U.S. FOOD & DRUG ADMINISTRATION

Standard Operation Procedure
Molecular Detection of *Cyclospora cayetanensis* in Fresh Produce Using Real-Time PCR

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1. Standard Operation Procedure –Molecular Detection of *Cyclospora cayetanensis* in Fresh Produce Using Real-Time PCR.

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 × 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

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 dH_2O 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 dH_2O 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 µL of the supernatant without disturbing the pellet. Note: If a pooled debris pellet sample is greater than approximately 850 µL, 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.

Detection of *Cyclospora cayetanensis* in Produce

Figure 1a

Figure 1b

Figure 1c

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
- 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).
- 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 × 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.
- Remove and discard a total of 1.4 mL of supernatant from each tube in two 700 µL aliquots.
- 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).

- Centrifuge at 14,000 × g for 1 minute. Empty catch tube and replace (FastDNA protocol step 13).
- 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).
- 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 µM 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 → 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 primers for amplification of the mitochondrial *C. cayetanensis* target	Mit1C-f	5'-TCTATTTTCACCATTCTTGCTCAC-3'
Reverse primers for amplification of the mitochondrial *C. cayetanensis* target	Mit1C-r	5'-TGGACTTACTAGGGTGGAGTCT-3'
Forward primers for amplification of the IAC target	dd-IAC-f	5'-CTAACCTTCGTGATGAGCAATCG-3'
Reverse primers for amplification of the IAC target	dd-IAC-r	5'-GATCAGCTACGTGAGGTCCTAC-3’

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" → 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.

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)" → 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

TACAGCACCCTAGCTTGGTAGAATCGATCAGCTACGTGAGGTCCTACGACGATCGCC
AAGCATGCCCTAGCTAAGATGCATCGATTGCTCATCACGTACGTTAGGTCGACTAGG
AGGACTGGAGTGCATCGACTAGCTAAGATGGTTCGATTGCTCATCACGAAGGTTAG
GTCGACTACGAACGAGTCGTATTGCAGGTT

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.

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**AA**TAGAGAAGTTGGTACTACATCAGCTTCTCTGGTTTCATCAATTTGTTTAGGTGTT
ATTAGTACTGAGTTACTACTATTTGTTAGCTTCTTCTGGGGTGCATACACCAGCATTC
TATCTCCTAGTTATGTAACAGACTCCACCCTAGTAAGTCCAACTGAGGGTCTTGTAA
GTATCTCTAGTAG

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 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).

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	

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 components	10.0	µL 2X PrimeTime™ 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).

C. 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 ¼ 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 ¼ DILUTION IN THE SAME INITIAL EXPERIMENTAL RUN (Controls are not tested at a ¼ dilution). Prepare diluted samples following instructions below.

E. ¼ Sample Dilution Protocol:

Transfer 2.5 µL of sample to a clean microcentrifuge tube containing 7.5 µL 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 × 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 ≤ 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 ¼ 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 ¼ dilution).

C. Negative Samples

a. If a sample *C. cayetanensis* Mit1C target reaction produces all replicates with undetermined Ct or no replicate with Ct ≤ 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 ≤ 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. 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/µL. Store dilutions and working solution at -20 degrees 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 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.

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 degrees 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.

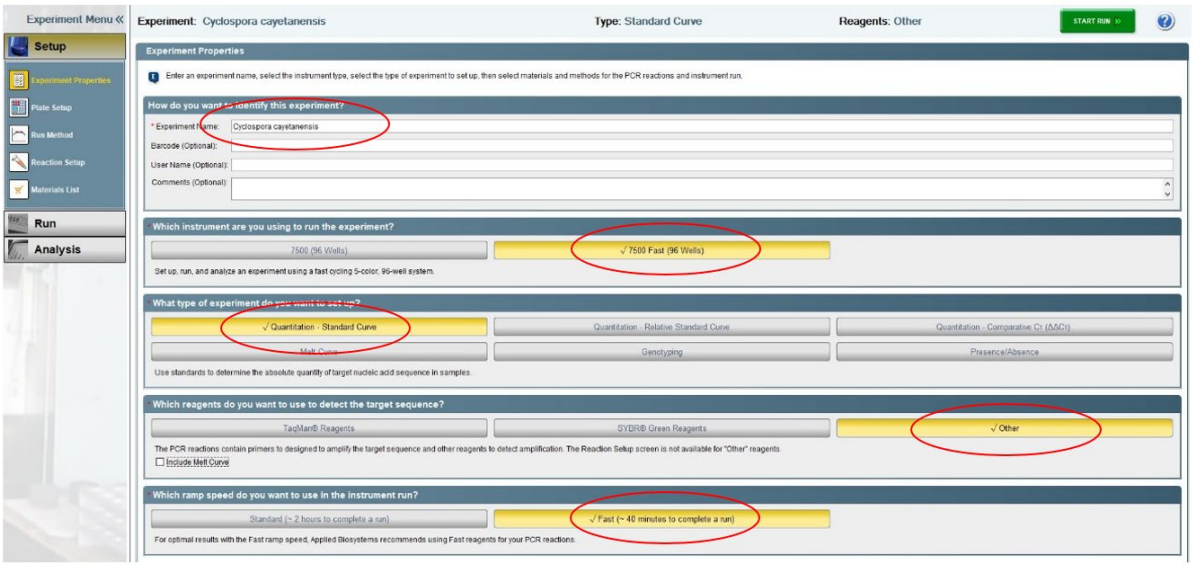
  Step 1: Use the DNA amount (250 ng) and the molecular weight of the sequence (151227.2) to compute the copy number. This will be approximately E12 copies (using 650 Da as the mass of a DNA base pair). Since the lyophilized target is dissolved in 500 µL TE, the approximate copies are 2E9 copies/µL.

- 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.

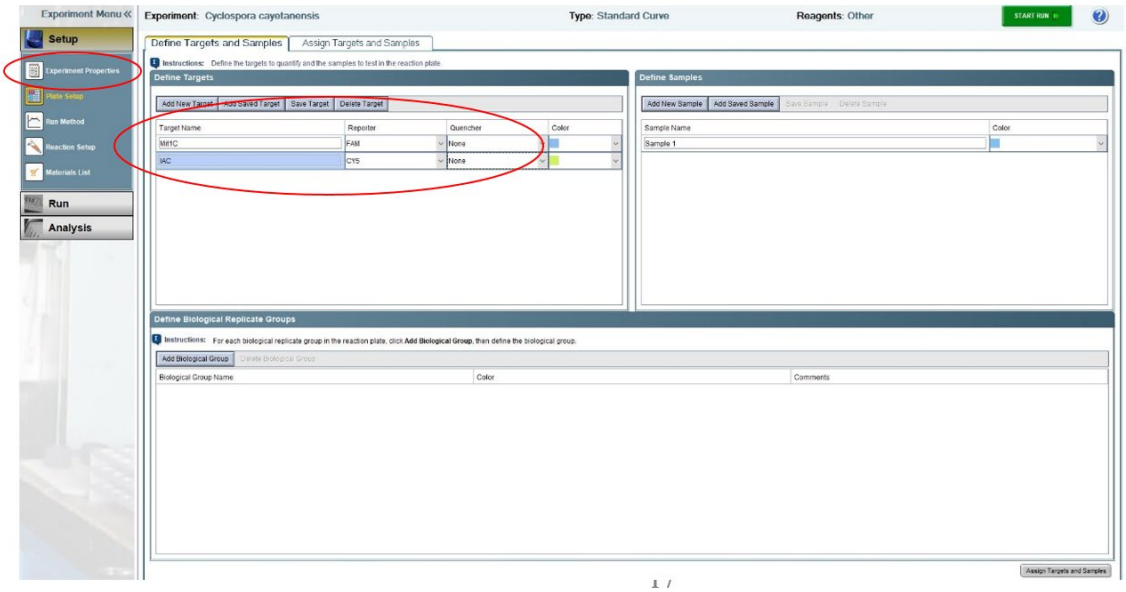
6. Appendix 5. ABI 7500 Fast v2.0 or 2.3 Method

(A) Define a Run Template Using Software v2.0 or 2.3 on the ABI 7500 Fast Instrument

