	2.0 μL
control	
Samples	2.0 μ L (1X and ¹ / ₄ dilution)
Positive Control	2.0 μL Mit1AA gBlock (5E2
	copies/µL)

D. Always briefly vortex and centrifuge controls and samples before adding to reaction

wells or tubes.

ALL UNKNOWN SAMPLES ARE TO BE ANALYZED AT 1X AND A ¹/₄ DILUTION IN THE SAME INITIAL EXPERIMENTAL RUN (Controls are not tested at a ¹/₄ dilution). Prepare diluted samples following instructions below.

E. ¹/₄ Sample Dilution Protocol:

Transfer 2.5 μ L of sample to a clean microcentrifuge tube containing 7.5 μ L of TE. Mix well and centrifuge briefly.

F. 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 seconds.

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 in Sections I and J below.

G. 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.

H. Run Methods on the ABI 7500 Fast Instrument:

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.

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

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

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

I. Interpretation of Results:

A. Positive Samples

a. 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.

b. ONLY ONE REPLICATE OF AN UNKNOWN SAMPLE OR ¹/₄ DILUTION OF THAT SAMPLE NEEDS TO BE POSITIVE FOR THE *C. cayetanensis* Mit1C TARGET IN ORDER TO CONSIDER A SAMPLE POSITIVE.

B. Samples for Further Analysis

a. 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 1/4 dilution).

C. Negative Samples

a. If a sample *C. cayetanensis* Mit1C target reaction produces all replicates with undetermined Ct or no replicate with $Ct \le 38.0$ and sample IAC target reaction produces an average Ct value which is not more than 3 cycles higher compared to the NTC:

Sample is NEGATIVE, no further action.

D. Invalid Results

- a. 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**.
- b. 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 using additional washed food samples if available.
- c. 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**.

E. Inconclusive Results

If on initial test (or after re-test if required), a sample produces no replicate with $Ct \le 38.0$ (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 \rightarrow consult CFSAN SME.

2. Appendix 1. Alconox[®] Produce Wash Solution Recipe.

- 1. Prepare 1.0% Alconox[®] stock solution:
 - a. Dissolve 10 grams Alconox® in 1 liter distilled water
- 2. Prepare 0.1% Alconox® for wash solution
 - a. Mix 200 mL 1.0% Alconox® stock with 1800 mL distilled water

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 degrees C.

Table 4-1. Intern	al Amplification	Control (IAC)	Target V	Vorking Solution.
	1			8

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

• 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 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 1E7 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 degrees Celsius. The working solution can be stored at -20 or 4 degrees C. A fresh working solution should be prepared from the frozen 5E3 dilution every 90 days.

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 degrees 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

Table 5-1. Positive Control 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: 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

(A) 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, then select	lect materials and methods for the PCR reactions and instrument run.			
Plate Setup	How do you want to identify this experiment?				
Run Method	* Experiment forme: Cyclospora cayetanensis				
Reaction Setup	Barcode (Optional):				
🛒 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, 96-well system.				
	What type of experiment do you want to set up?				
	√Quantitation - Standard Curve	Quantitation - Relative Standard Curve	Quantitation - Comparative Cr	(ΔΔC1)	
	Malt Curre	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?	the second s		_	
	TaqMan® Reagents	SYBRØ Green Reagents	√ Other		
	The PCR readions contain primers to designed to amplify the target sequence and other reagents to dete Include Meth.Curve	tect amplification. The Reaction Selup 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 reagents 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".

Experiment Menu «	Experiment: Cyclospora cayetar	nensis		Type: Standard Curve	Reagents: Other	START RUN	()
Setup	Define Targets and Samples	Assign Targets and Samples					
	Instructions: Define the targets to quart	IV and the samples to test in the reaction rist	14				
Experiment Properties	Define Targets			Define Samples	The Real Property lies and the	and the same state of the same state of the	
Plate Setup		Design Terret		Little Very Comple			
En Mathed	HOUNEW Talley HOU Saved Talger	pave raider Delete raider		Add mew Sample	And paved paintine of the paintine of the paintine		
	Target Name	Reporter	Quencher	Color Sample Name		Color	_
Reaction Setup	MittC	FAM	v None	v Sample 1			~
Matorials List	IAC	CY5	~ None ~	×			
Run							
Analysis							
1117							
	Define Biological Replicate Group:	5					
	Instructions: For each biological papirs	ate oroup in the reaction plate, click Add Biolo	nical Green, then define the biologic	al aroun			
	Add Biological Group	(Green					
	Biological Graup Name		Color		Comments		
			0.00		Contracting		
	<u> </u>					(margaret 1)	
				1 /		Assign Targets an	d Samples

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.



