AOAC SMPR® 2024.003

Standard Method Performance Requirements (SMPRs®) for Detection, Identification, and Characterization of *Cyclospora cayetanensis*

Intended Use: Surveillance and Monitoring by Trained Technicians

1 Purpose

What: AOAC *Standard Method Performance Requirements* (SMPRs®) are voluntary consensus standards developed in accordance with the AOAC policy, "AOAC Due Process for Development of AOAC Non-Method Consensus Standards and Documents." SMPRs describe the scientific community's recommended minimum method performance characteristics and analytical requirements for a specific method related intended use.

Who: Drafted by AOAC working groups, SMPRs are adopted by AOAC by a consensus of stakeholders affiliated with its integrated science programs and projects, which are composed of volunteer subject matter experts representing academia, government, industry, and nonprofit sectors from around the world.

Use: AOAC uses SMPRs in its core science programs in which they are a resource for AOAC method experts, including expert review panels, in the evaluation of validation study data for methods submitted to the AOAC *Official Methods of Analysis*℠ and AOAC *Performance Tested Methods*℠ programs. Additionally, AOAC SMPRs may be used to provide acceptance criteria for the verification of methods and serve as a resource to guide method development and optimization.

2 Applicability

Methods used to detect, identify, and characterize *Cyclospora cayetanensis* DNA from oocysts in fresh produce, agricultural water, and/or environmental matrices.

3 Analytical Technique

Any analytical technique that meets the requirements of this SMPR.

4 Definitions

Agricultural water.—Water that is used to grow fresh produce and sustain livestock. Intended to, or is likely to, contact produce or food contact surfaces, including water used in growing activities (including irrigation water applied using direct water application methods, water used for preparing crop sprays, and water used for growing sprouts) and in harvesting, packing, and holding activities (including water used for washing or cooling harvested produce and water used for preventing dehydration of produce after processing).

Candidate method.—Method submitted for validation.

Coccidia.—Obligate intracellular parasites in the phylum Apicomplexa that have complex life cycles and infect the intestinal tract of animals. They develop resistant oocysts that are shed with feces from an infected host.

Cyclospora.—A genus of coccidian parasites with a direct fecal-oral life cycle; members are characterized as having oocysts with two sporocysts, each containing two sporozoites.

Cyclospora cayetanensis.—A species of the genus *Cyclospora* and the cause of human cyclosporiasis. Current detection and characterization methods have been designed to include all currently designated *C. cayetanensis* lineages and future methods must incorporate all of these lineages regardless of any nomenclature changes; no animal reservoirs for *C. cayetanensis* have been identified.

Fresh fruits and vegetables.—Fresh produce that is likely to be sold to consumers in an unprocessed (i.e., raw) form. Fresh produce may be intact, such as whole strawberries, carrots, radishes, fresh cilantro, fresh parsley, or tomatoes, or cut from roots or stems during harvesting, such as celery, broccoli, lettuce, or cauliflower.

Fresh-cut produce.—Fresh fruits and vegetables for human consumption that have been minimally processed and altered in form by peeling, slicing, chopping, shredding, coring, or trimming, with or without washing, prior to being packaged for use by the consumer or a retail establishment (e.g., pre-cut, packaged, ready-to-eat salad mixes). Fresh-cut produce does not require additional preparation, processing, or cooking before consumption, with the possible exception of washing or the addition of salad dressing, seasoning, or other accompaniments.

Internal amplification control.—Exogenous noncompetitive amplification control DNA used to identify false-negative results due to reaction failure caused by PCR inhibitors commonly found in food matrices.

Negative PCR/qPCR or no template control.—Sample consisting of PCR reagents only run with unknown samples to provide evidence that cross contamination did not occur. This sample is not run through the extraction process.

Negative Process control.—Known negative, reagent-only sample run through the entire process and then tested to provide evidence that cross contamination did not occur.

Oocyst.—Hardy, thick-walled life cycle stage of coccidian parasites, which is shed in feces of infected host. Coccidian oocysts vary in size and shape. *Cryptosporidium* oocysts are spherical in shape and ~4–5 µm in diameter. *Cyclospora* oocysts are spherical in shape and 8–10 µm in diameter. Related species of *Eimeria* oocysts can be 10–30 µm in length and width and are more ovoid in shape. Each genus presents a characteristic sporulation pattern.

Positive extraction control.—Sample containing oocysts ran through the DNA extraction process and then tested to assure that DNA extraction is operating properly.

Positive PCR/qPCR control.—gDNA from target organism, or nucleic acid segment, possibly synthetic version of target nucleic acid, run alongside test samples to determine that reaction worked successfully.

