MEDICINAL GENOMICS®

Research & Development

Report

P0103-RPT-002-03

USP <1223> Alternative Method Equivalency Study for Detection of *Salmonella spp.* and *Escherichia coli* in Dietary Supplements Using Medicinal Genomics PathoSEEK® Multiplex Detection Assay with MaGiC Lysis

cambium analytica

an NSF company

Table of Contents

General Information 2

Introduction 2

Scope 4

Methods 5

Validation Criteria Summary 9

Specificity 10

Limit of Detection (LOD) 12

Repeatability 19

Ruggedness 21

Method Agreement Summary 26

Conclusion 27

Method Feedback & Recommendations 28

References 29

List of Appendices 30

Revision History 31

General Information

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Proposals: P0103-PRO-001-03 (Parent Project); P0211-PRO-001 (Change Order - Add Chocolate Matrix)

Companion Report: P0103-RPT-001

Introduction

Rapid microbiological methods (RMMs) including qPCR-based assays have been increasingly adopted in the dietary supplement industry to improve testing efficiency, reduce turnaround time, and enable greater automation within quality control laboratories. While compendial methods described in United States Pharmacopeia (USP) chapters remain the established standard, these culture-based approaches can be time- and resource-intensive, requiring selective media, extended incubation periods, and multiple handling steps.

In contrast, qPCR-based methods offer a rapid and sensitive alternative for the detection of specified microorganisms. To support their implementation, alternative methods must demonstrate equivalency to compendial procedures in accordance with USP <1223>.

This study was conducted to evaluate the equivalency of a multiplex qPCR assay for the qualitative detection of *Salmonella spp.* and *Escherichia coli* in dietary supplement matrices. These microorganisms are commonly included in microbiological testing specifications for dietary supplements, and the availability of reliable rapid detection methods is of significant relevance to industry testing needs. The alternative method evaluated in this study is designed to support efficient, automated workflows while maintaining performance comparable to compendial approaches.

Purpose

The purpose of this study is to evaluate the equivalency of Medicinal Genomics' PathoSEEK® *Salmonella* and *Escherichia coli* Multiplex Detection Assay with MaGiC Lysis Kit (herein referred to as the "alternative method") relative to USP compendial methods for the qualitative detection of *Salmonella spp.* and *Escherichia coli* in dietary supplement matrices.

This study was conducted in accordance with the framework established in USP <1223> Validation of Alternative Microbiological Methods. The study evaluated the specificity, limit of detection (LOD), robustness, repeatability, ruggedness, and equivalency of the qPCR-based alternative method across six representative dietary supplement matrices. The alternative method was proposed as a faster and more efficient approach to testing for these specified organisms compared to traditional plating methods. In addition, a matrix-independent LOD determination was performed using certified reference materials (CRMs) to establish the LOD_{50} for each target analyte.

Objectives

The objectives of this study were to:

- Demonstrate equivalency of the multiplex qPCR assay to compendial methods for the qualitative detection of *Salmonella spp.* and *Escherichia coli* in dietary supplement matrices, in alignment with USP <1223>.
- Determine the LOD_{50} (and LOD_{95}) of the alternative method for each target analyte using CRMs in a fractional-positive dilution design, independent of sample matrix.
- Compare in-matrix detection at low inoculation levels with the CRM-based LOD to assess whether matrix effects impair detection.
- Evaluate validation parameters relevant to qualitative microbiological methods, including specificity, repeatability, ruggedness, and robustness, across two qPCR thermocycler platforms and six matrices representing broad supplement categories.
- Compile comprehensive documentation including raw data, statistical analyses, and well-supported conclusions regarding method equivalency and LOD characterization.