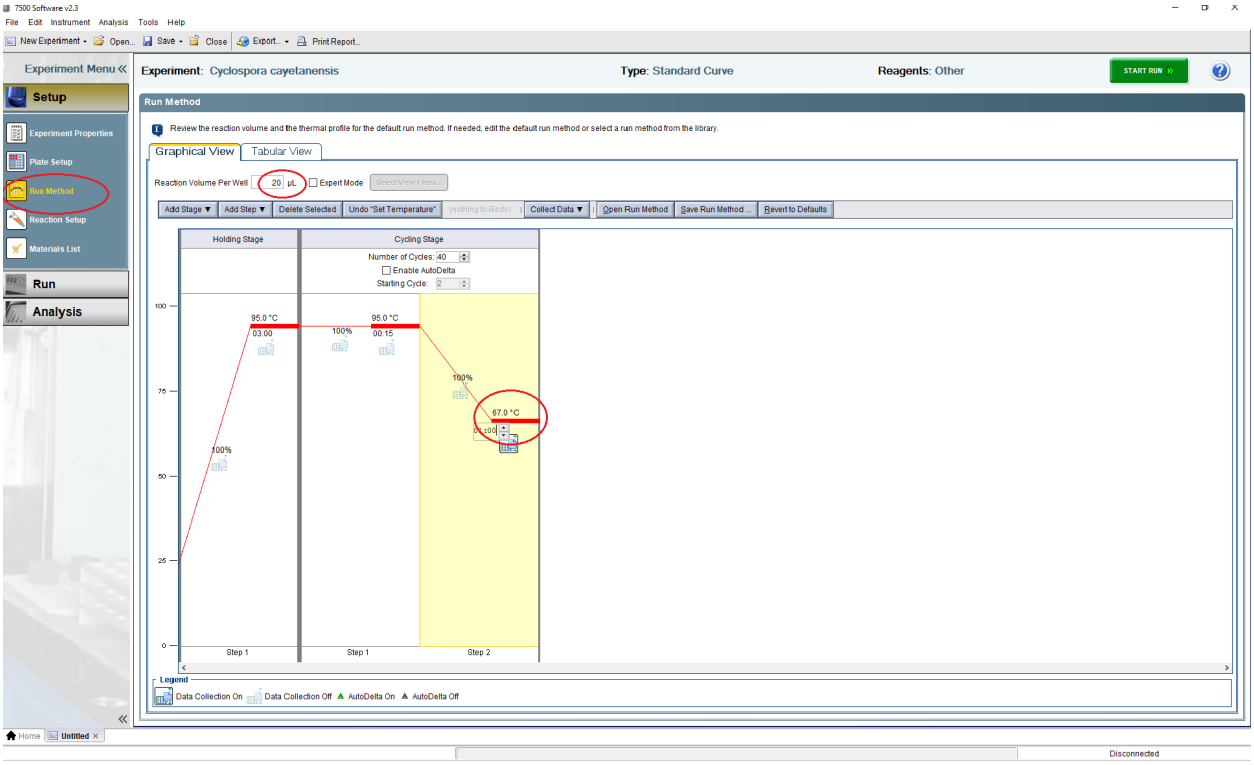
Turn on the computer and ABI 7500 FAST Real-Time PCR system. Open the 7500 Software v2.0 or 2.3 and click "New Experiment" (Advanced Setup). Define "Experimental Properties" as shown below with "Experiment Name"→ "Cyclospora cayetanensis"



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.

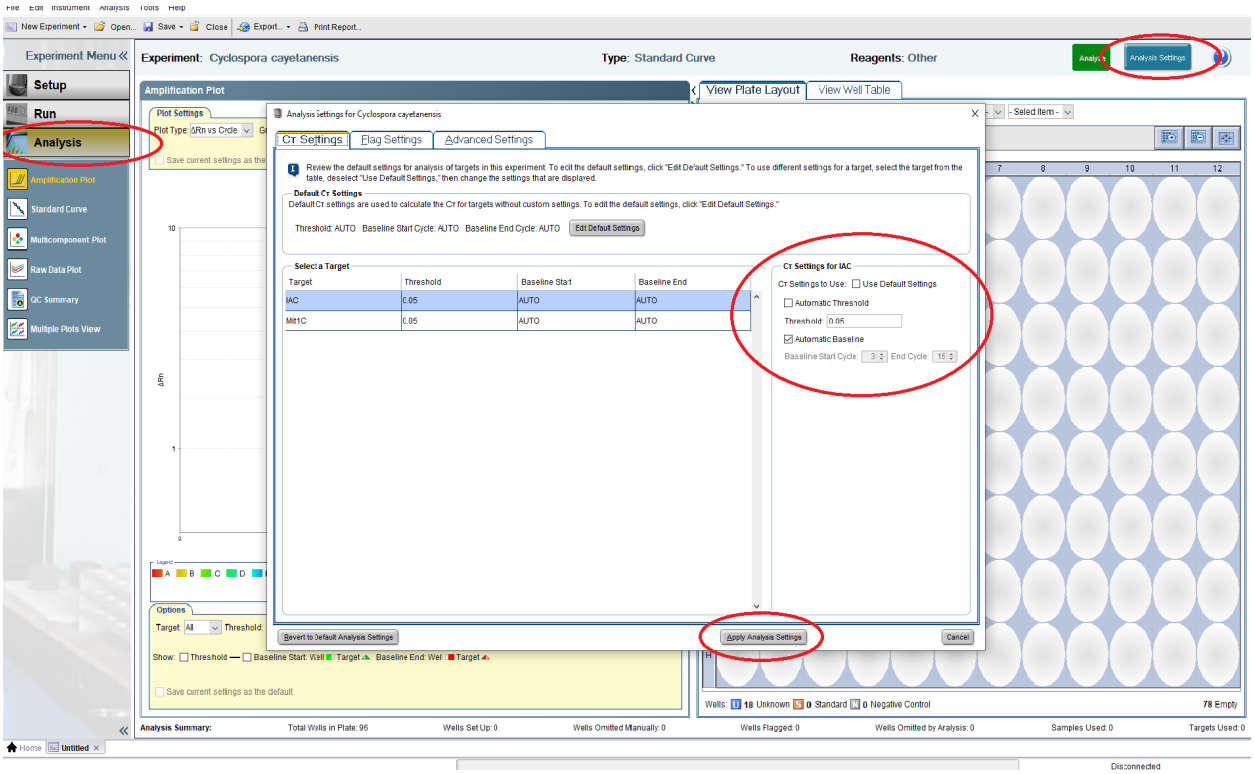


Choose "Analysis" on the left and click "Analysis Settings" in the upper right corner. Define target Ct settings in the pop up window:

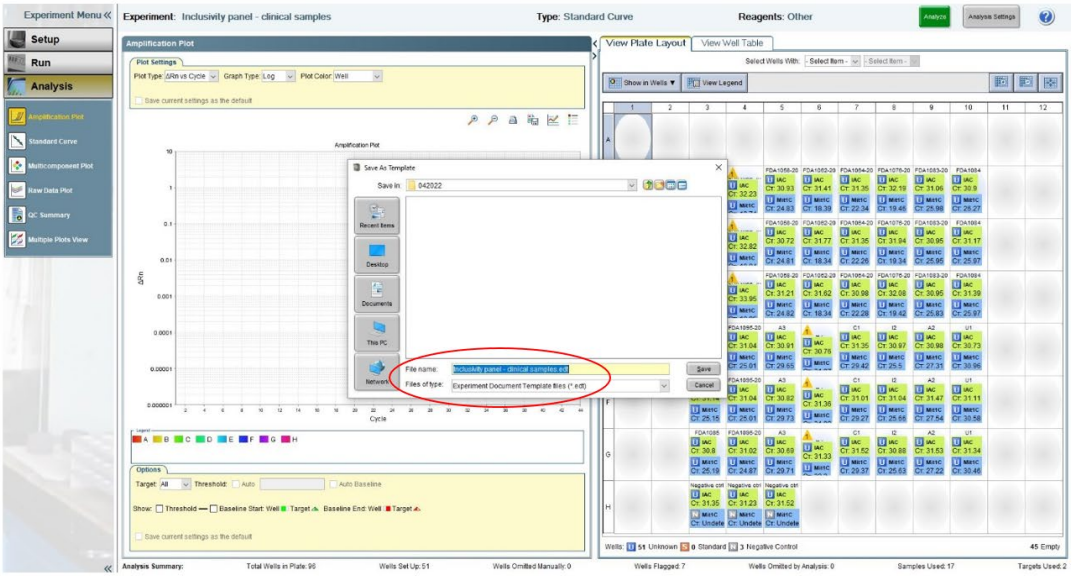
(A) Select the **Mit1C** target: Turn **off:** **"**Use Default Settings" and "Automatic Threshold". Set the Threshold to **0.05** and choose "Auto Baseline"**.**

(B) Select the **IAC** target: Turn **off:** **"**Use Default Settings" and "Automatic Threshold". Set the Threshold to **0.05** and choose "Auto Baseline"**.**

(C) Click "Apply Analysis Settings".