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".

File Edit instrument Analysis	roois Heip								
🔝 New Experiment + 📓 Open.	🛃 Save 🗸 當 Close 🏼 🌆 Expo	rt 🔹 📇 Print Report							
Experiment Menu «	Experiment: Cyclospora	cayetanensis		Тур	e: Standard Curve	е	Reagents: Other	Analys	Analysis Settings
Setup	Amplification Plot					/iew Plate La	ayout View Well Table		
Run	Plot Settings	Analysis Settings for Cyclospora	cayetanensis					X - V - Seled Item - V	
Analysis	Plot Type: <u>ARn vs Cycle</u> v Gr	CT Settings Flag Se	ettings Advanced Sett	tings				_	
Mamplification Plot		Review the default setting table, deselect "Use Default Cr Sottings	is for analysis of targets in this ex ult Settings," then change the set	periment. To ecit the default se ttings that are displayed.	ttings, click "Edit De'ault :	Settings." To use o	different settings for a target, select the target from the	7 8 9 1	0 11 12
Standard Curve	10	Default CT settings are used to Threshold: AUTO Baseline	o calculate the Cr for targets witho Start Cycle: AUTO Baseline End	out custom settings. To edit the I Cycle: AUTO Edt Defaut Set	default settings, click "Ed tings	lit Default Settings			
Raw Data Plot		Select a Target					CT Settings for IAC		
		Target	Threshold	Baseline Start	Baseline End	- A	CT Settings to Use: Use Default Settings		
Multiple Diete Mieur		MittC	0.05	AUTO	AUTO		Threshold 0.05		
							Automatic Baseline		
	Б ^р						Basenne Gantojde. Je Endojde, Te		
	1								
	0								
	Options					ų			
	Target: Al V Threshold:	Bevert to Default Analysis Settings)		(Apply Analysis	Settings		
	Show: Threshold — Base	iline Start: Well 🔳 Target 🔺 Basel	line End: Well 🔳 Target 📣						
	Save current settings as the c	lefault				/ells: 🚺 18 Unkn	iown 🛐 0 Standard 🔝 0 Negative Control		78 Empty
«	Analysis Summary:	Total Wells in Plate: 96	Wells Set Up:0	Wells Omitted	Manually: 0	Wells Fla	gged: 0 Wells Omitted by Aralysis	s: 0 Samples Used: 0	Targets Used: 0
Home Untitled ×									
								Disco	onnected

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

Experiment Menu «	Experiment: Inclusivity panel - clinical sample:		Type: Stand	ard Curve		Reag	ents: Ol	her			Analyzo	Analys	is Settings	
Setup	Amplification Plot			View Plate La	yout View	Well Table	•							
Run	Plot Settings			>		Sele	ct Wells With	- Select II	om- v -S	elect item -	SV.			
Analysis	Plot Type: ARn vs Cycle 🐱 Graph Type: Log 🐱 Plot Colo	r Well		O Show in Wells	View	egend							國	
	Save current settings as the default			3	2 3	4	5	6	7	8	9	10	11	1 3
Amplification Plot			▶ ₽ @ 幅 座 []											
Standord Curve	20.	Amplification Plot		*										
Nulticomponent Plot		Save As Template			>		FDA1058-20	FDA1062-20	FDA1034-20	FDA1076-20	FDA1083-20	FDA1084		
taw Data Plot	1	Save In: 042022		000		U uc	ш мс Ст: 30.93	CT 31.41	U MC CT 31.35	10 IAC CT: 32.19	U WC CT 31.06	CT: 30.9		
The Balance		Co				U MARIC	CT: 24.83	1 Metc CT 18.39	CT 22:34	CT 19.45	CT 28.98	CT 25.27		
	0.1	Recent terms				A	FDA1058-20	FDA1062-20	FDA1084-20	FDA1078-20	FDA1083-20	FDA1084		
taltiple Plots View						CT: 32.82	CT: 30.72	CT. 31.77	CT 31.35	CT 31.94	CT 30.95	Cr. 31.17		
	0.01 E	Desktop				Matte	Cr: 24.81	CT. 18.34	Cr. 22.26	CT. 19.34	CT 25.95	CT: 25.97		
	S	19 A				U wc	CT: 31.21	U MC Ct. 31.62	LI MAC CT. 30.98	L IAC CT. 32.08	Cr. 30.95	Cr: 31.39		
		Decumenta				CT: 33.95	U MILIC CT: 24.82	1 Mintc Cr. 18.34	Minc CT: 22.28	1 Masc Cr. 19.42	1 Matc Cr. 25.83	Cr. 25.97		
	0.0001					FDA1095-20	A3	A	C1	12	A2	01		
		This PC				CT: 31.04	CT: 30.91	Ц мс Ст. 30.76	CT 31.35	Ст. 30.97	CT 30.98	CT: 30.73		
	0.0001	Fite name:	ncluskily panel - dinical samples ed		Save	CT 25.01	CT 29.65	Metc	CT 29 42	CT 25.5	CT 27 31	CT 30.96		
		Network Files of type: E	xperiment Document Template files (* edt)	/ ·	Cancel	U VAC	U MC	1 1 wc	1 MC	I IAC	U WC	U WC		
	D.000001 2 4 6 8 10 12 14 10	N 20 22 24 28 28 20 Coria			CT 25 15	CT 25.01	Mitte	Ct: 31.36	T Metc	CT 25.68	T METC	CT 30.58		
		oper			FDA1085	FDA1895-20	43	4	C1	12	A2	UI		
				G	CT: 30.8	Cr: 31.02	Cr: 30.69	U MC Ct. 31.33	CT 31.52	CT: 30.88	CT: 31.53	Cr. 31.34		
	Options				Cr. 25.19	CT. 24.87	Cr: 29.71	U MHC	CT 29.37	CT 25 63	CT 27.22	Cr: 30.46		
	Target All V Threshold Auto	Auto Baseline			Negative c	I Negative co	Negative of							
	Show: Threshold - Baseline Start: Well Target & E	aseline End: Well 🔳 Target 📣		н	Matc	Matc	Minc California							
	Save current settings as the default				CT Unde	e CT: Undece	GEORGEN							
				Wells: 1 51 Unkno	iwn 🔝 0 Standa	d 🔝 3 Neg	alive Control	2						45