Scope

This study evaluates the alternative method for the qualitative detection (presence/absence) of *Salmonella spp.* and *Escherichia coli* in the following dietary supplement matrices:

- Matrix 1: Energy Shot
- Matrix 2: Vitamin Gummy
- Matrix 3: Vitamin Powder
- Matrix 4: Botanical Powder
- Matrix 5: MCT Oil
- Matrix 6: Chocolate Bar

The selected matrices represent a range of dietary supplement product types including aqueous liquids, gummies, powders, botanicals, oils, and a high-fat, polyphenol-rich confection. These were chosen to challenge the assay across different physical and chemical compositions and to assess the potential for matrix interference.

USP compendial plating was performed per USP <2022> Microbiological Procedures for Absence of Specified Microorganisms: Nutritional and Dietary Supplements (harmonized with USP <62>). The compendial reference methods used for comparison include plating on MacConkey Agar for *E. coli* and Xylose Lysine Deoxycholate (XLD) Agar for *Salmonella,* with confirmatory testing of recovered colonies.

Each matrix was evaluated under uninoculated conditions (unspiked blanks and non-target-spiked negatives) and at low, intermediate, and high inoculation levels, with eight replicates at the low and intermediate levels and two confirmatory replicates at the high level. All target organisms were co-inoculated into each positive sample. Testing of the alternative method was performed using automated DNA extraction (BioMolecular Systems Myra) with each extract analyzed on two qPCR thermocycler platforms (Bio-Rad CFX Opus and BioMolecular Systems Mic).

This assay was evaluated concurrently with the Medicinal Genomics *Salmonella*, *E. coli*, and *S. aureus* triplex assay (companion report P0103-RPT-001) using the same inoculated samples. Each positive sample was therefore co-inoculated with *Salmonella spp.*, *E. coli*, and *S. aureus*. *S. aureus* is not a target of the duplex assay and is not evaluated in this report.

Inclusivity and exclusivity: Inclusivity and exclusivity (primer/probe specificity across strain panels) were established independently by the Client and were not re-evaluated in this study. Specificity in this report is evaluated as the absence of false-positive results in uninoculated and non-target-spiked matrix samples.

Methods

Study Design Overview

This study was designed as a side-by-side comparison of the alternative qPCR method and USP compendial methods, preceded by a matrix-independent LOD determination using CRMs. Each matrix was tested at multiple inoculation levels to evaluate the detection capability and reliability of the alternative method. The study included uninoculated (blank) controls, non-target-spiked negatives, low-level spikes (target 1 to 10 CFU/test portion), intermediate-level spikes, and high-level spikes.

1. Sample Matrices

1.1. Six representative matrices were selected to reflect a range of dietary supplement compositions including:

1.1.1. Energy Shot (Aqueous Liquid)

1.1.2. Vitamin Gummy

1.1.3. Vitamin Powder

1.1.4. Botanical Powder

1.1.5. MCT Oil

1.1.6. Chocolate Bar

2. Reference Strains

2.1. The target organisms for this study include:

2.1.1. *Salmonella enterica* NCTC 6676 (CRM Lot 250140)

2.1.2. *Escherichia coli* NCTC 12923 / ATCC 8739 (CRM Lot 250807)

2.2. Inoculated samples were also co-inoculated with *Staphylococcus aureus* NCTC 10788 / ATCC 6538 (CRM Lot 240304) as part of the concurrent triplex evaluation. *S. aureus* is not a target of the duplex assay.

2.3. Non-target-spiked negative samples were inoculated with *Klebsiella oxytoca* NCTC 8167 (CRM Lot 251205) and *Citrobacter koseri* ATCC 25408 (CRM Lot 70061360), which are not detected by the alternative method or recovered as target organisms on the compendial media.

2.4. Reference strains were grown and enumerated, or CRMs were reconstituted per the manufacturer's certificate, to prepare stocks of known CFU/mL. Dilution series were prepared to achieve the desired contamination levels for spiking.

3. Limit of Detection (LOD) Determination

3.1. A standalone LOD determination was performed using CRMs for each target analyte independent of sample matrix. CRM stocks were serially diluted to target levels of 5, 2.5, 1, 0.5, 0.25, and 0.1 nominal CFU (approximate 2-fold series bracketing the expected detection threshold of 1 nominal CFU).

