U.S. FOOD & DRUG ADMINISTRATION

# Bacteriological Analytical Manual

# Chapter 19a: Detection of Cyclospora and Cryptosporidium from Fresh Produce: Isolation and Identification by Polymerase Chain Reaction (PCR) and Microscopic Analysis

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# Note

**Updated methods are available:**

- (2017) Chapter 19b: Detection of Cyclospora cayetanensis in Fresh Produce using real-time PCR
- (2020) Chapter 19c: Dead-end Ultrafiltration for the Detection of Cyclospora cayetanensis from Agricultural Water

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# 1. Materials and Equipment

a. BagPage®+ filter bags (400 ml) and Bag Clips (*Interscience*, St Nom, France)
b. Envirochek™ sampling capsule, 1 µm nominal (Pall Gelman Laboratory)
c. Rocker platform
d. Rotary shaker
e. Rotating wheel
f. 150 ml analytical filter units (Nalgene, Cat No. 130-4045)
g. 25 mm disposable filter funnels (Whatman Biosciences)
h. Dynal L10 tubes Prod. No. 740-03)
i. Dynal MPC®-1 (Prod. No 120.01)
j. Dynal MPC®-S (Prod. No 120.20)
k. Vacuum manifold (Vac-Man® Laboratory Vacuum Manifold 20-sample capacity; Promega)
l. FTA Filters, Classic Card of 960circle pre-printed card formats (Whatman Biosciences)
m. Photo laminating sheets (Scotch®)
n. Conical 250 ml centrifuge tubes
o. Sorvall RT7 Plus refrigerated centrifuge or equivalent (to centrifuge 250 ml conical centrifuge tubes)
p. Epi-illuminated Fluorescence Microscope-equipped with the following: UV 1A filter block (excitation Filter, EX 365/10; Dichroic mirror, DM 440; Barrier Filter, BA-400; or equivalent. Optics for differential interference contrast (DIC). For *Cryptosporidium* spp.: appropriate filter for viewing fluorescein isothiocyanate (FITC) conjugated oocysts. (*Table 1*)
q. Glass microscope slides and cover slips
r. Blotting paper
s. Heating block for incubation at 56°C
t. Single punch (6 mm diameter) hole puncher
u. Thin-walled 0.65 ml PCR tube (PGC)
v. ART pipette tips (Molecular BioProducts)
w. PTC-200 DNA Engine (MJ Research) or comparable thermal cycler.
x. Horizontal gel electrophoresis apparatus and power supply.
y. Polaroid camera or digital imaging system to capture ethidium bromide-stained gels
z. Polaroid Type 667 film
aa. UV Transilluminator

# 2. Reagents

a. Water
    i. Deionized water (for washing produce [$dH_2O$])
    ii. Sterile deionized water (for PCR procedure)
b. Envirochek™ elution buffer (0.01M Tris, pH 7.4 ; 0.001 M EDTA; 1% SDS)

c. Silicone vacuum grease
d. Albumin, bovine (BSA) (Sigma, A-7030)
e. Celite (Sigma, C-8656)
f. Polyvinyl polypyrrolidone (PVPP) (Sigma, P-6755)
g. NET buffer: 0.1 M Tris, pH 8.0, 0.15M NaCl, 0.001M EDTA
h. NET-BSA buffer-NET buffer containing 1% (w/v) BSA
i. 20% celite in NET-BSA buffer (w/v)
j. 10% PVPP (w/v) in $dH_2O$
k. Dynabeads anti-Cryptosporidium Kit (Dynal Biotech Inc, USA)
l. Hydrofluor Combo Detection Kit for *Cryptosporidium* and *Giardia* (Strategic Diagnostics, Inc.)
m. 0.1 N HCl
n. 0.1 N NaOH
o. Immersion oil
p. Clear fingernail polish, slide compound, paraffin wax or equivalent
q. FTA Purification Buffer (Whatman Biosciences)
r. FTA filter wash buffer: 0.01 M Tris, Ph 8.0; 0.1 mM EDTA
s. DNA Primers-See Table 1 in PCR Section
t. HotStartTaq™ Master Mix Kit (Qiagen)
u. 0.5x Tris-acetate-EDTA buffer (0.5x TAE)
v. Molecular biology-grade agarose (BioRad)
w. Ethidium bromide
x. 6x gel loading solution
y. 100 bp and 25 bp DNA ladders (Invitrogen)
z. *Vsp I* restriction endonuclease (Promega)
aa. *Dra II* restriction endonuclease (Hoffman-La Roche)
bb. NuSieve® 3:1 agarose (Biowhitaker Molecular Applications)