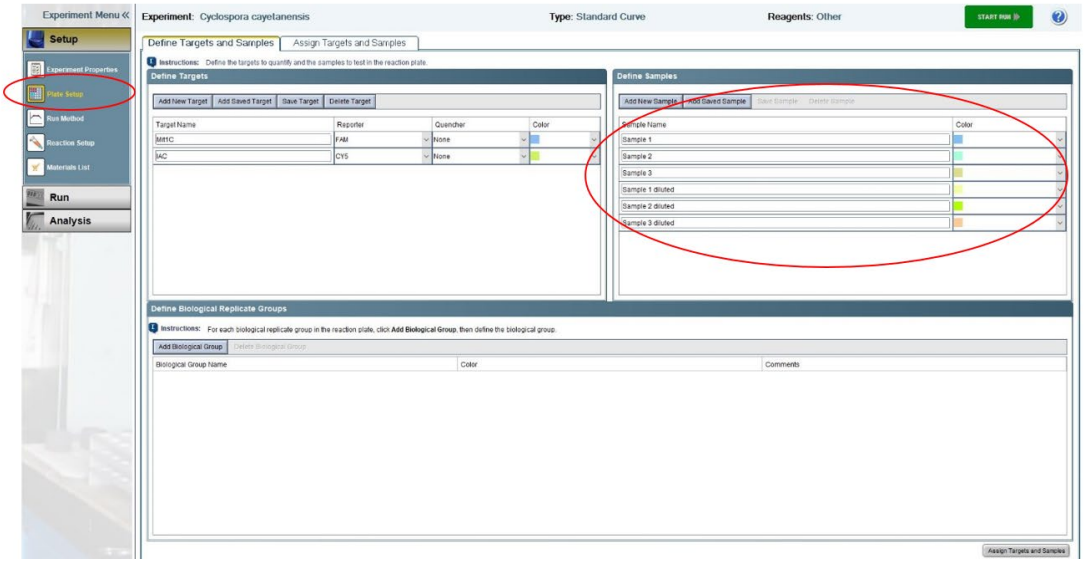
Click "File" → "Save as template…" → "Save"



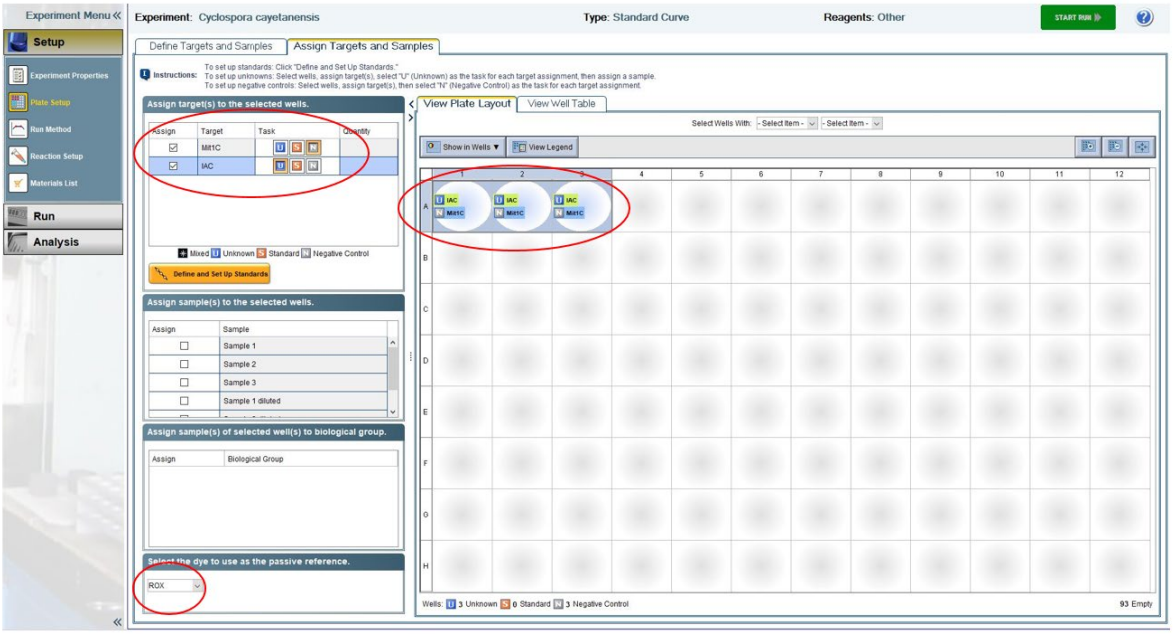
(B) Run Method Using Software v2.0 or 2.3 on the ABI 7500 Fast Instrument

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.

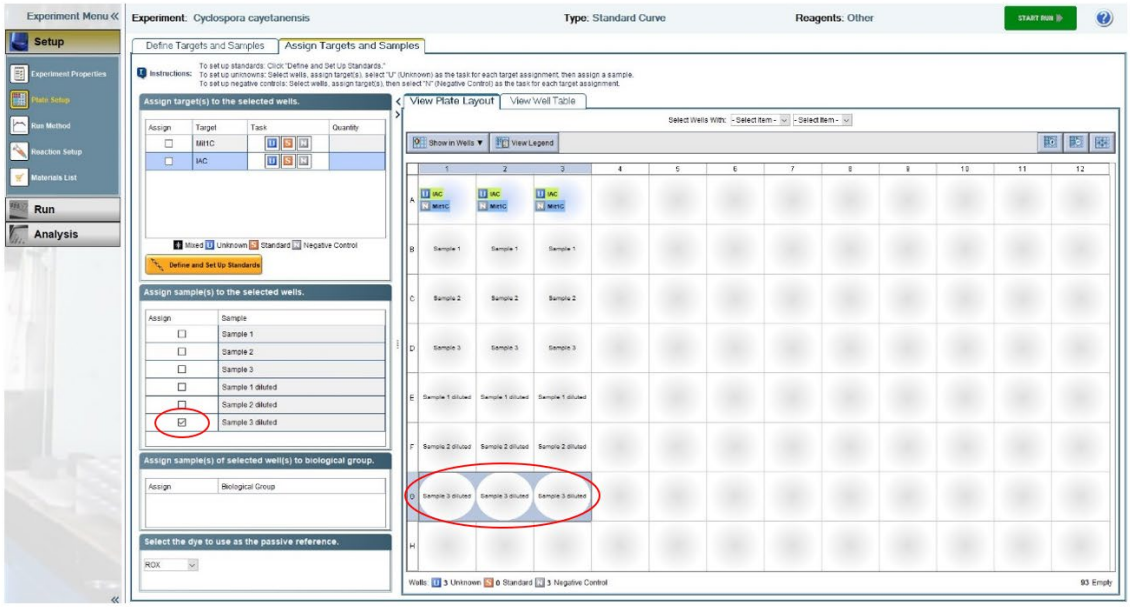


Click the "Assign Targets and Samples" tab to define well assignments. Define the NTC wells by selecting three wells and checking the box next to the Mit1C target choosing "N" as task. Also, assign the internal amplification control by selecting all reaction wells and checking the box next to the IAC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