Positive process control.—Sample known to be positive (i.e., containing oocysts) ran through the entire process and then tested to demonstrate that all phases of method were performed successfully. May include extraction, amplification, and detection.

Probability of detection (POD).—Portion of positive analytical outcomes for qualitative method for given matrix at given analyte level or concentration. dPOD is the difference between any two POD values.

Sporulated oocyst.—Mature, infective form of coccidian oocyst.

Sporulation.—Process by which immature (noninfective) coccidian oocysts develop into mature, infective form. *Cyclospora* oocysts are shed unsporulated in feces of infected individuals and must mature in the environment (outside the host), under favorable conditions, to become infective to someone else. Sporulation of *Cyclospora* oocysts takes an average of 7 to 14 days, or even longer, under temperatures between 22 to 32°C.

Surrogate organism.—Non-*Cyclospora cayetanensis* protozoan oocyst used as indicator that extraction and detection method worked successfully. It can also be used as a tool to validate a method. Consideration should be given if this organism is genetically or physiologically similar to *C. cayetanensis* (e.g., *Eimeria, Toxoplasma, Cryptosporidium).*

Unsporulated oocyst.—Noninfective form of the oocyst.

5 Method Performance Requirements

See Tables 1 and 2.

6 System Suitability Tests and/or Analytical Quality Control

Positive and negative controls shall be embedded in assays as appropriate. Inhibition controls should be used for method verification for each new matrix. Manufacturer must provide written justification if controls are not appropriate to an assay.

See Table 3 for list of recommended controls.

7 Inclusivity and Exclusivity Reference Material(s)

Inclusivity.—Molecular marker must accurately detect all human-associated *Cyclospora* sequences available on public databases (*in silico*).

Shall include sequences from at least 10 geographically and temporally diverse isolates of *C. cayetanensis* (*in vitro*).

Synthetic sequences may be obtained based on desired target.

Exclusivity.—Shall include gDNA or commercially produced DNA sequences from at least 10 closely related species.

Must include minimum of three different genera, one of which shall be *Eimeria.*

See Table 4.

8 Validation Guidance

FDA *Guidelines for the Validation of Analytical Methods for the Detection of Microbial Pathogens in Foods and Feeds,* Edition 3.0, Table 2: General Guidelines for the Validation of Qualitative Detection Methods for Microbial Analytes—Unique Isolation and/or Enrichment Challenges, https://www.fda.gov/media/83812/download

FDA *Bacteriological Analytical Manual* (BAM) Chapter 19b: Molecular Detection of *Cyclospora cayetanensis* in Fresh Produce Using Real-Time PCR, https://www.fda.gov/food/laboratory-methods-food/bam-chapter-19a-detection-cyclospora-and-cryptosporidium-fresh-produce-isolation-and-identification

FDA *Bacteriological Analytical Manual* (BAM) 19c: Dead-end Ultrafiltration for the Detection of *Cyclospora cayetanensis* from Agricultural Water, https://www.fda.gov/media/140309/download?attachment

Appendix I: *AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Biological Threat Agent Methods and/or Procedures*, George W. Latimer, Jr. (Ed.), *Official Methods of Analysis of AOAC INTERNATIONAL*, 22nd Ed. (New York, 2023; online edition, Oxford Academic, Jan. 4, 2023), https://doi.org/10.1093/9780197610145.005.009

Appendix J: *AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces*, George W. Latimer, Jr. (Ed.), *Official Methods of Analysis of AOAC INTERNATIONAL*, 22nd Ed. (New York, 2023; online edition, Oxford Academic, Jan. 4, 2023), https://doi.org/10.1093/9780197610145.005.010

Appendix Q: *Recommendations for Developing Molecular Assays for Microbial Pathogen Detection Using Modern In Silico Approaches*, George W. Latimer, Jr. (Ed.), *Official Methods of Analysis of AOAC INTERNATIONAL*, 22nd Ed. (New York, 2023; online edition, Oxford Academic, Jan. 4, 2023), https://doi.org/10.1093/9780197610145.005.017

Note: For commercial methods, robustness and product consistency and stability testing is required.

9 Maximum Time-to-Result

None.

Approved by stakeholders interested in and affiliated with the AOAC Analytical International Methods and Standards (AIMS) Program and the affiliated AOAC Microbiology Community. Version 23. Final version: April 22, 2024. Effective date: June 30, 2024.

Table 1. Inclusivity/exclusivity performance requirements

Parameter	Requirement	Final test concentration	Minimum acceptable results
Inclusivity	gDNA or sequences from at least 10 patient samples of *C. cayetanensisa*	2 × LOD of candidate method	100% Positive results[b]
Exclusivity	gDNA or sequences from at least 10 closely related species, representing at least 3 different genera (including those in Table 4)	10 × LOD of candidate method	100% Negative results[b]

[a] It is anticipated that tests that detect *C. cayetanensis* will detect other species that cause human cyclosporiasis, based on currently published genomic data. If this situation changes, the SMPR may be updated. Previously validated tests should then be reevaluated to determine whether they detect newly characterized species and labeled appropriately.

