FDA U.S. FOOD & DRUG ADMINISTRATION

Bacteriological Analytical Manual

Chapter 19B: Molecular Detection of Cyclospora cayetanensis in Fresh Produce Using Real-Time PCR

April 2022 Edition

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Revision History

- April 2022: *C. cayetanensis* Blueberry Extension Report has been added.
- April 2022: Method Modification Due to the Discontinuation of the Current/Original QuantiFast Multiplex PCR kit and Replacement with TaqMan™ Fast Advanced Master Mix Verification Study has been added.
- December 2020: *Cyclospora cayetanensis* Blackberry and Shredded Cabbage Reports have been added.
- November 2019: *Cyclospora cayetanensis* Romaine lettuce Extension Report has been added.
- September 2017: Modified FastDNA Spin Extraction Protocol section: Duplicate text removed from step C.; The word "shaking" has been replaced with the word "inverting" in step G.
- September 2017: Posted a Published Journal Article (PDF) and a Supplemental Data File (PDF).
- August 2017: *Cyclospora cayetanensis* Basil Extension Report, C. cayetanensis Parsley Extension Report, and *C. cayetanensis* Carrot Extension Report have been added.
- Six Appendices (PDF format) are available at the end of this method.
- New BAM Chapter 19b; June 2017: Replaces all aspects of the *Cyclospora* methodology in BAM Chapter 19a related to detection of *C. cayetanensis* in produce.

Introduction

Cyclospora cayetanensis is a protozoan parasite responsible for waterborne and foodborne human diarrheal illness called cyclosporiasis. This disease occurs in developed and developing nations with certain seasonality. *C. cayetanensis* is considered the only species of this genus that causes cyclosporiasis in humans. Human cyclosporiasis is a significant public health concern in the U.S. where foodborne outbreaks have occurred since the mid-1990s, and are frequently linked to consumption of imported fresh produce including leafy greens and berries (Hall *et al*., 2012; Herwaldt, 2000). According to the Centers for Disease Control and Prevention, infection by *C. cayetanensis* has affected 1481 patients in the U.S. during the large multi-state outbreaks that took place from 2013 to 2015 (http://www.cdc.gov/parasites/cyclosporiasis/outbreaks/index.html). Infected individuals shed spherical (8-10 µm in diameter) unsporulated oocysts into the environment with feces. Person-to-person transmission is considered unlikely because oocysts seem to require a week or longer in the environment to sporulate and become infective (Ortega and Sanchez, 2010). The infectious dose of *C. cayetanensis* is not known.

C. cayetanensis oocysts are not culturable, which makes the application of viability assays impractical. Identification of *Cyclospora* oocysts in food matrices by microscopy using currently available methodologies is challenging: 1. These methods lack the required sensitivity for the detection of low concentration of oocysts in food matrices; 2. These methods do not allow discrimination of *Cyclospora* species that are morphological identical to *C. cayetanensis*. A method including molecular detection by PCR was developed and published in the FDA BAM in 2004 but recently its use has been hindered because certain aspects of its execution are technically problematic. FDA's regulatory oversight related to surveillance and outbreak investigations was severely impacted during the cyclosporiasis outbreaks from 2013 to 2015 due to lack of fully validated sensitive detection methods and molecular epidemiological tools for source tracking (Abanyie *et al*., 2015). The development and validation of an improved and streamlined FDA method for recovery and identification of *C. cayetanensis* from produce by real-time PCR will allow FDA to begin to close investigational gaps and support regulatory actions when food commodities are shown to be contaminated with the parasite, particularly in outbreak situations. The process for molecular identification of *C. cayetanensis* in fresh produce by PCR can be broken down into the following steps: 1. Produce wash procedure, 2. Isolation of DNA from produce wash, and 3. Identification by PCR amplification. The 2004 method found in Chapter 19A of the FDA BAM entitled "Detection of *Cyclospora* and *Cryptosporidium* from Fresh Produce: Isolation and Identification by Polymerase Chain Reaction (PCR) and Microscopic Analysis" became impractical to use in 2012 when certain supplies required for the oocyst recovery and DNA extraction methodology were no longer commercially available through the original or alternative manufacturers. In addition, the Chapter 19A method relied on amplification by nested PCR for molecular identification which is time consuming, laborious, and prone to producing false-positive results. These factors elicited an urgent need for a replacement method that could be used for regulatory testing of fresh produce. Therefore, a new and improved produce washing and DNA extraction protocol for detection of *C. cayetanensis* in fresh produce was developed and validated in conjunction with a new optimized real-time PCR method in July of 2016. The new validated method is appropriate for the detection of *C. cayetanensis* in leafy greens such as lettuces, cilantro, and basil; soft fruit such as raspberries, blackberries, or strawberries; and whole vegetables such as beans or peas. The new method has been validated for cilantro and raspberries in a multi-laboratory validation study and for shredded carrots, parsley, and basil in matrix extension studies. Validation studies were conducted in accordance with the OFVM Guidelines for the Validation of Analytical Methods for the Detection of Microbial Pathogens in Foods and Feeds, 2nd Edition and approved by the Microbiology Methods Validation Subcommittee. The MLV was approved 7/6/2016 and the matrix extension studies were on 2/7/2017 and 6/16/2017.