# 3. Optional Reagents, Supplies, and Equipment

a. LightCycler®-FastStart DNA Master SYBR Green I kit (Roche Diagnostics)
b. LightCycler® Capillaries (Roche Diagnostics)
c. LightCycler® System (Roche Diagnostics)

# 4. Wash Procedure for Fresh Produce

This procedure can be used to analyze for potential contaminants on fresh leafy produce (lettuce, herbs, etc) and berries (*e.g.* raspberries) and may be applicable to other fresh produce.

a. Place produce to be analyzed (10-25 g of fresh leafy produce or 50g of fresh berries) in a BagPage®+ filter bag, add 100 ml $dH_2O$ and seal with Bag Clip.
b. Place on rocker platform and gently agitate for 30 minutes at room temperature, inverting the bag after 15 minutes.
c. Decant supernatant from the BagPage®+ filter bag into clean 50 ml conical centrifuge tubes and centrifuge for 20 minutes at 2,000xg.

**Isolation of *Cyclospora* from Fresh Produce Washes**

a. Aspirate supernatants (without disturbing debris pellets) to a volume not to exceed 45 ml when combined.
b. Suspend pellets in remaining supernatants and combine.
c. Add 2.5 ml 20% (w/v) celite in NET-BSA suspension (ensure that celite is thoroughly suspended prior to its addition to sample washes). Mix samples on a rotating wheel at room temperature for 15 minutes.
d. Add 1.0 ml of 10% PVPP suspension (w/v). Mix samples on a rotating wheel at room temperature for 15 minutes.
e. Prepare a 150 ml analytical filter unit by removing the membrane filter (0.45 µm or 0.2 µm) but retaining the grade 4 filter backing. Attach to a vacuum source.
f. Pre-wet the analytical filter with a small volume (~10ml) of NET buffer.
g. Decant celite/PVPP-containing sample wash into a prepared 150 ml analytical filter unit and vacuum filter. Ensure that the liquid passes through the filter septum to remove particulates and the celite particles from the suspension as it is filtered. The adsorbant celite should prevent filter clogging.
h. Rinse the container with 10 ml NET to recover as much of the residual celite-containing sample and decant into the filter unit. Then rinse the celite and particulate material trap onto the filter with an additional 10 ml volume of NET.
i. Prior to FTA filtration in **step 4l**, save ~10% of the filtered sample for microscopic examination.
j. Prepare filter funnel unit(s) containing FTA filter disk and attach to vacuum manifold.
k. Under vacuum, pre-wet the FTA filter assembly.
l. Slowly decant filtrate from **step 4i** into filter funnel unit while under vacuum until entire sample has passed through FTA filter.
m. In succession while filter funnel unit is still attached to vacuum manifold, rinse filter twice with 10 ml of FTA purification buffer and twice with 10 ml of FTA filter wash buffer.
n. Remove filter funnel unit from vacuum manifold, disassemble unit and dry FTA filter disk on 56°C heating block.