3.2. CRM stocks were reconstituted per the manufacturer's certificate and serially diluted to each target level. Five replicates per level were extracted directly using MaGiC Lysis (no enrichment) and analyzed on both thermocyclers.

4. Inoculation Levels (Equivalency Study)

4.1. Each matrix was tested at the following inoculation levels; each extract was analyzed on both thermocyclers.

4.1.1. **Uninoculated:** 4 samples per matrix (2 unspiked blanks, 2 non-target-spiked negatives)

4.1.2. **Low level (target 1 to 10 CFU/test portion):** 8 samples per matrix

4.1.2.1. Low inoculation levels were within 1 to 10 CFU/test portion and were not adjusted to achieve fractional positive results.

4.1.3. **Intermediate level:** 8 samples per matrix

4.1.4. **High level:** 2 samples per matrix

4.2. All target organisms were co-inoculated into each low-, intermediate-, and high-level sample. Spiked samples were homogenized thoroughly to ensure even distribution of organisms.

5. Alternative Method

5.1. The alternative method utilizes the PathoSEEK® *Salmonella* and *Escherichia coli* Multiplex Detection Assay with MaGiC Lysis Kit. This is a qualitative real-time PCR assay that simultaneously detects *E. coli* (FAM) and *Salmonella* spp. (ROX), and an internal amplification control (HEX) in a single multiplexed reaction.

5.2. Sample preparation and enrichment were performed per the manufacturer's User Guide (Table 1 of this report). Each test portion was homogenized in buffered peptone water (1:10 w/v) in a filter enrichment bag and incubated for 20-24 hours at 37 ± 2 °C.

5.3. DNA was extracted from 10 µL of each homogenized enrichment using the MaGiC Lysis protocol (50 µL MaGiC Lysis Reagent plus 5 µL internal control; 95 °C for 10 minutes followed by 25 °C for 10 minutes; MaGiC Stabilization Buffer). The internal control was added at a 1:50,000 dilution for all matrices except Chocolate, which received a 1:5,000 dilution. Stabilized lysates were diluted in nuclease-free water prior to qPCR where specified in Table 1. Each extract was analyzed on both qPCR instruments (Bio-Rad CFX Opus and BioMolecular Systems Mic).

5.4. qPCR cycling conditions: Hot start at 95 °C for 5 minutes, followed by 40 cycles of 95 °C for 15 seconds and 65 °C for 90 seconds with data capture. Each qPCR run included one negative control, one media blank (BPW incubated), and one assay positive control.

5.5. Per the User Guide, a target result (FAM, ROX) is positive when amplification crosses the threshold at Cq < 40. In this study, each target amplification curve was additionally reviewed by a qualified microbiologist and accepted as positive only if the respective amplification curve passed through the threshold during its exponential growth phase and had a strong ΔRn. Curves with late, non-exponential, or low-amplitude signal were not called positive. Curves meeting these criteria generally crossed the threshold at Cq ≤ 37. This review is more conservative than the User Guide criterion alone, so any effect would be to reduce, not increase, the number of positive results. All calls, including curves not accepted on review, are documented in Appendix A.

5.6. Each reaction includes an internal amplification control (IC, HEX channel) added at a fixed concentration. A result is valid only if the IC amplifies at Cq ≤ 35, per the User Guide.

Table 1. Matrix-Specific Sample Preparation (per Manufacturer's User Guide)

Matrix	Test Portion	BPW Volume (1:10)	Enrichment	Lysate Dilution Before qPCR
MCT Oil	25 g	225 mL	20-24 h, 37 ± 2 °C	None
Vitamin Gummy	10 g	90 mL	20-24 h, 37 ± 2 °C	None
Vitamin Powder	10 g	90 mL	20-24 h, 37 ± 2 °C	None
Botanical Powder	10 g	90 mL	20-24 h, 37 ± 2 °C	1:10
Energy Shot	1 g	9 mL	20-24 h, 37 ± 2 °C	1:10
Chocolate	5 g	45 mL	20-24 h, 37 ± 2 °C	1:100

6. Reference (Compendial) Methods

6.1. USP compendial plating methods were performed in parallel for each sample per USP <2022> Microbiological Procedures for Absence of Specified Microorganisms — Nutritional and Dietary Supplements (harmonized with USP <62>):

6.1.1. ***E. coli:*** Plating on MacConkey Agar, incubated at 30°C - 35°C for 18-72 hours.