Multi-laboratory Validation Study Publication:

The new method has been validated for cilantro and raspberries in a multi-laboratory validation study and for shredded carrots, parsley, basil, romaine lettuce in matrix extension studies. Validation studies were conducted in accordance with the OFVM Guidelines for the Validation of Analytical Methods for the Detection of Microbial Pathogens in Foods and Feeds, 2nd Edition and approved by the Microbiology Methods Validation Subcommittee. The MLV was approved 7/6/2016 and the matrix extension studies were on 2/7/2017 and 6/16/2017.

1. Article, PDF, Interlaboratory validation of an improved method for detection of *Cyclospora cayetanensis* in produce using a real-time PCR assay. 893Kb

2. Supplementary Data, PDF, 384Kb

Extension Reports for Basil. Parsley, Carrot, Romaine Lettuce, Blackberry, Shredded Cabbage and Blueberry:

1. *C. cayetanensis* Basil Extension Report, PDF, 65Kb
2. *C. cayetanensis* Parsley Extension Report, PDF, 66Kb
3. *C. cayetanensis* Carrot Extension Report, PDF, 67Kb
4. *C. cayetanensis* Romaine Lettuce Extension Report, PDF, 232Kb
5. *C. cayetanensis* Blackberry Extension Report, PDF, 60Kb
6. *C. cayetanensis* Shredded Cabbage Extension Report, PDF, 157Kb
7. *C. cayetanensis* Blueberry Extension Report, PDF, 54.5Kb

Method Modification Due to the Discontinuation of the Current/Original QuantiFast Multiplex PCR kit and Replacement with TaqMan™ Fast Advanced Master Mix (PDF)

Produce Washing and DNA Extraction Procedures

This analytical procedure provides improved steps for isolation of *C. cayetanensis* oocysts from produce by washing and subsequent DNA template preparation from produce washes. The method replaces the FTA filter based methodology employed in Chapter 19A for detection of *C. cayetanensis*. Improvements include the use of Alconox® detergent in a produce wash solution which significantly improves recovery of parasitic protozoa from food (Shields *et al.*, 2012) and DNA isolation from food wash debris pellets using a commercial DNA extraction kit to prepare DNA templates for use in subsequent PCR detection (Shields *et al.*, 2013).

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®+ 400ml 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 15 and 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

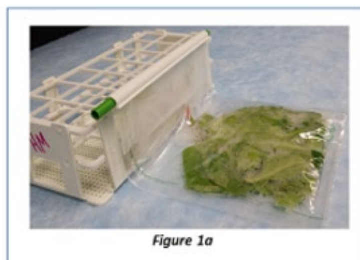
Figure 1a

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

A. Assemble the samples to be extracted from wash procedure step *4J* and add an empty FastPrep tube as a DNA extraction control.
B. Carefully transfer into each tube in **step 5A** the beads from a Lysing Matrix E tube (supplied with the FastDNA Spin Kit).
C. Add 122 µl MT buffer (FastDNA protocol step 3).