**Isolation of *Cryptosporidium* spp. from Fresh Produce Washes**

a. Aspirate supernatants (without disturbing debris pellets) to a volume not to exceed 10 ml when combined.
b. Suspend pellets in remaining supernatants and combine. **NOTE**: pellet volume should not exceed 0.5 ml packed volume as it will interfere with subsequent steps that employ immunomagnetic adsorption of *Cryptosporidium* oocysts.
c. Follow directions accompanying Dynabead anti-*Cryptosporidium* Kit for immunomagnetic separation (IMS) of *Cryptosporidium* oocysts from fresh produce washes using recommend tubes and magnetic capturing devices.
d. Elute captured *Cryptosporidium* oocysts in a 0.1 ml volume of 0.1N HCl for 5 minutes a room temperature.
e. Neutralize acid eluates with 0.01 ml 1N NaOH. Save ~10% of the sample for microscopic examination.
f. Dilute with 10 ml NET buffer.
g. Proceed as in **Section 4A, steps j-n.**

# 5. Isolation of Parasitic Contaminants from Juices, Cider and Milk

This procedure can be used to analyze for contaminants in liquid samples such as orange juice, apple juice, apple cider, milk and milk products.

**Isolation of *Cyclospora* from Juices, Cider and Milk**

a. Adjust a 25 ml aliquot of liquid sample to pH 8.0.
b. Add an equal volume of NET buffer and mix well.
c. Proceed as in **Section 4A, steps c-n**

**Isolation of *Cryptosporidium* spp. from Juices, Cider and Milk**

A 10 ml volume of cider, juice or milk product is directly sampled by IMS using the Dynabead anti-*Cryptosporidium* Kit along with the recommend tubes and magnetic capturing devices as in **Section 4B, steps c-f.**

# 6. Isolation of Parasitic Contaminants from Large Volumes of Water

This procedure is designed to isolate contaminants from a designated water source (stream, river, reservoir, standing water, runoff, etc).

a. Place a single Envirochek™ sampling capsule in line with the water source to be sampled. Ensure that the flow rate does not exceed the standards established by the manufacturer.
b. Collect a water sample from 10 L of flow through.
c. Remove contaminants captured by the filter using 125 ml of elution buffer. Filters should be treated with elution buffer, sealed and agitated on a rotating wheel (moderate speed) for at least 5-10 minutes following the manufacturer's recommendations. Decant filter rinse into a 250 ml conical centrifuge tube.
d. Repeat **step 6c** and combine filter rinses.
e. Centrifuge the sample at 1,500-2,000 x g for 20 minutes allowing the centrifuge to coast to a stop (do not use the brake)
f. Proceed as described in **Sections 4A and 4B** for the isolation of *Cyclospora* and *Cryptosporidium* spp. respectively.

# 7. Slide Preparation and Microscopic Analysis – *Cyclospora cayetanensis*

*Cyclospora* oocysts emit a cobalt blue autofluorescence with the UV-1A emission filter or blue-green with broader emission spectra filters under ultraviolet illumination. Prepare slides in duplicate and examine slides under ultraviolet illumination as described below.

Laboratories should use a microscope reticle capable of measuring organism in the 8-10 µm range to verify oocyst size when organisms are recovered. Compare morphological characteristics of presumptive oocysts to those in a known standard.

**Slide Preparation**

Centrifuge the volume set aside for microscopic analysis (**Section 4A, step i**), at 1,500 × *g* for 10-15 min at 4°C. Aspirate supernatant to within 0.5 ml of debris pellet. Uniformly suspend pellet material by gentle, repeated pipetting.

a. Apply silicone vacuum grease to edge of cover slip
b. Place 10 µl of suspended debris to a clean glass microscope slide and prepare a wet mount using pre-greased cover slip

**Microscopy**

a. Examine slide under UV light at 400× magnification. *Cyclospora* oocysts autofluoresce cobalt blue. **Examine slide at multiple planes under UV. Occasionally, debris in slide preparations make it difficult to only view the slide on one plane.**
b. Switch from epifluorescence microscopy to bright field microscopy or differential interference contrast microscopy. Determine oocyst size of any presumptive oocysts at 1000X magnification. Compare to standards. Confirm internal structures of presumptive *Cyclospora* oocysts as compared to a standard.
c. Seal cover slips to the glass slides of presumptive positives with fingernail polish, slide compound or paraffin wax.
d. Document presumed positive samples with photographs taken at multiple planes.