6.1.2. ***Salmonella spp.:*** Plating on XLD Agar, incubated at 30°C - 35°C for 18-48 hours.

6.2. Media blanks were included daily and for each new lot of media.

7. Confirmatory Methods

7.1. Confirmatory tests were performed on colonies from compendial plating to verify organism ID:

7.1.1. ***E. coli* Confirmation:** Indole Test (Red ring = positive) and Citrate Test (green = negative)

7.1.2. ***Salmonella spp.* Confirmation:** Citrate Test (blue = positive) and Indole Test (no red ring = negative)

7.2. All presumptive colonies from inoculated samples were confirmed as the target organism.

8. Instruments

8.1. BioRad CFX Opus Real-Time PCR System

8.2. BioMolecular Systems Mic 4-Channel PCR Instrument

8.3. Automated extraction platform (BioMolecular Systems Myra)

Validation Criteria Summary

Table 2 provides a summary of the results for the six USP <1223> parameters evaluated in this study; equivalency met the USP <1223> non-inferiority criterion.

Table 2. Summary of USP <1223> Validation Parameters

Validation Parameter	Result Summary	Status
Specificity	No false positives for off-target organisms or uninoculated blanks across all matrices and both instruments.	**Reported**
Limit of Detection	CRM LOD_{50} 0.27 to 0.38 and LOD_{95} 1.16 to 1.63 nominal CFU (CRM dilution series, direct lysis). In-matrix detection 100% at the low level in all six matrices.	**Reported**
Repeatability	Qualitative results agreed across all replicate samples; average IC Cq RSD ≤ 5.00%	**Reported**
Ruggedness	Identical qualitative results on both qPCR instruments for every sample and target (100% concordance).	**Reported**
Robustness	Internal amplification control (HEX) detected in all samples; method tolerates matrix variability across six diverse product types.	**Reported**
Equivalency	Non-inferiority demonstrated ($Z > 1.645$ at $\alpha = 0.05$, $\delta = 0.20$) at all levels; 100% agreement with the compendial method.	**Met**

Specificity

Evaluation Approach

USP <1223> describes specificity as the ability to detect a range of challenge microorganisms, demonstrated by comparable recovery by both methods and, for non-growth-based methods, by showing that extraneous material (e.g., DNA) does not interfere. In this study, specificity was evaluated as the absence of positive results in uninoculated and off-target-spiked samples; target recovery at the low, intermediate, and high inoculation levels is reported under the Limit of Detection section.

Table 3. Method Specificity in Uninoculated and Non-Target-Spiked Samples

Matrix	Instrument	NA	PD	FPR%	SPalt%	SPref%
Energy Shot	Bio-Rad CFX Opus	4	0	0	100	100
	Mic	4	0	0	100	100
Vitamin Gummy	Bio-Rad CFX Opus	4	0	0	100	100
	Mic	4	0	0	100	100
Vitamin Powder	Bio-Rad CFX Opus	4	0	0	100	100
	Mic	4	0	0	100	100
Botanical Powder	Bio-Rad CFX Opus	4	0	0	100	100
	Mic	4	0	0	100	100
MCT Oil	Bio-Rad CFX Opus	4	0	0	100	100
	Mic	4	0	0	100	100
Chocolate	Bio-Rad CFX Opus	4	0	0	100	100
	Mic	4	0	0	100	100

NA = Negative Agreement (true negatives, counted per sample; both target channels were negative in every uninoculated and non-target-spiked sample), PD = Positive Deviation (alternative positive, reference negative). In uninoculated and non-target-spiked samples, a PD represents a false positive.