D. 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

E. 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.
F. Remove the tubes from the sample holder and centrifuge at 14,000 × g for 15 minutes (FastDNA protocol step 5).
G. 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).
H. 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.
I. 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.
J. Remove and discard a total of 1.4 ml of supernatant from each tube in two 700 µl aliquots.
K. 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.
L. Add 500 µl prepared SWES-M to each filter. Gently resuspend each by pipetting up and down (FastDNA protocol step 12).
M. Centrifuge at 14,000 × g for 1 minute. Empty catch tube and replace (FastDNA protocol step 13).
N. 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).
O. Air dry the filter for 5 minutes at room temperature (FastDNA protocol step 15).
P. 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).
Q. Centrifuge at 14,000 × g for 1 minute to recover the eluted DNA and then discard the SPIN Filter (FastDNA protocol step 17).

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R. 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 conventional and real-time PCR methods found in the FDA BAM Chapter 19A. The real-time PCR protocol described below offers several advantages including increased sensitivity, specificity, and throughput, and it significantly reduces analytical time to obtain results. 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 method used by the Centers for Disease Control for clinical samples (Qvarnstrom, 2016; Verweij *et al*., 2003). The real-time PCR assay is a duplex reaction which targets the C. cayetanensis multicopy 18S ribosomal RNA gene and uses an internal amplification control (Deer *et al*., 2010) to monitor for potential matrix derived inhibition of the reaction. The method also provides a traceable synthetic positive control, allowing sequence verification to identify false positives stemming from inadvertent laboratory contamination.

1. **Laboratory Facilities**

The *Cyclospora cayetanensis* Real-Time qPCR assay is extremely sensitive. To eliminate the potential for positive results due to contamination it is imperative to design separate workstations for each step in the qPCR analysis with an efficient workflow.

A. Mastermix reagents, samples, and positive controls should always be stored in separate locations and handled separately with clean gloves and wearing clean lab coats. Utmost care should be taken when handling and opening tubes to avoid cross-contamination. Tubes should always be vortexed and briefly centrifuged prior to opening.

B. Dilution of samples should be done in a separate clean area.

C. Surfaces, hoods, pipets and other equipment should be cleaned routinely with freshly prepared 10% bleach solution and UV sterilized for 20 minutes if possible. Equipment should be dedicated to one workstation and not transferred from one to another. Gloves should be changed frequently. Aerosol-barrier pipet tips must always be used.

D. The mastermix preparation workstation should be strictly separated from other workstations. Ideally this workstation should be in a separate room if space allows using a dedicated bench top and a PCR hood ideally under positive pressure. Addition of mastermix to 96 well plates or tube strips should be performed at this workstation. Water is added to the NTC reactions in this area.

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E. Test samples should be added to the mastermix plate in a second dedicated clean area, preferably in a PCR hood as well.

F. Positive controls should be handled with the utmost caution and added to the reaction plate as the last step in a separate clean dedicated area if possible.

2. **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. Micropipettors

   F. Aerosol resistant micropipette tips

   G. Latex or nitrile gloves

   H. Vortex Mixer

   I. Dnase-free microcentrifuge tubes, 1.5 mL, low retention

3. **Reagents**

   A. Qiagen QuantiFast Multiplex PCR Kit (400), Cat No. 204654 After discontinuation of Qiagen QuantiFast Multiplex PCR Kit by the manufacturer, please use TaqMan™ Fast Advanced Master Mix, ThermoFisher Scientific Applied Biosystem, Cat #4444556 (1 x 1 mL) or Cat #4444557 (1 x 5 mL).

   B. Sterile Dnase-free TE buffer pH 7.5 (commercially prepared or see Appendix 2).

   C. Primers, 500 µM working solutions (See Table 1).

   D. Probe working solutions (See Table 2).

   E. IAC Target (HMultra130-synIAC), 1E7 copies/µL, see Reagent Ordering and Preparation Instructions, Section 3.

   F. Positive Control (HMgBlock135m), 5E2 copies/µL. see Reagent Ordering and Preparation Instructions, Section 4.

   G. Negative control (Water, included in the QuantiFast Multiplex PCR Kit above).

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4. **Reagent Ordering and Preparation Instructions**

All Primers, Probes, and Target 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:

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

Table 1. Primer Names and Sequences

	Item Name	**Sequence**
Primers for amplification of the *C. cayetanensis* target	Cyclo250F	5'-TAGTAACCGAACGGATCGCATT-3'
Primers for amplification of the *C. cayetanensis* target	Cyclo350RN	5'-AATGCCACGGTAGGCCAATA-3'
Primers for amplification of the IAC target	dd-IAC-f	5'-CTAACCTTCGTGATGAGCAATCG-3'
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 quencher. The IAC probe is labeled with 5’ Cy5 reporter dye and 3’ Iowa Black® RQ-Sp quencher. Probes are ordered from IDT and hydrated to working concentrations as described below and stored at -20 degrees 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 2. (No "Services" options are required.)