# 8. Slide Preparation and Microscopic Analysis & *Cryptosporidium* spp.

Microscopic examination of produce washings and other liquid samples for the presence of *Cryptosporidium* spp. oocysts will be conducted using commercially available immuno-magnetic bead separation (IMS) kits and immunofluorescence labeling kits.

A. An aliquot (~10µl) of the IMS-derived sample **Section 4B, step e**, is FITC-labeled using the Hydrofluor Combo Detection Kit for *Cryptosporidium* and *Giardia* (Ensys Inc., Research Triangle Park, NC) per the manufacturer's instructions and examined in conventional DIC and epifluorescence mode.

**Table 1: Parameters for Epifluorescence Microscopy†**
INCIDENT LIGHT
Light Source-Mercury Vapor
200W, 100W, or 50W

| Excitation Filter | Dichroic Filter | Barrier Filter | Red Suppression Filter |
|---|---|---|---|
| KP500 | TK510 | K510 or K530 | BG38 |
| FITC | TK510 | K530 | BG38 |
| D. Tungsten-Halogen 50 and 100 W | | | |
| KP500 | TK510 | K510 or K530 | BG38 |
| FITC | TK510 | K530 | BG38 |

†*Taken from protocol provided with HYDROLFUOR-Combo Detection Kit for Giardia cysts and Cryptosporidium oocysts (Ensys Inc., Research Triangle Park, NC)*

# 9. PCR Analysis

The molecular detection of *Cyclospora* spp. and *Cryptosporidium* spp. is independently accomplished using nested PCR protocols. The differential identification of *Cyclospora cayetanensis* from other closely related non-human pathogenic parasites (*i.e. Eimeria* spp.) employs a nested multiplex PCR assay. This assay can be accomplished using either a conventional thermal cycler with heated lid or a real-time PCR platform using the Roche LightCycler®.

The detection of *Cryptosporidium* spp. also involves nested PCR amplification. However, differentiation and speciation of *Cryptosporidium* spp. requires further analysis by a restriction fragment length polymorphism (RFLP) assay. Please note the following: *C. parvum* genotype I has been renamed *C. hominis*; *C. parvum* genotype II (bovine strain) is now referred to as *C. parvum*

### A. DNA Primers

**Table 2: DNA Primer Sequences for Cyclospora-specific PCR Amplification†**

| Primer Designation | Primer Specificity | Primer Sequence (5'-3') | Amplicon Size (bp) | Designated Application |
|---|---|---|---|---|
| F1E (forward) | *Cyclospora and Eimeria* spp. | TACCCAATGAAAACAGTTT | 636 | Primary Amplification |
| R2B (reverse) | *Cyclospora and Eimeria* spp. | CAGGAGAAGCCAAGGTAGG | 636 | Primary Amplification |
| CC719 (forward) | *C. cayetanensis* | GTAGCCTTCCGCGCTTCG | 298 | Nested Amplification |

| | | | | |
|---|---|---|---|---|
| PLDC661 (forward) | *C. cercopitheci, C. colobi, C. papionis* | CTGTCGTGGTCATCGTCCGC | 361 | Nested Amplification |
| ESSP841 (forward) | *Eimeria spp.* | GTTCTATTTTGTTGGTTTCTAGGACCA | 174 | Nested Amplification |
| CRP999 (reverse) | *Cyclospora and Eimeria spp.* | CGTCTTCAAACCCCCTACTGTCG | | Nested Amplification |

†All primer sequences were derived from the published sequences for the 18S rRNA genes of the respective organisms.

**Table 3: DNA Primer Sequences for Cryptosporidium Genus-specific PCR Amplification†**

| Primer Designation | Primer Specificity | Primer Sequence (5'-3') | Amplicon Size (bp) | Designated Application |
|---|---|---|---|---|
| ExCry1 (forward) | *Cryptosporidium* spp. | GCCAGTAGTCATATGCTTGTCTC | 844 | Primary Amplification |
| ExCry2 (reverse) | *Cryptosporidium* spp. | ACTGTTAAATAGAAATGCCCCC | 844 | Primary Amplification |
| NesCry3 (forward) | *Cryptosporidium* spp. | GCGAAAAAACTCGACTTTATGGAAGGG | 590-593 | Nested Amplification |
| NesCry4 (reverse) | *Cryptosporidium* spp. | GGAGTATTCAAGGCATATGCCTGC | 590-593 | Nested Amplification |

†All primer sequences were derived from the published sequences for the 18S rRNA genes of the respective organisms.