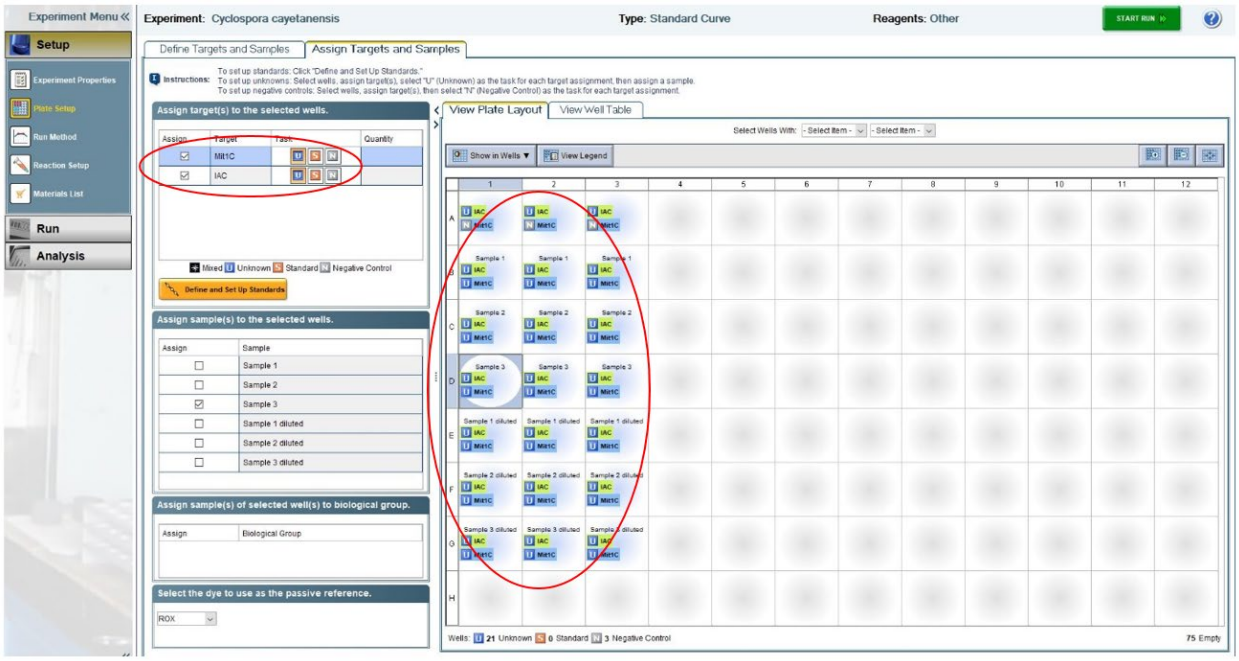


Define all unknown samples or DNA extraction controls one at a time by selecting three wells for each and checking the box next to the sample name in the "Assign sample(s) to the selected

wells" panel.

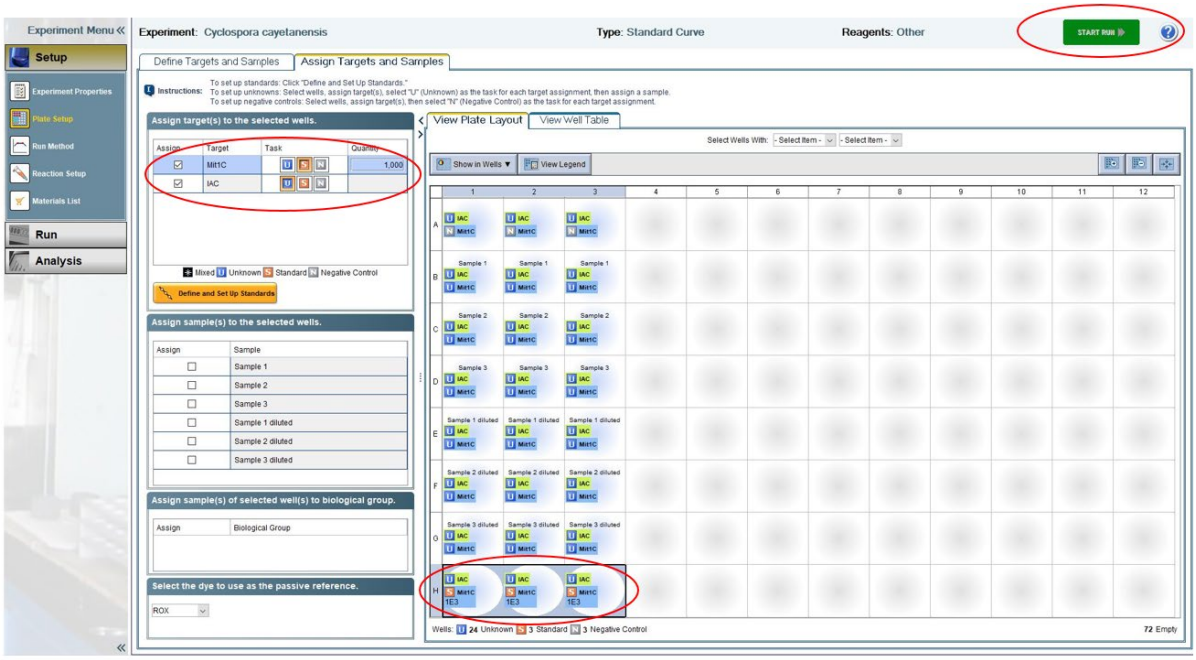


Then select all unknown samples or DNA extraction controls and check the box next to Mit1C and IAC targets choosing "U" as task.



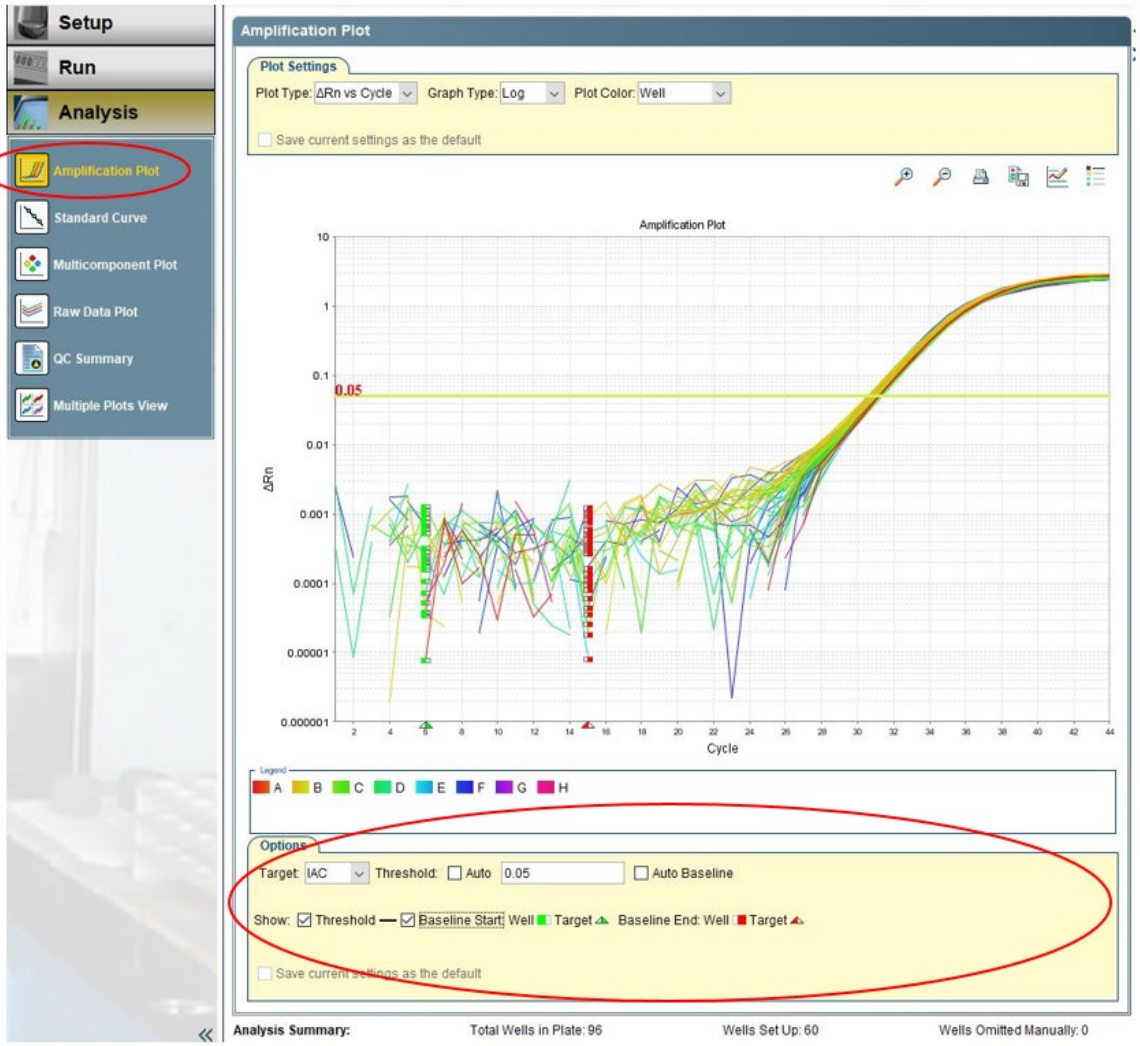
Define the Positive control (Standard) wells by selecting three wells and checking the box next to the Mit1C target choosing "S" as task and "1000" as quantity. "File"□"Save as"□ Experiment