(B) 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 cayetanensi	is	Type: Standard Curve	Reagents: Other	START PUR ())-	()
	Setup	Define Targets and Samples As	sign Targets and Samples				
		I lastructions: Define the targets to quantify and	The samples to test in the reaction state				
	Experiment Properties	Define Targets		Define Samples	A REAL PROPERTY AND ADDRESS OF TAXABLE PARTY.		
C	Plate Setup	I manufacture data				~	_
-		Add New Target Add Saved Target Save Ta	arget Delete Target	Add New Sample	Rod Saved Sample Sove Complex Detete Comple		
	Run Method	Target Name	Reporter Quencher	Color Semple Name		Color	
	🔦 Reaction Setup	MIC	FAM ~ None	v v Sample 1			~
	Materiale List	MC .	CY5 Vone	Sample 2			
	Assurements List			Sample 3			~
	Run			Sample 1 diuted			1
	E			Sample 2 diuted			/
	Analysis			Sample 3 diuted			~
							- 1
							- 1
		Define Biological Replicate Groups					
		Instructions: For each biological replicate gro	up in the reaction plate, click Add Biological Group, then	define the biological group.			
		Add Biological Group Delete Biological Group					
		Biological Group Name	Color		Comments		
							- 11
							- 11
						Assign Targets an	d 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." Instructions: To set up unknowns: Select wells, assign target(s), select "U" (Unknown) as th To set up negative controls: Select wells, assign target(s), then select "V" (Neg	e task for each target assignment, then assign a sample. altve Control) as the task for each target assignment.		
Plate-Setup	Assign target(s) to the selected wells.	te Layout View Well Table		
Run Method	Assign Target Task Obenity	Select Wells W	inn Select tem - 💟 - Select tem - 💟	8001 8001 mm
Reaction Setup		n Wells View Legend		
Materials List		2 4 5	6 7 8 9 10	11 12
1000				
Run	Marc	Maric Maric		
Analysis	Mired III Linknown Standard Stendard Nenative Centrol			a state of the sta
	The Define and Set Up Standards			
	Assign sample(s) to the selected wells.			
	Assign Sample			
	Sample 1			
	Sample 3			
	Sample 1 diluted			1000
	Assign sample(s) of selected well(s) to biological group.			
	Assign Brondical Group			
	0			1.000
	Select the dye to use as the passive reference.			1000
	ROX			
	Wells: 1 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.