**B. General Sample Preparations for Primary PCR Amplifications**

a. Punch marked triplicate areas (6 mm diameter) from dried FTA filter disk using a single punch hole-puncher. **Decontamination of the hole-puncher is not necessary as cross contamination between samples from the hole-puncher has been found to be negligible. However, the individual researcher may wish to swab the punch with ethanol between sample disks if it is deemed appropriate.**
b. Insert filter punches snuggly into bottom of 0.65 ml thin-walled PCR tubes.
c. Dispense 50 µl HotStartTaq™ Master Mix into each PCR tube.
d. Prepare reagent master mix (see *Table 4*) with the appropriate forward and reverse DNA primers (see *Tables 2 and 3*) and dispense into each PCR tube.
e. All PCR analyses must include positive and negative controls (see *Table 5*).
f. Mix tubes with gentle tapping.
g. Follow the appropriate thermal cycling protocol (*Table 6 and 7*) for primary PCR amplification.

**Table 4: General PCR Conditions for Primary PCR Amplification**

| Component | | lume (µl)* | Final Concentration |
|---|---|---|---|
| FTA Filter Disk (DNA Template) | | | |
| HotStartTaq™ Master Mix | | 50.0 | † |
| Reagent Master Mix | $MgCl_2$, 25 mM | 2.0 | 2.0 ‡ |
| | Forward Primer, 10 µM | 2.0 | 0.2 µM |
| | Reverse Primer, 10 µM | 2.0 | 0.2 µM |
| | Sterile deionized water | 44.00 | |

*100 µl total volume
†Final concentrations for components in the HotStartTaq™ Master Mix are as follows: 200 µM of each dNTP, 1.5 mM $MgCl_2$ and 2.5 U HotStarTaq™ DNA Polymerase
‡Final $MgCl_2$ concentration is that contributed by both the HotStartTaq™ Master Mix and 25 mM $MgCl_2$stock

**Table 5: Controls for PCR amplifications**

| Control Type | Condition/Organism | |
|---|---|---|
| Negative Control-1 | Reagent blank-no filter | |
| Negative Control-2 | Reagent blank + unspotted, washed filter | |
| †Positive Controls: | *Cyclospora* Analysis: | *C. cayetanensis* |
| †Positive Controls: | *Cyclospora* Analysis: | ‡¶*Cyclospora* spp. (NHP) |
| †Positive Controls: | *Cyclospora* Analysis: | **Eimeria* spp. |
| †Positive Controls: | *Cryptosporidium* Analysis: | ¶*C. hominis* (formerly know as *C. parvum* genotype 1) |
| †Positive Controls: | *Cryptosporidium* Analysis: | *C. parvum*, (formerly know as *C. parvum* genotype II (Bovine) |
| †Positive Controls: | *Cryptosporidium* Analysis: | ¶*C. baileyi* |
| †Positive Controls: | *Cryptosporidium* Analysis: | ¶*C. serpentis* |

†Whenever possible, positive control FTA filters should be spotted with at least $10^3$ organisms
‡Non-human primate-derived *Cyclospora* spp.
¶Not routinely available.
*Most available *Eimeria* spp. are suitable.