Discussion

Uninoculated blanks and non-target-spiked negatives were tested across all six matrices on both qPCR instruments. The alternative method produced no positive results in any target channel (PD = 0), and the internal amplification control met the validity criterion (Cq ≤ 35; Methods 5.6) in every sample, so all negative results were valid. The compendial methods likewise recovered no target organisms from these samples.

Because both targets were co-inoculated into each positive sample, no loss of detection was observed when the targets were present together at the levels tested. Full inclusivity and exclusivity were determined independently by the Client.

Conclusion

The alternative method demonstrated 100% specificity in uninoculated and non-target-spiked samples across all matrices and both instruments. No false-positive detections were observed.

Limit of Detection (LOD)

Evaluation Approach

Per USP <1223>, the LOD of the alternative method should be comparable to that of the compendial method. LOD was evaluated in two parts: (1) LOD_{50} and LOD_{95} were estimated from a CRM dilution series (5 to 0.1 nominal CFU, five replicates per level) run by direct lysis without matrix or enrichment, for the alternative method only; and (2) detection at low, intermediate and high inoculation levels in six matrices is reported descriptively, with per-matrix POD and dPOD (95% CI). At n = 8 per level, per-matrix data cannot establish non-inferiority; method comparison is evaluated on pooled paired data (see Equivalency).

Part 1: CRM-Based LOD Determination

Table 4 presents the number of positive replicates out of five at each nominal CRM level, and Table 5 presents the resulting LOD estimates.

Table 4. CRM Fractional-Positive Results (Positive Replicates / 5)

Nominal CFU	*E. coli*		*Salmonella spp.*	
	Bio-Rad CFX Opus	Mic	Bio-Rad CFX Opus	Mic
5	5/5	5/5	5/5	5/5
2.5	5/5	5/5	5/5	5/5
1	5/5	5/5	5/5	5/5
0.5	4/5	4/5	3/5	3/5
0.25	2/5	2/5	2/5	1/5
0.1	0/5	0/5	0/5	0/5

Positive per the criteria in Methods 5.5. Raw Cq values are provided in Appendix A.

Table 5. LOD_{50} and LOD_{95} Estimates from CRM Dilution Series (Nominal CFU, Direct Lysis)

Target	Instrument	LOD_{50}	*95% CI*	LOD_{95}	*95% CI*
E. coli	Bio-Rad CFX Opus	0.27	0.14, 0.51	1.16	0.62, 2.19
	Mic	0.27	0.14, 0.51	1.16	0.62, 2.19
Salmonella spp.	Bio-Rad CFX Opus	0.33	0.17, 0.61	1.41	0.75, 2.65
	Mic	0.38	0.20, 0.71	1.63	0.87, 3.05

LOD_{50}, LOD_{95}, and associated confidence intervals (CIs) were computed using the PODLOD program referenced in ISO 16140-2.

Discussion

All targets were detected in all five replicates at nominal levels of 1 CFU and above on both instruments. Fractional responses occurred at 0.5 and 0.25 nominal CFU, and no target was detected at 0.1 nominal CFU, bracketing the detection threshold as intended. LOD_{50} estimates ranged from 0.27 to 0.38 nominal CFU and LOD_{95} estimates from 1.16 to 1.63 nominal CFU across all targets and instruments.

For context, a culture-based method requiring at least one culturable cell has a theoretical LOD_{50} of 0.69 nominal CFU. Observed detection rates at 0.5 and 1 nominal CFU exceeded those expected under this model, indicating that the assay detects target DNA from cells not counted as culturable CFU on the CRM certificate, as expected for a DNA-based method. LOD values are therefore reported in nominal CFU and are not a per-cell detection limit. With five replicates per level, confidence intervals are wide (Table 5). The 95% confidence intervals for both instruments overlap for each target.