Table 2. Probe Ordering Information

	Probe for detection of *C. cayetanensis* target	**Probe for detection of IAC target**
Item Name	Cyclo281T	dd-IAC-Cy5
Sequence	5'-CCGGCGATAGATC ATTCAAGTTTCTGACC-3'	5'-AGCTAGTCGATGC ACTCCAGTCCTCCT-3'
5' Code	/56-FAM/	/Cy5/

3' Quencher	ZEN-3' Iowa Black® FQ	3' Iowa Black® RQ-Sp
3' Code	/3IABkFQ/	/3IAbRQSp/

Preparation of Probe Working Solutions:

100 µM Cyclo281T: 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.

50 µM dd-IAC-Cy5: Hydrate the lyophilized probe in sterile Dnase free TE buffer by adding 2× 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:

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

TACAGCACCCTAGCTTGGTAGAATCGATCAGCTACGTGAGGTCCTACGACGATCGCCAAGCA
TGCCCTAGCTAAGATGCATCGATTGCTCATCACGTACGTTAGGTCGACTAGGAGGACTGGAG
TGCATCGACTAGCTAAGATGGTTCGATTGCTCATCACGAAGGTTAGGTCGACTACGAACGAG
TCGTattgcaGGTT

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 degrees C.

D. **Positive Control**: The positive control DNA (**HMgBlock135m**) is a 998bp double stranded synthetic gBlocks® Gene Fragment synthesized by IDT. The sequence corresponds to *C. cayetanensis* 18S rRNA gene nucleotides 203-1200 but contains traceable mutations (T885A and C886G) 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:: HMgBlock135m

Sequence:

TTTATTAGATACAAAACCAACCCACTTTGTGGAGCCTTGGTGATTCATAGTAACCGAACGGATCGCATTTGG
CTTTAGCCGGCGATAGATCATTCAAGTTTCTGACCTATCAGCTTAGGACGGTAGGGTATTGGCCTACCGTGG
CATTGACGGGTAACGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGG
AAGGCAGCAGGCGCGCAAATTACCCAATGAAAACAGTTTCGAGGTAGTGACGAGAAATAACAATACAGGGCA
TTTAATGCTTTGTAATTGGAATGATAGGAATTTAAAATCCTTCCAGAGTAACAATTGGAGGGCAAGTCTGGT
GCCAGCAGCCGCGGTAATTCCAGCTCCAATAGTGTATATTAGAGTTGTTGCAGTTAAAAAGCTCGTAGTTGG

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ATTTCTGTCGTGGTCATCCGGCCTTGCCCGTAGGGTGTGCGCCTGGGTTGCCCGCGGCTTTCTTCCGGTAGC
CTTCCGCGCTTCGCTGCGTGCGTTGGTGTTCCGGAACTTTTACTTTGAGAAAAATAGAGTGTTTCAAGCAGG
CTTGTCGCCCTGAATACTGCAGCATGGAATAATAAGATAGGACCTTGGTTCTATTTTGTTGGTTTCTAGGAC
CGAGGTAATGATTAATAGGGACAGTTGGGGGCATAGGTATTTAACTGTCAGAGGTGAAATTCTTAGATTTGT
TAAAGACGAACTACTGCGAAAGCATTTGCCAAGGATGTTTTCATTAATCAAGAACGACAGTAGGGGGTTTGA
AGACGATTAGATACCGTCGTAATCTCTACCATAAACTATGCCGACTAGAGATAGGGAAACGCCTACCTTGGC
TTCTCCTGCACCTCATGAGAAATCAAAGTCTCTGGGTTCTGGGGGGAGTATGGTCGCAAGGCTGAAACTTAA
AGGAATTGACGGAGGGGCACCACCAGGCGTGGAGCCTGCGGCTTAATTTGACTCAACACGGG

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 1000 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.