**Table 6: PCR Thermal Cycling Parameters for Cyclospora and Eimeria spp.**

| | Step | Number of Cycles | Temperature and Time |
|---|---|---|---|
| ***Primary PCR*** | Initial Activation | 1 | 95°C; 15 min |
| | Amplification | 35 | Denaturation: 94°C; 30 sec |
| | Amplification | 35 | Annealing: 53°C; 30 sec |
| | Amplification | 35 | Extension: 72°C; 90 sec |
| | Final Extension | 1 | 72°C; 10 min |
| ***†Nested Multiplex PCR*** | Initial Activation | 1 | 95°C; 15 min |
| | Amplification | 25 | Denaturation: 94°C; 15 sec |
| | Amplification | 25 | Annealing: 66°C; 15 sec |

†This is a stringent, 2-step amplification program (simultaneous annealing and extension at 66°C). Likewise, it does not require a final extension step.

**Table 7: PCR Thermal Cycling Parameters for Cryptosporidium spp.**

| | Step | Number of Cycles | Temperature and Time |
|---|---|---|---|
| ***Primary PCR*** | **Initial Activation** | **1** | |
| | Amplification | 40 | Denaturation: 94°C; 45 sec |
| | Amplification | 40 | Annealing: 53°C; 75 sec |
| | Amplification | 40 | Extension: 72°C; 45 sec |
| | Final Extension | 1 | 72°C; 7 min |
| ***Nested PCR*** | Initial Activation | 1 | 95°C; 15 min |
| | Amplification | 35 | Denaturation: 94°C; 25 sec |
| | Amplification | 35 | Annealing: 65°C; 25 sec |
| | Amplification | 35 | Extension: 72°C; 25 sec |
| | Final Extension | 1 | 72°C; 7 min |

**Conventional Nested Multiplex PCR Amplification for the Differential Identification of *Cyclospora* and *Eimeria* spp.**

1. Dispense 25 µl of HotStartTaq™ Master Mix into each PCR tube.
2. Prepare reagent master mix (*Table 8*) and dispense into all tubes
3. Complete reaction samples with the addition of the desired volume (1-3 µl) of primary amplicon solution.
4. Be sure to include all positive and negative controls as in the primary amplification reactions.
5. Mix tubes with gentle tapping.
6. Follow the appropriate thermal cycling protocol listed in *Table 6*.

**Table 8: Assay Conditions for the Conventional Nested Multiplex PCR Amplification of Cyclospora and Eimeria spp.**

| Component | Volume (µl)* | Final Concentration |
|---|---|---|
| HotStartTaq™ Master Mix | 25.0 | † |
| $MgCl_2$, 25 mM | 1.0 | 2.0 mM‡ |
| CC719 (forward primer), 10 µM | 1.0 | 0.2 µM |
| PDCL661 (forward primer), 10 µM | 1.0 | 0.2 µM |
| ESSP841 (forward primer), 10 µM | 1.0 | 0.2 µM |
| CRP999 (reverse primer), 10 µM | 1.0 | 0.2 µM |
| Sterile deionized water | 19.00 | |
| DNA Template (primary amplicon) | 1.0 | |

*50 µl total volume

†Final concentrations for components in the HotStartTaq™ Master Mix are as follows: 200 µM of each dNTP, 1.5 mM $MgCl_2$ and 2.5 U HotStarTaq™ DNA Polymerase

‡Final $MgCl_2$ concentration is that contributed by both the HotStartTaq™ Master Mix and 25 mM $MgCl_2$ stock.

**OPTIONAL-Real Time Multiplex PCR Amplification for the Differential Identification of *Cyclospora* and *Eimeria* spp. using the Roche LightCycler® System**

a. Place the requisite number of glass capillaries in a pre-chilled cooling block (with accompanying centrifuge adapters)
b. Prepare a reagent master mix (*Table 9*) and dispense into individual 0.65 ml PCR tubes

c. Complete reaction samples with the addition of the 1 µl of primary amplicon solution.
d. Be sure to include all positive and negative controls as in the primary amplification reactions.
e. Mix tubes with gentle tapping.
f. Dispense reaction mixture into glass capillaries, cap, and centrifuge briefly (5-10 sec at 3000 rpm) in a bench-top microcentrifuge.
g. Transfer glass capillaries to LightCycler® carousel.
h. Follow the appropriate thermal cycling protocol for (*Table 10* ).
i. Real time confirmation of pathogen can be made by melting curve analyis (*see Table 11*)
j. For final confirmation, samples can be recovered from each glass capillary.
k. Uncap, invervet capillary into a 0.65 ml PCR tube containing 5 µl of gel loading solution, and briefly centrifuge (5-10 sec at 3000 rpm) in a bench-top microcentrifuge.
l. Following instruction for agarose gel electrophoresis **(Part 9, Section F, steps b-f)**