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

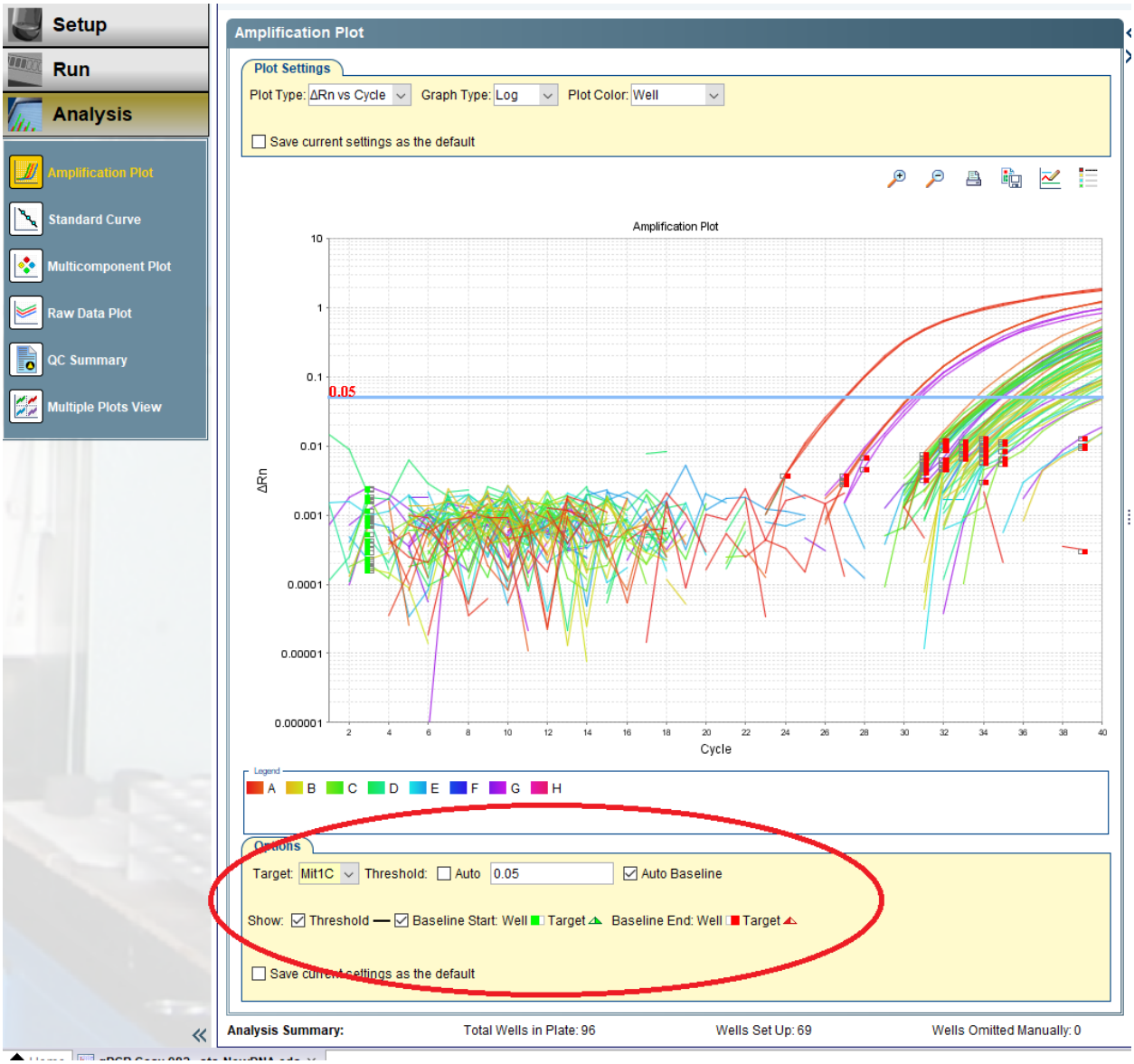


(C) Analysis Using Software v2.0 or 2.3 on the ABI 7500 Fast Instrument

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.


Setup
Run
Analysis
Amplification Plot
Standard Curve
Multicomponent Plot
Raw Data Plot
QC Summary
Multiple Plots View
Amplification Plot
Plot Settings
Plot Type: ΔRn vs Cycle
Graph Type: Log
Plot Color: Well
Save current settings as the default
Amplification Plot
0.05
ΔRn
Cycle
Legend
A B C D E F G H
Options
Target: IAC
Threshold:
Auto
0.05
Auto Baseline
Show: Threshold
Baseline Start: Well Target
Baseline End: Well Target
Save current settings as the default
Analysis Summary:
Total Wells in Plate: 96
Wells Set Up: 60
Wells Omitted Manually: 0

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.

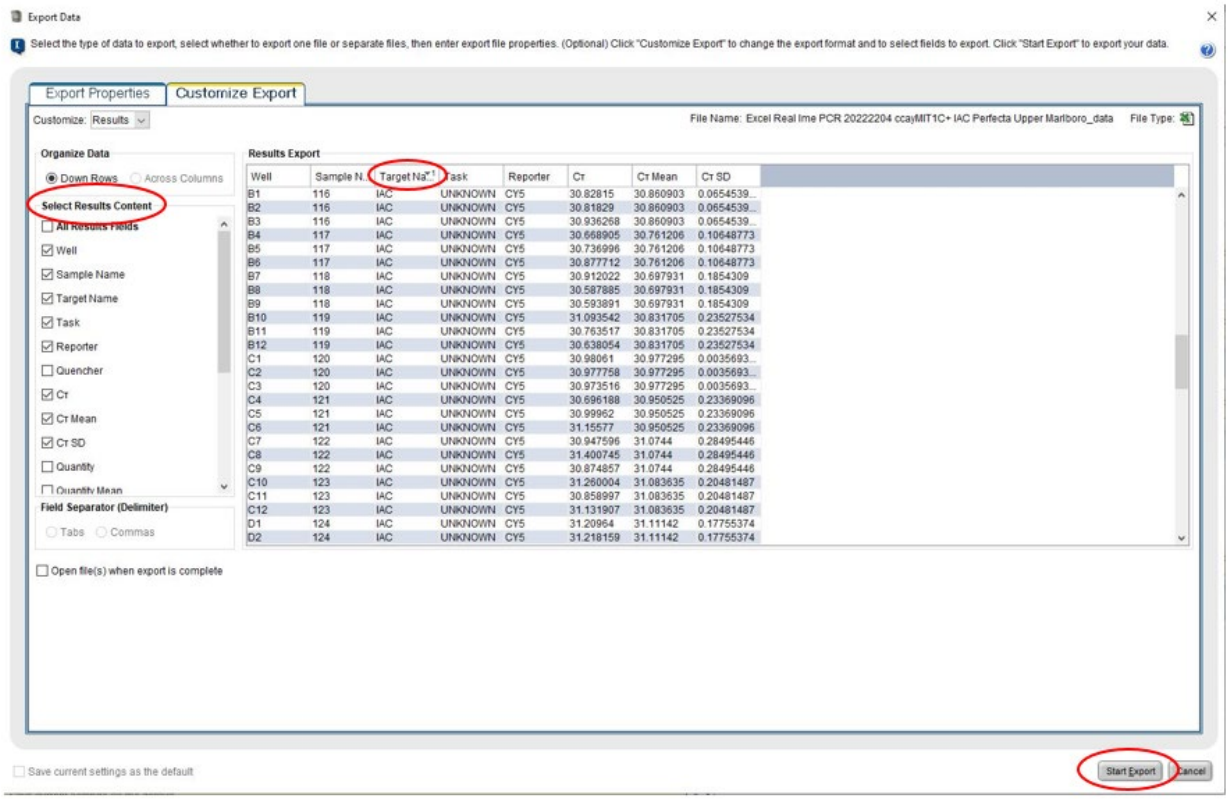
Then, assure that all reaction wells on the plate are selected by clicking the upper left corner of the plate layout. Click “Export” to open the Export Tool window.