5. 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)

20X Ccay18S Pr/Pro (10 µM each primer, 2.0 µM probe)	
10.0 µL 500 µM Cyclo250F	0.5 µM final in reaction
10.0 µL 500 µM Cyclo350RN	0.5 µM final in reaction
10.0 µL 100 µM Cyclo281T	0.1 µM final in reaction
470 µL TE	
500 µL final volume	

20X synIAC Pr/Pro (2.0 µM each primer,4 µM probe, 2E5 copies/µL synIAC target)	
2 µL 500 µM dd-IAC-f	100 nM final in reaction
2 µL 500 µM dd-IAC-r	100 µM final in reaction
40 µL 50 µM dd-IAC-Cy5	200 nM final in reaction
10 µL 1E7 copies/µl HMultra130-synIAC	1E4/µL final in reaction
446 µL TE	
500 µL final volume	

B. <u>Real-Time PCR reaction mix for 20 µl volume reactions</u>:

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 mastermix 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 mastermix between N+1 and N+3 to assure sufficient reagent for all replicates.

Master Mix Component	10.0	µL 2X Qiagen QuantiFast Multiplex PCR Master Mix
Master Mix Component	1.0	µL 20X Ccay18S Pr/Pro Mix
Master Mix Component	1.0	µL 20X synIAC Pr/Pro Mix
Master Mix Component	6.0	µL H_2O (provided in PCR Master Mix Kit)
	2.0	µL sample or control
	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).

After discontinuation of Qiagen QuantiFast Multiplex PCR Kit (400), Cat No. 204654 by the manufacturer, please use TaqMan™ Fast Advanced Master Mix, ThermoFisher Scientific Applied Biosystem as:

Master Mix Component	10.0	µL 2X AB TaqMan™ Fast Advanced Master Mix
Master Mix Component	1.0	µL 20X Ccay18S Pr/Pro Mix
Master Mix Component	1.0	µL 20X synIAC Pr/Pro Mix
Master Mix Component	6.0	µL H_2O (provided in PCR Master Mix Kit)
	2.0	µL sample or control
	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).

Note: TaqMan™ Fast Advanced Master Mix is stored at 4°C.

C. **Samples and Controls:**

NTC	2.0 µL H_2O (provided in PCR Master Mix Kit)
DNA extraction control	2.0 µL
Samples	2.0 µL (1X and ¼ dilution)
Positive Control	2.0 µL HMgBlock135m (5E2 copies/µL)

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.

¼ Sample Dilution Protocol: Transfer 2.5 µL of sample to a clean microfuge tube containing 7.5 µL of TE. Mix well and centrifuge briefly.

After addition of samples and controls to reaction wells or tubes, seal the plate with the adhesive film or seal the tube strips with cap strips and centrifuge at 400×g for 30 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 D and E below.

D. **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.

E. **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 Appendix 5 or 6 are followed:

a. Manual Threshold = 0.020 (C. cayetanensis target)
b. Manual Threshold = 0.010 (IAC target)
c. Manual Baseline = cycle 6 to cycle 15 (both targets)

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

6. Interpretation of Results

A. <u>Positive Samples</u>:

a. Samples are only considered Positive for the presence of *C. cayetanensis* if on initial test or re-test, one (1) or more sample replicates produces a positive result with Ct ≤ 38.0 for the Ccay18S target reaction and the IAC target reaction is either Negative or Positive.

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b. ONLY ONE REPLICATE OF AN UNKNOWN SAMPLE OR ¼ DILUTION OF THAT SAMPLE NEEDS BE POSITIVE FOR THE Ccay18S TARGET IN ORDER TO CONSIDER A SAMPLE POSITIVE.

B. Samples for Further Analysis:

a. Any sample producing one (1) or more replicates for the Ccay18S target reaction crossing the threshold with Ct(s) > 38.0 and the IAC target reaction is either Negative or Positive:
Re-test sample one time (in triplicate) at both 1X and ¼ dilution.

C. Negative Samples:

a. If a sample Ccay18S 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 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 Ccay18S target reactions produces a positive result crossing the threshold, the experimental run is invalid and must be repeated.