**Table 9: Assay Conditions for the Real-time Nested Multiplex PCR Amplification of Cyclospora and Eimeria spp.**

| Component | Volume (µl)* | Final Concentration |
|---|---|---|
| LightCycler®-FastStart DNA Master SYBR Green | 2.0 | - |
| $MgCl_2$, 25 mM | 1.6 | 3.0 mM |
| CC719 (forward primer), 10 µM | 1.0 | 0.5 µM |
| PDCL661 (forward primer), 10 µM | 1.0 | 0.5 µM |
| ESSP841 (forward primer), 10 µM | 1.0 | 0.5 µM |
| CRP999 (reverse primer), 10 µM | 1.0 | 0.5 µM |
| Sterile deionized water | 11.40 | - |
| DNA Template (primary amplicon) | 1.0 | - |

*20 µl total volume

**Table 10: LightCycler® Thermal Cycling Parameters for Real-time Multiplex PCR Amplification of Cyclospora and Eimeria spp.**

| Step | Number of Cycles | Temperature and Time | Comments |
|---|---|---|---|
| Hot Start | 1 | 95°C; 10 min | |
| Amplification | 30 | Denaturation: 95°C; 15 sec | |
| | 30 | Annealing: 66°C; 15 sec | Single Fluorescence Acquisition |
| Melting Curve Analysis | 1 | 95°C; 15 sec | |
| | 1 | 65°C; 15 sec | |
| | 1 | 98°C; 0.1°C/sec | Continuous Fluorescence Acquisition |

**Table 11: Expected Results for Real-time Multiplex PCR Amplification of Cyclospora and Eimeria spp. by Melting Curve Analysis**

| Primer Designation | Primer Specificity | Primer Sequence (5'-3') | Amplicon Size (bp) | Amplicon $T_m$ (°C) |
|---|---|---|---|---|
| CC719 | *C. cayetanensis* | GTAGCCTTCCGCGCTTCG | 298 | 85°C |
| PLDC661 | *C. cercopitheci, C. colobi, C. papionis* | CTGTCGTGGTCATCGTCCGC | 361 | 91°C |
| ESSP841 | *Eimeria* spp. | GTTCTATTTTGTTGGTTTCTAGGACCA | 174 | 81°C |

**Nested PCR Amplification for the Differential Identification of *Cryptosporidium* spp.**

a. Dispense 25 µl of HotStartTaq™ Master Mix into each PCR tube.
b. Prepare reagent master mix (*Table 12*) and dispense into all tubes
c. Complete reaction samples with the addition of the desired volume (1-3 µl) of primary amplicon solution.
d. Be sure to include all positive and negative controls as in the primary amplification reactions.
e. Mix tubes with gentle tapping.
f. Follow the appropriate thermal cycling protocol listed in *Table 7*.

**Table 12: Assay Conditions for Nested Amplification of Cryptosporidium spp.**

| Component | Volume (µl)* | Final Concentration |
|---|---|---|
| HotStartTaq™ Master Mix | 25.0 | † |
| $MgCl_2$, 25 mM | 1.0 | 2.0 ‡ |
| NesCry3 (forward primer), 10 µM | 1.0 | 0.2 µM |
| NesCry4 (reverse primer), 10 µM | 1.0 | 0.2 µM |
| Sterile deionized water | 21.0 | |
| DNA Template (primary amplicon) | 1.0 | |

*50 µl total volume
†Final concentrations for components in the HotStartTaq™ Master Mix are as follows: 200 µM of each dNTP, 1.5 mM $MgCl_2$ and 2.5 U HotStarTaq™ DNA Polymerase
‡ Final $MgCl_2$ concentration is that contributed by both the HotStartTaq™ Master Mix and 25 mM $MgCl_2$ stock

