

**Real-Time PCR Assay for Detection of
Cyclospora cayetanensis on Fresh Produce:**

**Bagged pre-cut romaine lettuce salad
Matrix Extension Study Results**

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1. Background:

Cyclospora cayetanensis is a protozoan parasite causing an intestinal illness in humans called cyclosporiasis. The transmission of this parasite has been associated with the consumption of contaminated fresh produce or water (1). Human cyclosporiasis is a significant public health concern in the U.S. where large foodborne outbreaks and several sporadic cases, affecting hundreds of persons, have occurred since the mid-1990s. These cyclosporiasis outbreaks have been frequently associated with consumption of imported fresh produce including leafy greens and berries, among others. The epidemiological investigations conducted during several multi-state outbreaks underscored the need for improved laboratory detection and characterization methodologies to identify and properly track sources of produce contamination (2). A method for the detection of *C. cayetanensis* in produce was validated for cilantro and raspberries based on a multi-laboratory validation study (approved on 7/6/2016) and published in the FDA *Bacteriological Analytical Manual* (BAM) as Chapter 19B (<https://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm553445.htm>). Subsequently, the method was validated in shredded carrots, basil and parsley by matrix extension studies (approved on 2/7/2017 and 6/16/2017, respectively).

In 2013, imported salad mix was linked to restaurant-associated cyclosporiasis cases in Iowa and Nebraska. The results of epidemiologic investigations indicated that imported contaminated romaine lettuce in the mix was the most probable source of the infections (3).

In 2018, 511 laboratory-confirmed cases of *C. cayetanensis* spanning 16 states were reported in an outbreak associated with people who had consumed a variety of salads containing Romaine lettuce from McDonald's restaurants in the Midwest (4). During the investigation two samples of domestically grown Romaine lettuce tested positive for *C. cayetanensis*.

The events reported above indicate a need to extend and validate FDA detection methods for *C. cayetanensis* to include commodities such as romaine lettuce in advance of potential future outbreak investigations. Although the BAM Chapter 19B method has been validated for shredded carrots and for some leafy greens, such as cilantro and parsley, it has not been validated for bagged pre-cut Romaine lettuce. The outcome of a matrix extension study performed to assess the previously validated method for detection of *C. cayetanensis* seeded on bagged pre-cut romaine salad is described below.

2. Method:

The matrix extension was performed through a single laboratory validation study following guidelines for organisms posing unique isolation challenges, found in the FDA OFVM "Guidelines for the Validation of Analytical Methods for the Detection of Microbial Pathogens in Foods and Feeds" (see Table 2; <https://www.fda.gov/downloads/ScienceResearch/FieldScience/UCM298730.pdf>), published in 2015. The MMVS previously specified that 10 replicates should be tested at the fractional level for matrix extension studies for BAM Chapter 19B. The matrix extension was performed by examination of 25 g samples of ready-to-eat bagged pre-cut Romaine lettuce salad, either unspiked or spiked with 5 or 200 *C. cayetanensis* oocysts. The BAM Chapter 19B sample preparation

and detection methods were used with no modifications to wash produce, extract DNA, and perform molecular detection using a qPCR analysis specific for *C. cayetanensis*.

3. Results:

Table 1 shows a summary of the results obtained for the bagged pre-cut Romaine salad matrix extension study. The detection rate for the bagged pre-cut Romaine salad samples seeded with 5 oocysts was 80.0%. All bagged pre-cut Romaine salad samples seeded with 200 oocysts were positive; all unseeded bagged pre-cut Romaine salad samples were negative. No inhibited qPCR reactions were identified based on the performance of the internal amplification control (IAC). See Table 4 for detailed qPCR detection data including the number of positive qPCR replicates, as well as C_T values for *C. cayetanensis* and IAC targets for each sample. Following the data analysis protocol established for BAM Chapter 19B, reactions producing C_T 's greater than 38.0 were considered negative.

For comparison, summaries of the results obtained from the multi-laboratory validation study (MLV) and the parsley matrix extension study are provided in Tables 2 and 3, respectively. Results for detection of *C. cayetanensis* in bagged pre-cut romaine salad using the validated method were similar to results obtained in the matrix extensions for basil, parsley and carrots, and in the MLV study for cilantro and raspberries, with 5 *C. cayetanensis* oocysts identified as the limit of detection.

TABLE 1. Summary of bagged, pre-cut, romaine salad matrix extension results.

Matrix	Oocysts seeded	No. of Samples tested	No. of samples positive by qPCR:	
Bagged pre-cut	0	8	0	0%
romaine salad	5	10	8	80.0%
(25 grams)	200	8	8	100.0%

TABLE 2. *MLV results for cilantro and raspberries.*

Matrix	Seeding Level	Positive samples (80 tested)	% positives
cilantro	0	0	0.0%
	5	25	31.3%
	10	64	80.0%
	200	80	100.0%
raspberries	0	0	0.0%
	5	40	50.0%
	10	72	90.0%
	200	80	100.0%

TABLE 3. *Parsley matrix extension results*

Matrix	Oocysts seeded	No. of Samples tested	No. of samples positive by qPCR:	
Parsley (25 grams)	0	8	0	0%
	5	10	8	80.0%
	10	10	9	90.0%
	200	8	8	100.0%

TABLE 4. Bagged, pre-cut, romaine salad matrix extension qPCR data.

# oocysts	18S No. positive qPCR reactions (out of 3 replicates)	18 S C _T value	IAC C _T value*
0	0	Und	23.8±0.1
0	0	Und	24.0±0.1
0	0	Und	25.4±0.0
0	0	Und	25.8±0.2
0	0	Und	25.7±0.2
0	0	Und	25.6±0.2
0	0	Und	25.0±0.1
0	0	Und	25.3±0.3
5	1	36.7	23.8±0.1
5	1	37.6	23.7±0.0
5	3	36.8±1.1	23.6±0.1
5	3	36.5±0.7	23.7±0.1
5	2	37.2±1.1	23.6±0.1
5	1	36.5	23.5±0.1
5	0	41.7**	25.1±0.3
5	0	Und**	24.9±0.1
5	1	37.5	24.7±0.2
5	3	37.7±0.3	24.7±0.2
200	3	32.2±0.2	23.3±0.1
200	3	32.5±0.3	23.3±0.4
200	3	32.2±0.1	23.4±0.0
200	3	31.8±0.3	23.4±0.1
200	3	32.7±0.3	24.6±0.1
200	3	32.9±0.0	24.6±0.1
200	3	32.8±0.3	24.4±0.3
200	3	32.7±0.4	24.3±0.2

Und: Undetermined

* All positive IAC qPCR reactions (out of 3 replicates)

** Undetermined when DNA diluted ¼ (as per protocol described in BAM Chapter 19B)

4. References:

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<https://www.cdc.gov/parasites/cyclosporiasis/outbreaks/foodborneoutbreaks.html>
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