Part 2: In-Matrix Probability of Detection (POD)

The following tables (Tables 6-9) present the POD analysis for each target across all six matrices at the uninoculated, low, intermediate, and high inoculation levels on the Mic (Tables 6-7) and Bio-Rad CFX Opus (Tables 8-9). Per-matrix POD and dPOD are reported descriptively; at n = 8 per level (n = 2 at the high level), per-matrix data cannot establish non-inferiority. See Equivalency.

Table 6. Probability of Detection (POD) Analysis: Duplex Assay, Mic, E. coli Presumptive vs. Confirmed

			Candidate/Presumptive			Confirmed				
Matrix	**CFU/Test Portion**	**N**	**x**	**POD_{CP}**	**95% CI**	**x**	**POD_{CC}**	**95% CI**	**$dPOD_{CP}$**	**95% CI**
Energy Shot	0	4	0	0.00	0.00, 0.49	0	0.00	0.00, 0.49	0.00	-0.57, 0.57
	8	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	33	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	900	2	2	1.00	0.34, 1.00	2	1.00	0.34, 1.00	0.00	-0.98, 0.98
Vitamin Gummy	0	4	0	0.00	0.00, 0.49	0	0.00	0.00, 0.49	0.00	-0.57, 0.57
	5	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	82	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	950	2	2	1.00	0.34, 1.00	2	1.00	0.34, 1.00	0.00	-0.98, 0.98
Vitamin Powder	0	4	0	0.00	0.00, 0.49	0	0.00	0.00, 0.49	0.00	-0.57, 0.57
	2	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	59	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	400	2	2	1.00	0.34, 1.00	2	1.00	0.34, 1.00	0.00	-0.98, 0.98
Botanical Powder	0	4	0	0.00	0.00, 0.49	0	0.00	0.00, 0.49	0.00	-0.57, 0.57
	2	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	45	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	150	2	2	1.00	0.34, 1.00	2	1.00	0.34, 1.00	0.00	-0.98, 0.98
MCT Oil	0	4	0	0.00	0.00, 0.49	0	0.00	0.00, 0.49	0.00	-0.57, 0.57
	1	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	29	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	250	2	2	1.00	0.34, 1.00	2	1.00	0.34, 1.00	0.00	-0.98, 0.98
Chocolate	0	4	0	0.00	0.00, 0.49	0	0.00	0.00, 0.49	0.00	-0.57, 0.57
	3	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	56	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	400	2	2	1.00	0.34, 1.00	2	1.00	0.34, 1.00	0.00	-0.98, 0.98

POD = Probability of Detection, POD_{CP} = POD of the candidate presumptive method, POD_{CC} = POD of the compendial confirmed method, CI = Confidence Interval, dPOD = Difference between POD_{CP} and POD_{CC}. Values were computed using the PODLOD program referenced in ISO 16140-2.

Table 7. Probability of Detection (POD) Analysis: Duplex Assay, Mic, Salmonella spp. Presumptive vs. Confirmed

			Candidate/Presumptive			Confirmed				
Matrix	**CFU/Test Portion**	**N**	**x**	**PODCP**	**95% CI**	**x**	**PODCC**	**95% CI**	**dPODCP**	**95% CI**
Energy Shot	0	4	0	0.00	0.00, 0.49	0	0.00	0.00, 0.49	0.00	-0.57, 0.57
	2	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	30	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	150	2	2	1.00	0.34, 1.00	2	1.00	0.34, 1.00	0.00	-0.98, 0.98
Vitamin Gummy	0	4	0	0.00	0.00, 0.49	0	0.00	0.00, 0.49	0.00	-0.57, 0.57
	2	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	76	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	325	2	2	1.00	0.34, 1.00	2	1.00	0.34, 1.00	0.00	-0.98, 0.98
Vitamin Powder	0	4	0	0.00	0.00, 0.49	0	0.00	0.00, 0.49	0.00	-0.57, 0.57
	4	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	31	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	250	2	2	1.00	0.34, 1.00	2	1.00	0.34, 1.00	0.00	-0.98, 0.98
Botanical Powder	0	4	0	0.00	0.00, 0.49	0	0.00	0.00, 0.49	0.00	-0.57, 0.57
	1	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	29	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	400	2	2	1.00	0.34, 1.00	2	1.00	0.34, 1.00	0.00	-0.98, 0.98
MCT Oil	0	4	0	0.00	0.00, 0.49	0	0.00	0.00, 0.49	0.00	-0.57, 0.57
	6	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	55	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	550	2	2	1.00	0.34, 1.00	2	1.00	0.34, 1.00	0.00	-0.98, 0.98
Chocolate	0	4	0	0.00	0.00, 0.49	0	0.00	0.00, 0.49	0.00	-0.57, 0.57
	1	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	46	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	250	2	2	1.00	0.34, 1.00	2	1.00	0.34, 1.00	0.00	-0.98, 0.98