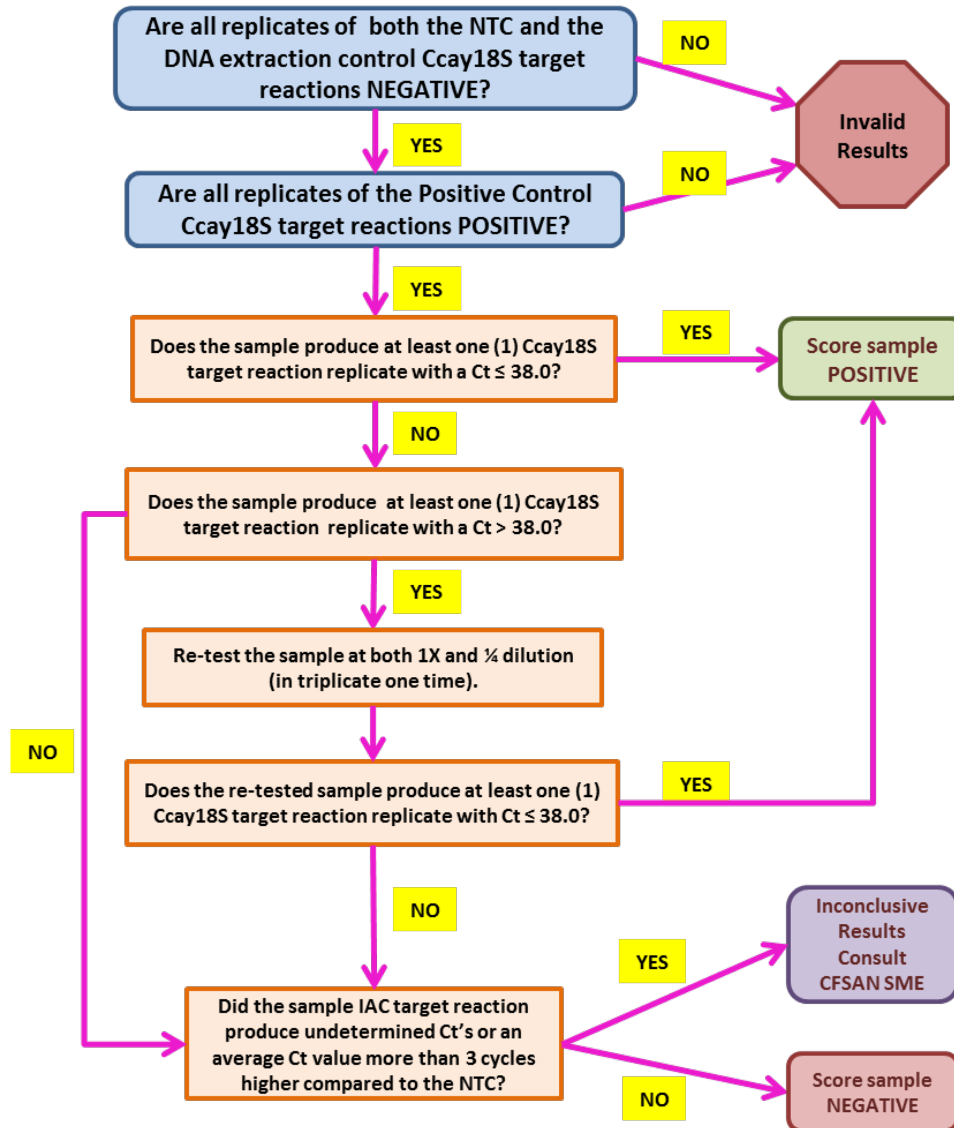
b. If after repeat of an invalid experimental run, the DNA extraction control repeatedly produces a positive result and the NTC sample is negative, the DNA extraction procedure was likely contaminated. The DNA extraction procedure must be repeated for the entire set of samples using additional washed food samples if available.

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

E. Inconclusive Results:

a. If on initial test (or after re-test if required), a sample Ccay18S target produces no replicate with Ct ≤ 38.0 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.

F. qPCR Data Analysis Flowchart:



Appendices (PDF Format)

1. **Appendix 1**: Alconox® Produce Wash Solution Recipe
2. **Appendix 2**: Tris EDTA (TE) pH 7.5 Primer Dilution Buffer Recipe
3. **Appendix 3**: Preparation of the Internal Amplification Control (IAC) Target Working Solution
4. **Appendix 4**: Preparation of the Positive Control Target Working Solution
5. **Appendix 5**: ABI 7500 Fast v2.0 or 2.3 Method
6. **Appendix 6**: ABI 7500 Fast v1.4 Method

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