enment Menu «	Experiment: Cyclospora cayetanensis		Туре:	Standard C	urve		Reag	gents: Other		START RUN	
etup	Define Targets and Samples Assign Targets and	Samples							 		
periment Properties	To set up standards: Click 'Define and Det Up Standards To set up unicrowns: Select wells, assign target(s), selec To set up negative controls: Select wells, assign target(s)	t"U" (Unknown) as the task for eac then select "N" (Negative Control)	th target assignment, then assi as the task for each target ass	gn a sample. Ignment							
te Solop	Assign target(s) to the selected wells.	View Plate Layout	View Well Table						 		
n Method	Assign Target Task Quantity				Select Well	s With: - Select ite	m - 🖂 - Seled	tilem- 🗸			
action Setup	I MIIC III	Show in Wells 🔻	View Legend							HO.	题
		1	2 3	4	5	6	7	8	 10	11	12
terials List		III ac III a	ac III ac								
un		A Metc N	ARTC METC								
alvsis											
	Mixed 🗓 Unknown 🔝 Standard 🔝 Negative Control	B Sample 1	Sample 1 Sample 1								
	Try Define and Set Up Standards										
	Assign sample(s) to the selected wells.	C Sample 2	Sample 2 Sample 2								
	Assign Sample										
	Sample 1										
	Sample 2	2 D Sample 3	Sample 3 Sample 3								
	Sample 3										
	Sample 1 diuted	E Sample 1 diubed Sam	ple 1 diuted Sample 1 diuted								
	Sample 3 diluted										
		F Sample 2 diuted Sam	ole 2 diluted Sample 2 diluted								
	Assign sample(s) of selected well(s) to biological group.										
	Assign Biological Group			N							
		D Cample 3 diuter Can	pre 3 diuted Eample 3 diuted								
	Select the dye to use as the passive reference.	H									
	ROX 🛩										

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: Cyclospora cayetanensis		Type: Standa	ard Curve	Reagents: Other	START	un (s. 🕜
Setup	Define Targets and Samples Assign 1	Targets and Samples					
Experiment Properties	To set up standards: Click "Define and To set up unknowns: Select wells, ass To set up negative controls: Select wells	I Set Up Standards." iign target(s), select "U" (Unknown) as the task fo lis, assign target(s), then select "V" (Negative Co	r each target assignment, then assign a sam ntrol) as the task for each target assignment.	sta.			
Plate Setup	Assign target(s) to the selected wells.	View Plate Lay	yout View Well Table				
Run Method	Assign Target Task	Quantity		Select Wells With: - Select	tem - 🗸 - Select item - 🗸		
Reaction Setup		Show in Wells	View Legend				
Materiats List			2 3 4	5 6	7 8	9 10 11	12
		U MC					
Run		K Merc	MINETC INVESTO				
Analysis		Sample 1	Sample 1 Sample 1				
1.1961	Three of Unknown Standard Nega	ave Control					
	A remit and set of seminarios	Sample 2	Samela 2 Samela 2				
	Assign sample(s) to the selected wells.						
	Assign Sample						
	Sample 1	i D I MC	Sample 3 Sample 3				
	Sample 2	I Marc	Matc Matc				
	Sample 1 diluted	Sample 1 diluted	Sample 1 diluted Sample 1 diluted				
	Sample 2 diluted	E U Metc	Matc Matc				
	Sample 3 diluted						
		F MAC	Inc Inc				
	Assign sample(s) of selected well(s) to biole	ogical group.					
	Assign Biological Group	Sample 3 diluted	Sample 3 diluted Sample diluted				
		1 Auto	MetC MetC				
	Select the dye to use as the passive refere	H					
	ROX	Maller IT of Links	un 🔽 6 Chesters 💟 2 Maantus Control				75 Emit
		Wells: 1 21 Unknor	wn M o standard M 3 Negative Control				r5 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" 🗆 "Save as" 🗆 Experiment

Document Single file (*.eds) with a unique name. Insert plate or tube strips and start the run.



(C) 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.