POD = Probability of Detection, POD_{CP} = POD of the candidate presumptive method, POD_{CC} = POD of the compendial confirmed method, CI = Confidence Interval, dPOD = Difference between POD_{CP} and POD_{CC}. Values were computed using the PODLOD program referenced in ISO 16140-2.

Table 8. Probability of Detection (POD) Analysis: Duplex Assay, Bio-Rad CFX Opus, E. coli Presumptive vs. Confirmed

			Candidate/Presumptive			Confirmed				
Matrix	**CFU/Test Portion**	**N**	**x**	**POD_{CP}**	**95% CI**	**x**	**POD_{CC}**	**95% CI**	**$dPOD_{CP}$**	**95% CI**
Energy Shot	0	4	0	0.00	0.00, 0.49	0	0.00	0.00, 0.49	0.00	-0.57, 0.57
	8	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	33	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	900	2	2	1.00	0.34, 1.00	2	1.00	0.34, 1.00	0.00	-0.98, 0.98
Vitamin Gummy	0	4	0	0.00	0.00, 0.49	0	0.00	0.00, 0.49	0.00	-0.57, 0.57
	5	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	82	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	950	2	2	1.00	0.34, 1.00	2	1.00	0.34, 1.00	0.00	-0.98, 0.98
Vitamin Powder	0	4	0	0.00	0.00, 0.49	0	0.00	0.00, 0.49	0.00	-0.57, 0.57
	2	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	59	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	400	2	2	1.00	0.34, 1.00	2	1.00	0.34, 1.00	0.00	-0.98, 0.98
Botanical Powder	0	4	0	0.00	0.00, 0.49	0	0.00	0.00, 0.49	0.00	-0.57, 0.57
	2	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	45	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	150	2	2	1.00	0.34, 1.00	2	1.00	0.34, 1.00	0.00	-0.98, 0.98
MCT Oil	0	4	0	0.00	0.00, 0.49	0	0.00	0.00, 0.49	0.00	-0.57, 0.57
	1	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	29	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	250	2	2	1.00	0.34, 1.00	2	1.00	0.34, 1.00	0.00	-0.98, 0.98
Chocolate	0	4	0	0.00	0.00, 0.49	0	0.00	0.00, 0.49	0.00	-0.57, 0.57
	3	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	56	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	400	2	2	1.00	0.34, 1.00	2	1.00	0.34, 1.00	0.00	-0.98, 0.98

POD = Probability of Detection, POD_{CP} = POD of the candidate presumptive method, POD_{CC} = POD of the compendial confirmed method, CI = Confidence Interval, dPOD = Difference between POD_{CP} and POD_{CC}. Values were computed using the PODLOD program referenced in ISO 16140-2.