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.



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" →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

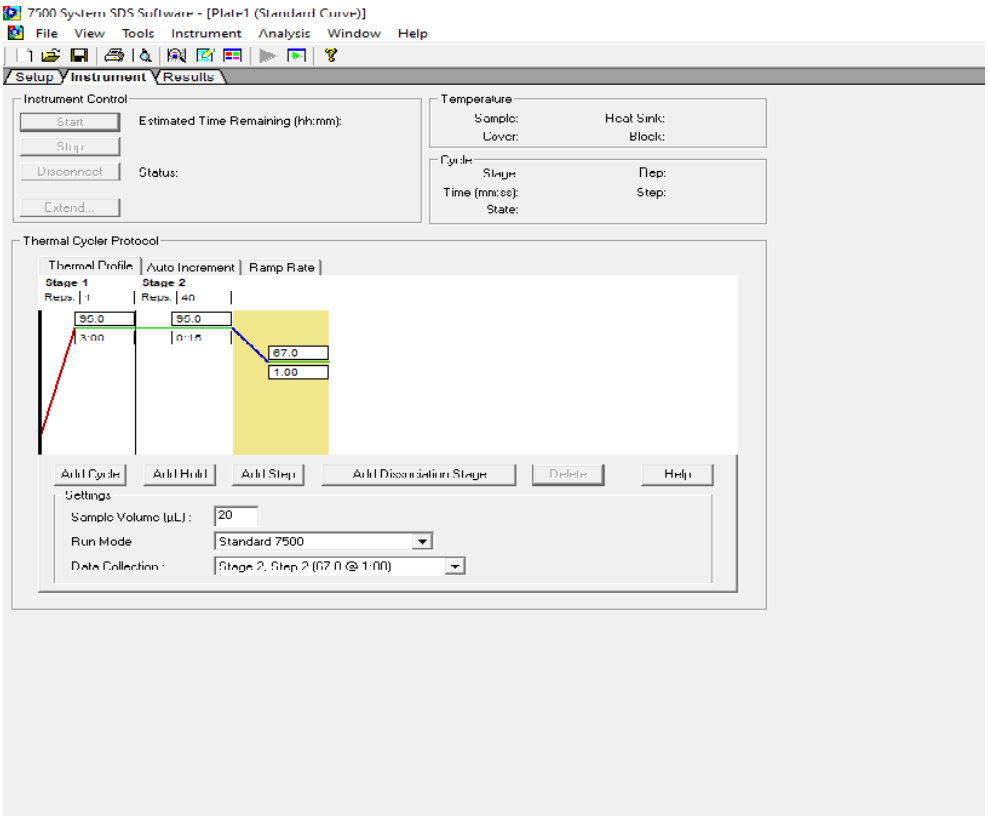
95 °C; 3:00
Stage 2: Reps: 40

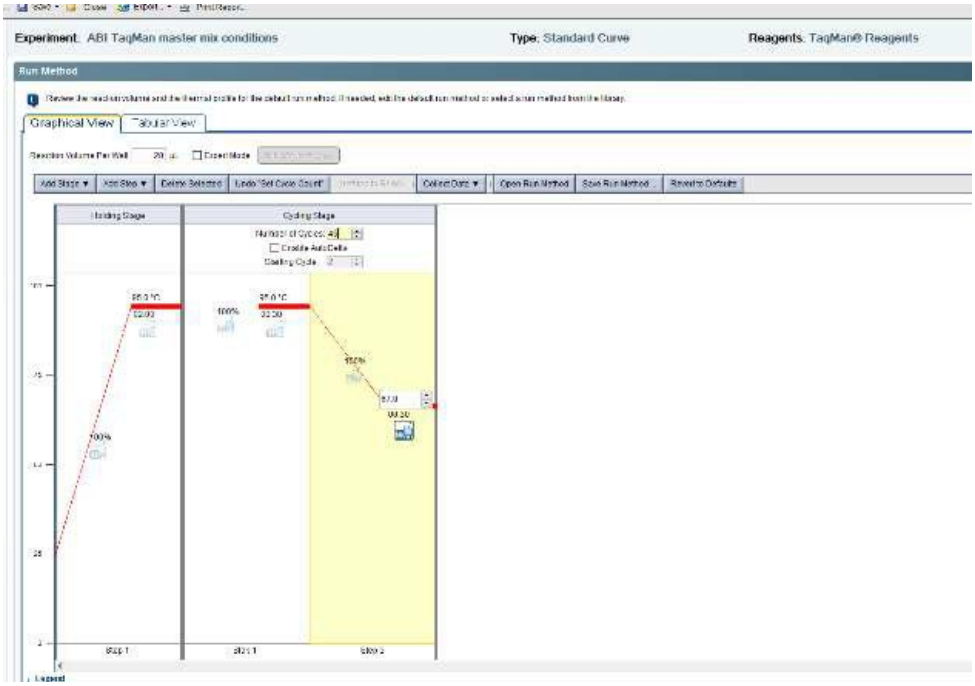
95 °C; 0:15

67 °C; 1:00

2. Sample Volume (µL): **20**

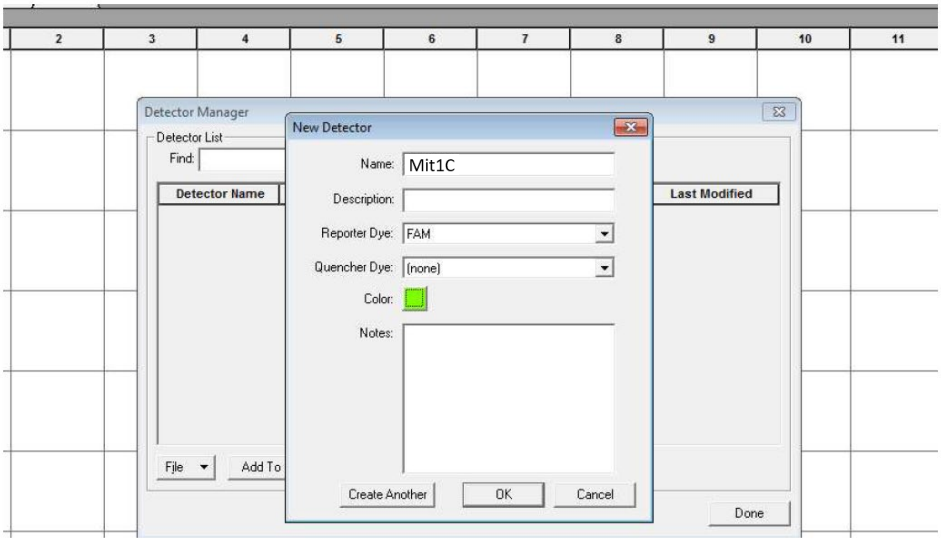
Run Mode: Fast **7500**
Data collection: **Stage 2, step 2 (67 °C @ 1:00)**



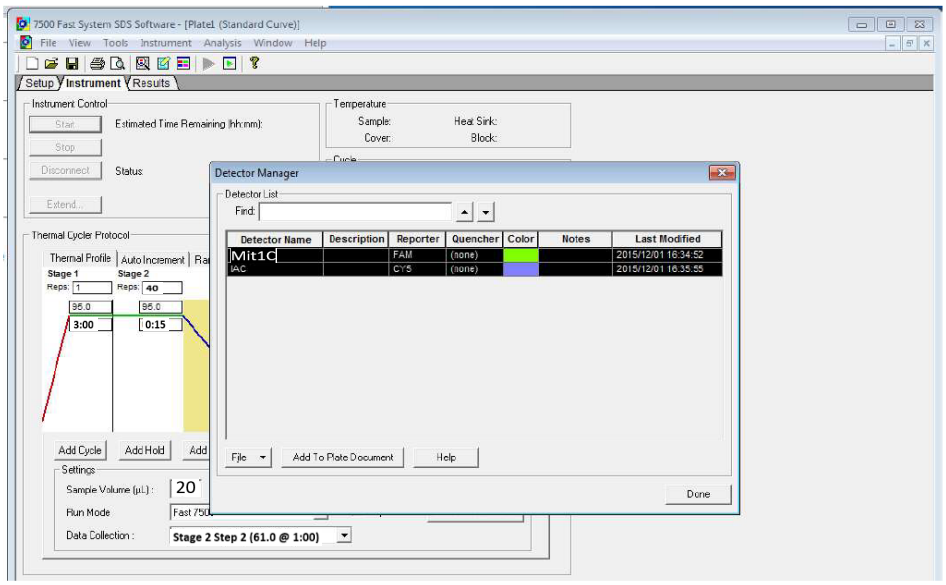


(B) Create Detectors: Mit1C & IAC (internal amplification control)

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”.