Results Expo						rife realitie. Exce		14.15.00.0000000000000000000000000000000
	ort	-						
Well	Sample N.	Target Na ¹ ask	Reporter	Ст	Ct Mean	CT SD		
B1	116	IAC UNKNOV	N CY5	30.82815	30.860903	0.0654539		
82	116	IAC UNKNOV	N CY5	30.81829	30.860903	0.0654539		
83	116	IAC UNKNOV	N CY5	30.936268	30.860903	0.0654539		
04	117	IAC LINKNOW	N CYS	30.008905	30.761206	0.10040773		
86	117	IAC LINKNOW	N CY5	30.877712	30 761206	0 10648773		
87	118	IAC UNKNOW	N CY5	30,912022	30,697931	0.1854309		
88	118	IAC UNKNOW	N CY5	30.587885	30.697931	0.1854309		
89	118	IAC UNKNOW	N CY5	30.593891	30.697931	0.1854309		
810	119	IAC UNKNOW	N CY5	31.093542	30.831705	0.23527534		
B11	119	IAC UNKNOW	N CY5	30.763517	30.831705	0.23527534		
B12	119	IAC UNKNOV	N CY5	30.638054	30.831705	0.23527534		
C1	120	IAC UNKNOW	N CY5	30.98061	30.977295	0.0035693		
C2	120	IAC UNKNOW	N CY5	30.977758	30.977295	0.0035693		
C3	120	IAC UNKNOW	N CY5	30.973516	30.977295	0.0035693		
C4	121	IAC UNKNOV	N CY5	30.696188	30.950525	0.23369096		
C5	121	IAC UNKNOV	N CY5	30.99962	30.950525	0.23369096		
C6	121	IAC UNKNOV	N CY5	31.15577	30.950525	0.23369096		
00	122	IAC UNKNOV	N CY5	30.947596	31.0744	0.28495446		
00	122	IAC UNKNOW	N CYS	31.400745	31.0744	0.28495446		
C10	122	IAC LINKNOW	N CV5	31,260004	21.002625	0.20495440		
C11	123	IAC LINKNOW	N CV5	30.959907	31.083635	0.20481487		
C12	123	IAC UNKNOW	N CY5	31 131907	31 083635	0 20481487		
D1	124	IAC UNKNOW	N CY5	31,20964	31 11142	0 17755374		
D2	124	IAC UNKNOW	N CY5	31,218159	31.11142	0.17755374		
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7. Appendix 6. ABI 7500 Fast v1.4 Method.

(A) 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 (μ L): **20**

Run Mode: Fast 7500 Data collection: Stage 2, step 2 (67 °C @ 1:00)

7500 System SDS Software - [Plate1 (Standard Curve)]			
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Reps. 1 Reps. 40			
35.0 55.0 3:00 0:15 67.0 1.00			
Add Cycle Add Hold Add Step Add Dissa Settings	icialion Stage Delete	Нер	
Sample Volume (µL): 20			
Bun Mode Standard 7500	T		
Data Lollection : [Stage 2, Step 2 (62 H @ 1900)	<u> </u>		
			1

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Method				
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(B) 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.



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: \rightarrow Applied Biosystems \rightarrow 7500 system \rightarrow templates \rightarrow File Name: "Cyclospora cayetanensis".

(C) 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"

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			A
				÷
Into Mamai	Gudennerr Text 1			

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".

7500 Fast Sys	tem SDS Softwar	e - [Plate1 CStandard	(Curve)								1.8	-
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dy							Discon	nected				NUM

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.

ietup V Ir	nstrument y Result			_							
late \1	2	3	4	5 0		7	8	9	10	11	1
NTC N	NTC N U	NTC N U									
Sample U U	1 Sample 1 U U	Sample 1 U U									
Sample : U U	2 Sample 2 U U	Sample 2 U									
Sample : U U	3 Sample 3 U U	Sample 3 U U	Well(s): A Sample N	A3,B1-B3,C1-C3, ame: (mixed)	D1-D3,E1-E3,F1	F3,G1-G3,H1-	НЗ				
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Sample : U U	2 diluted Sample 2 di U U	uted Sample 2 diluted U U	 0	mit Well					Passiv	e Reference:	
Sample : U U	3 diluted Sample 3 di U U	luted Sample 3 diluted U U	Add D	etector	Remove		ose		ROX	<u>.</u>	-

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

😰 7500 Fast System SDS Software - Cyclospora cayetanens	is (Standard Curve)]		
File View Tools Instrument Analysis Window	Help		- 5 X
Setup YInstrument VResults			
- Instrument Control	Temperature		
Start Estimated Time Remaining (hh:nni):	Sample: Heat Sink:		
Stop	Cover: Elock:		
Disconnect Status	Cycle Rev		
	Stage: Hap		
Extend	State:		
Thermal Cycler Protocol			
Thermal Profile Auto Increment Ramp Rate			
Stage 1 Stage 2 Reps 1 Reps 40			
96.6 [103] 8.00 0.15 [41.6 [1:00]			
Add Cycle Add Hold Add Step Add D	Viscociation Stage Delete Help		
Settings			
Sample Volume (µL) : 24			
Run Mode Fast 7500	Expert Mode Select Arienv Filters		
Data Collection : Stage 2 Step 2 (61.0 @ 1:00))		
Ready		Disconnected	NUM /

(D) 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:

"Auto Baseline" Threshold = 0.05

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

"Auto Baseline" Threshold = 0.05

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.

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".