**Agarose Gel Electrophoresis**

a. Mix 10 µl of nested amplification product with 2-3 µl of gel loading solution.
b. Load samples into wells of a 1.5% agarose gel prepared with 0.5 x TAE containing 0.2 µg/ml ethidium bromide. Include at least one lane containing 100 bp DNA ladder to approximate the size of any amplicon present.
c. Run the gel at 125 volts (constant voltage) for at least 30 min.
d. PCR products on the agarose gel can be visualized by using a UV transilluminator. Photograph the gel to have a permanent record of the results using a Polaroid Type 667 film (or a digital system, if you decide to include that in the material & methods).
e. The primary amplicon from primer pair F1E/R2B for *Cyclospora* PCR may not be visible; therefore, only product from the nested reaction should be electrophoresed.
f. Predicted sizes of PCR amplicons from *Cyclospora* spp., *Eimeria* spp. and *Cryptosporidium* spp. are listed in *Tables 1 and 2*

**Restriction Fragment Length Polymorphism (RFLP) Analysis of *Cryptosporidium* spp. Nested PCR Amplicons to Determine the Presence of *C. parvum* Oocysts and Distinguish *C. hominis* from *C. parvum***

a. A 590 bp (genotype I) or a 593 bp product (genotype II) following nested PCR is a presumptive positive for the presence of *Cryptosporidium* spp.
b. The restriction patterns resulting from the digestion of the nested amplicon with restriction endonucleases *VspI* and *DraII* will distinguish between *C. parvum* and *C. hominis* (*VspI* digestion) and *C. parvum* from *C. baileyi* and *C. sepentis* (*DraII* digestion).
c. Combine 15 µl of the *Cryptosporidium* nested PCR amplicon with on unit *VspI*, 2.0 µl of 10x enzyme buffer, and 0.2 µl BSA solution (include with enzyme). Adjust final volume to 20 µl with sterile $dH_2O$.
d. For digestion with *DraII*, combine 15 µl of the *Cryptosporidium* nested PCR amplicon with one unit *DraII*, 2.0 µl of 10x enzyme buffer, and adjust final volume to 20 µl.
e. Positive controls for *C. parvum* and other species of *Cryptosporidium* must be digested in the same manner and alongside test samples.
f. Incubate digestion samples for at least 2 hr at 37°C.
g. Analyze samples by gel electrophoresis using a 3% NuSieve gel prepared with 0.5x TAE and 0.2% ethidium bromide.
h. Mix 10-15 µl of nested amplification product with 2-3 µl of gel loading solution and load into wells of gel. Include at least one lane containing 25 bp DNA ladder to estimate the size of restriction fragments present.
i. Run the gel at 125 volts (constant voltage) for at least 45 min.
j. RFLP patterns can be visualized on the agarose gel by using a UV transilluminator. Photograph the gel to have a permanent record of the results using a Polaroid Type 667 film (or a digital system).
k. Predicted banding patterns confirmatory for the presence of *Cryptosporidium* spp. are listed in *Table 13.*

**Table 13: Evaluation of Nested PCR Amplification and RFLP Analysis to Differentiate Cryptosporidium spp.**

| **Organism** | **PCR Amplicon** | | **RFLP Digestion Products (bp)** | |
|---|---|---|---|---|
| | **Primary (bp)** | **Nested (bp)** | ***VspI*** | ***DraII*** |
| *C. hominis* (formerly *C. parvum*, genotype I) | 844 | 593 | 503 and 90 | - |
| *C. parvum* (formerly *C. parvum*, genotype II) | 840 | 590 | - | - |
| *C. baileyi* | 831 | 579 | - | 295 and 284† |
| *C. serpentis* | 836 | 583 | - | 298 and 284† |
| *C. muiris* | - | - | - | - |
| *C. wrairii* | - | - | - | - |

†Indistinguishable within an agarose gel

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