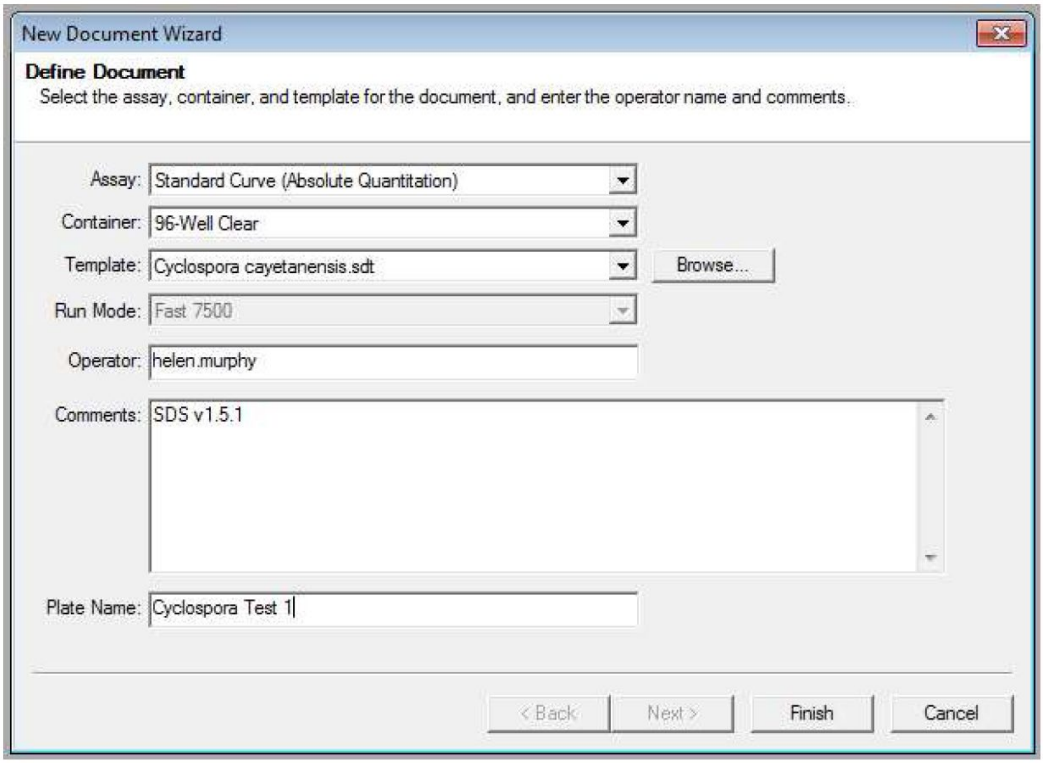
(C) Start a new Run

Turn on the ABI 7500 FAST Real-Time PCR instrument and computer. Open 7500 Fast System Software v1.4
From "Quick Startup" → Click "Create New Document" to open "New Document Wizard" and "Define Document".

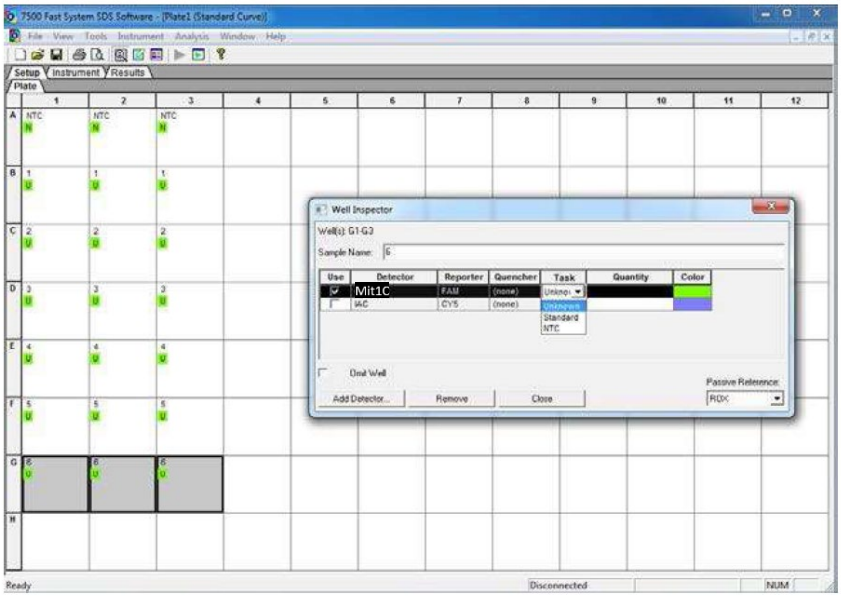
Assay: Standard Curve (Absolute Quantitation)

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

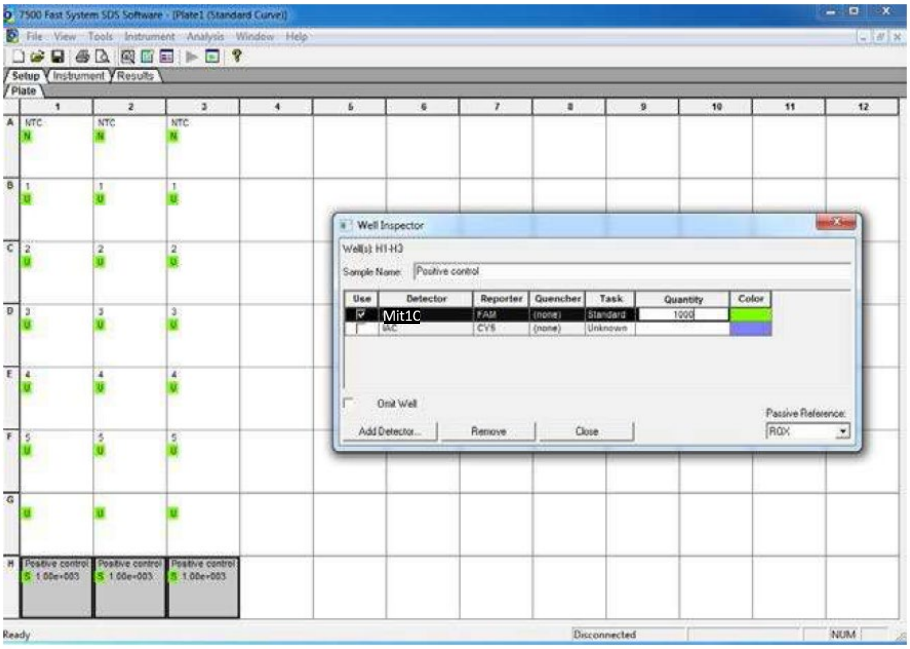
Plate Name: "Cyclospora Test 1" → "Finish"



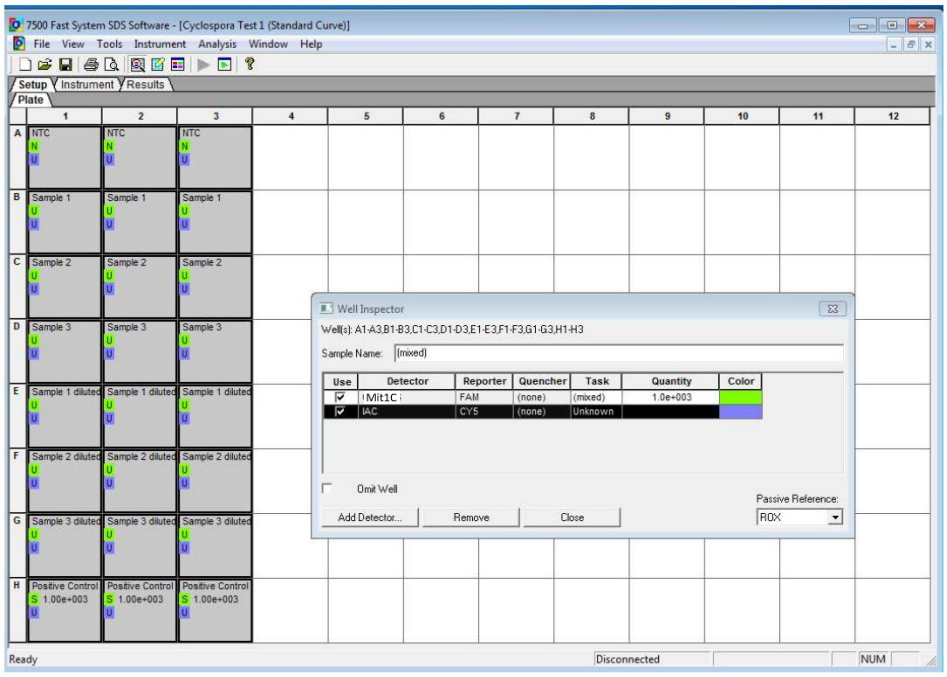
From "View" → Select "Well inspector" to define well assignments. Define the no template control wells by selecting three wells and type "**NTC**" in the "Sample Name" field on the "Well Inspector" window. Check the "Mit1C" detector choosing "NTC" as "Task". Define all unknown samples and DNA extraction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