[b] 100% Correct analyses are expected. All aberrations are to be retested following the *AOAC Methods Committee Guidelines for Validation of Biological Threat Agent Methods and/or Procedures* (OMA Appendix I). Some aberrations may be acceptable if the aberrations are investigated, and acceptable explanations can be determined and communicated to method users.

Table 2. Method performance requirements

Validation material[a]	Minimum replicate test portions[b]	Target test concentration	Acceptance criterion[c]
Single-laboratory validation			
Pure target DNA (no matrix)	20–High	POD[d] 1.00 ex. 10–100 copies[e]/test portion	
	20–Low	Fractional positive results POD 0.25–0.75 ex. 5–10 copies/test portion	$dPOD_C$[f] 95% CI[g]: LCL[h] < 0 < UCL[i]
	20–Negative	POD 0.00 noninoculated control	
Artificially contaminated matrix[j] (all claimed matrices), e.g., fresh produce (25 g), berries (50 g), agricultural water (10 L)	5–High	POD 1.00 ex. 25–100 oocysts/test portion[k]	$dPOD_C$ 95% CI: LCL < 0 < UCL
	12–Low	Fractional positive results POD 0.25–0.75 ex. 5–10 oocysts/test portion	
	5–Negative	POD 0.00 noninoculated control	
Multilaboratory validation (minimum 5 collaborators with valid data[l])			
Artificially contaminated matrix[j] (minimum 1 matrix)	4–High	LPOD ≥ 0.95 ex. 25–100 oocysts/test portion	$dLPOD_C$ 95% CI: LCL < 0 < UCL
	8–Low	LPOD 0.2–0.8 ex. 5–10 oocysts /test portion	
	4–Negative	LPOD ≤ 0.05 noninoculated control	

[a] Pure target DNA and matrix study are both required for submission.

[b] Minimum replicate test portions are per method (candidate method and reference method). *Note*: Number of replicate test portions for multilaboratory validation study may be subject to change based on recommendations from the AOAC expert review panel. Applicable reference methods for sample preparation and DNA extraction include, e.g., current FDA BAM methods for detection of *Cyclospora* in fresh produce and agricultural water.

[c] Range between lower and upper confidence interval should encompass 0. If not, results must be investigated and explanation provided.

[d] POD = Probability of detection.

[e] May be expressed as genomic units.

[f] $dPOD_C$ = Difference in probability of detection between candidate and reference method results.

[g] CI = Confidence interval.

[h] LCL = Lower confidence limit.

[i] UCL = Upper confidence limit.

[j] Multiple molecular methods can be compared using same centrally prepared reference materials that have been subjected to appropriate sample preparation method.

[k] Test portion sizes include 25 g for fresh produce, 50 g for berries, and 10 L for agricultural water.

[l] Maximum of two participants at one organization and should represent balance of industry, academic, and government laboratories.

Table 3. Recommended controls

Control	Description	Implementation
Positive extraction	Designed to demonstrate effective extraction of oocyst DNA (target or surrogate) from sample matrix[a]	Single use per sample (or sample set) run
Positive process	Designed to demonstrate appropriate test response; positive control should be included at low, but easily detectable, concentration and should monitor performance of entire assay[a]	Single use per sample (or sample set) run
Negative process	Designed to demonstrate that assay itself does not produce detection in absence of target organism; purpose of this control is to rule out contamination in assay or test	Single use per sample (or sample set) run
Inhibition/matrix	Designed to specifically address impact of sample or sample matrix on assay's ability to detect target organism	Single use per sample run/per matrix

[a] Though controls are generally required in molecular methods, *Cyclospora* presents unique challenges due to lack of commercially available reference materials. If non-*Cyclospora* parasites are used as positive process controls, they should be evaluated for morphological and physiological similarity to *Cyclospora*, and the proposed method must be able to detect them.

Table 4. Exclusivity panel: Examples

Species
Eimeria tenella
Eimeria bovis
Eimeria acervulina
Eimeria maxima
Eimeria brunetti
Eimeria mitis
Eimeria praecox
Eimeria necatrix
Isospora suis
Isospora lunaris
Toxoplasma gondii
Neospora caninum
Other *Cyclospora* species, excluding *cayetanensis* (i.e., *Cyclospora cercopitheci, Cyclospora colobi, Cyclospora macacae, Cyclospora papionis*)
Other closely related species, as appropriate (*Cryptosporidium* and other *Isospora* species)