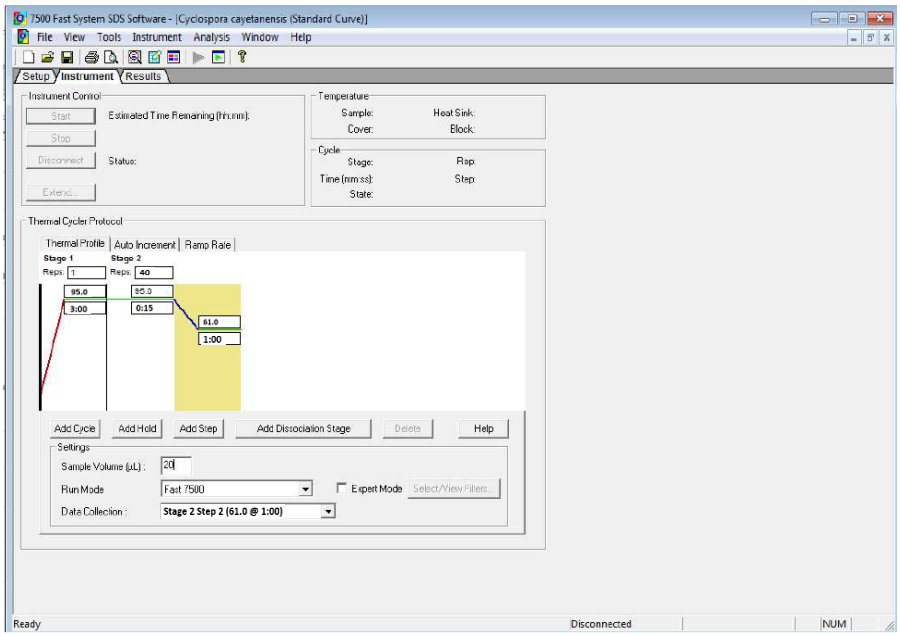
Define the positive control in the same manner but choose "Standard" as "Task" and enter "1000" as "Quantity".



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".



(D) 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:

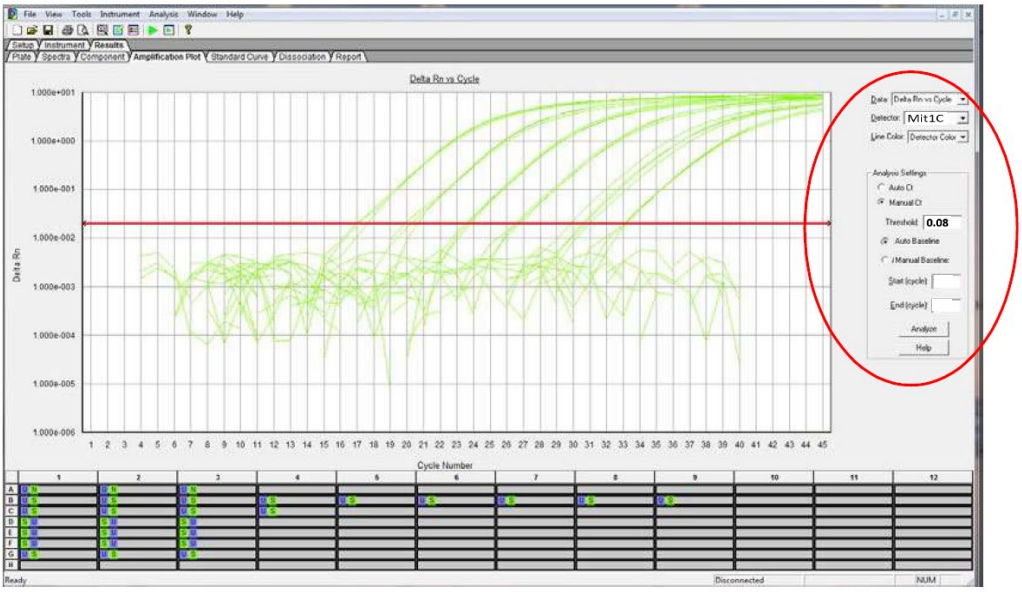
Select Detector "**Mit1C**" and Line Color "**Detector Color**". In the "Analysis Settings" box select:

"**Auto Baseline**" Threshold = **0.05**

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

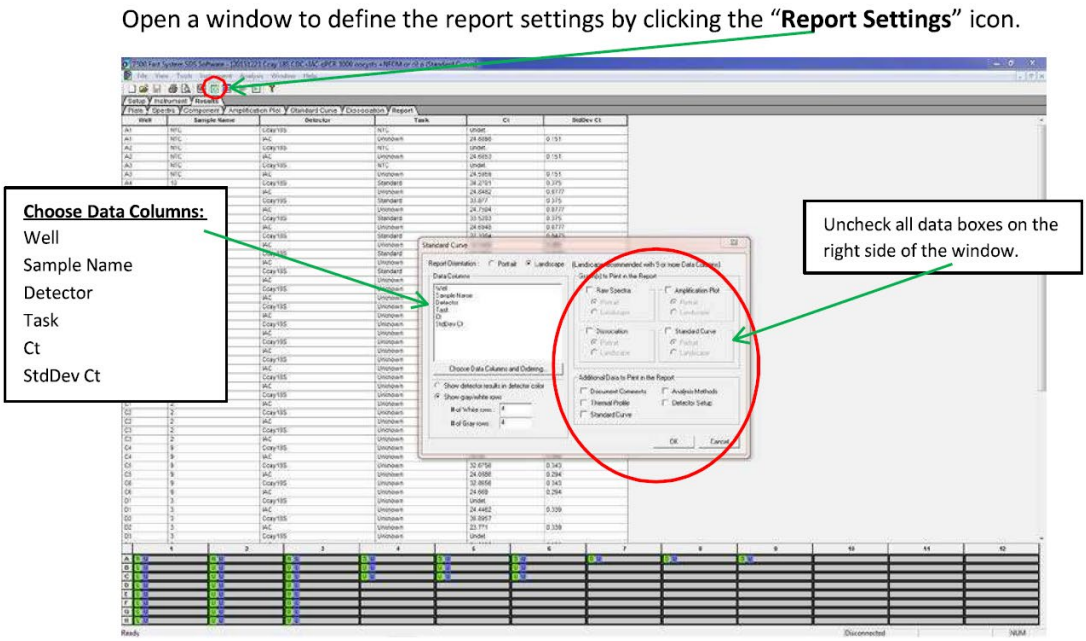
"Auto Baseline" Threshold = **0.05**

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.

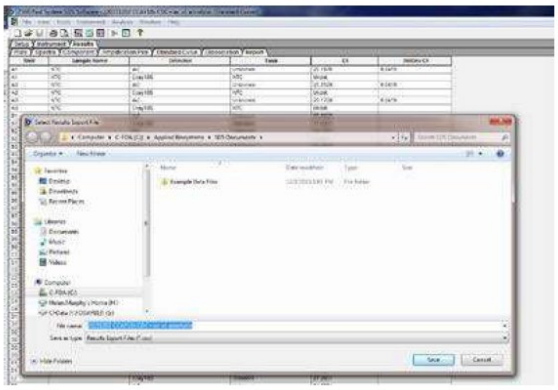
Click on the "Report" tab. Assure that all wells are still detected in the lower portion of the window.



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".