Table 9. Probability of Detection (POD) Analysis: Duplex Assay, Bio-Rad CFX Opus, Salmonella spp. Presumptive vs. Confirmed

			Candidate/Presumptive			Confirmed				
Matrix	**CFU/Test Portion**	**N**	**x**	**POD_{CP}**	**95% CI**	**x**	**POD_{CC}**	**95% CI**	**$dPOD_{CP}$**	**95% CI**
Energy Shot	0	4	0	0.00	0.00, 0.49	0	0.00	0.00, 0.49	0.00	-0.57, 0.57
	2	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	30	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	150	2	2	1.00	0.34, 1.00	2	1.00	0.34, 1.00	0.00	-0.98, 0.98
Vitamin Gummy	0	4	0	0.00	0.00, 0.49	0	0.00	0.00, 0.49	0.00	-0.57, 0.57
	2	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	76	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	325	2	2	1.00	0.34, 1.00	2	1.00	0.34, 1.00	0.00	-0.98, 0.98
Vitamin Powder	0	4	0	0.00	0.00, 0.49	0	0.00	0.00, 0.49	0.00	-0.57, 0.57
	4	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	31	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	250	2	2	1.00	0.34, 1.00	2	1.00	0.34, 1.00	0.00	-0.98, 0.98
Botanical Powder	0	4	0	0.00	0.00, 0.49	0	0.00	0.00, 0.49	0.00	-0.57, 0.57
	1	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	29	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	400	2	2	1.00	0.34, 1.00	2	1.00	0.34, 1.00	0.00	-0.98, 0.98
MCT Oil	0	4	0	0.00	0.00, 0.49	0	0.00	0.00, 0.49	0.00	-0.57, 0.57
	6	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	55	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	550	2	2	1.00	0.34, 1.00	2	1.00	0.34, 1.00	0.00	-0.98, 0.98
Chocolate	0	4	0	0.00	0.00, 0.49	0	0.00	0.00, 0.49	0.00	-0.57, 0.57
	1	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	46	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	250	2	2	1.00	0.34, 1.00	2	1.00	0.34, 1.00	0.00	-0.98, 0.98

POD = Probability of Detection, POD_{CP} = POD of the candidate presumptive method, POD_{CC} = POD of the compendial confirmed method, CI = Confidence Interval, dPOD = Difference between POD_{CP} and POD_{CC}. Values were computed using the PODLOD program referenced in ISO 16140-2.

Discussion

At the uninoculated level (0 CFU/test portion), POD_{CP} and POD_{CC} were 0.00 for all matrices and organisms; no positive results were observed by either method. At the intermediate and high inoculation levels, POD_{CP} and POD_{CC} were 1.00 across all matrices and targets (dPOD = 0.00). The high level (n = 2 per matrix) was included as a confirmatory positive condition, and its confidence intervals are correspondingly wide.

At the low inoculation level (mean compendial recoveries of 1 to 8 CFU/test portion), both methods detected every inoculated sample for all targets, matrices, and instruments (POD_{CP} and POD_{CC} = 1.00; dPOD = 0.00). No matrix-related loss of detection was observed at the levels tested, consistent with the CRM-based LOD_{95} (1.16 to 1.63 nominal CFU). Qualitative results were identical on both qPCR instruments for every sample and organism.

Conclusion

Per-matrix POD and dPOD (95% CI) are reported descriptively. At the sample size used in this study (n = 8 per low/intermediate levels, n = 2 at the high level), the POD analysis is presented as supporting data. Method comparison is evaluated on pooled paired data (see Equivalency section).

Repeatability

Evaluation Approach

Repeatability is the closeness of agreement between independent test results obtained under stipulated conditions. Repeatability was evaluated as qualitative agreement among replicate samples at each level, with internal control (HEX) Cq precision reported as supporting data.

Repeatability was assessed by comparing log-transformed Cq values between paired conditions across all six matrices. The internal control channel (HEX) was used as a supporting indicator of method consistency as it is present in every valid sample regardless of target detection status. Target-channel Cq values were not used for this assessment because they also reflect the inoculum delivered to each sample and growth during enrichment.

Each DNA extract was analyzed on both the Bio-Rad CFX Opus and the Mic. For every sample, the RSD of the two log-transformed HEX Cq values was calculated, and the results were summarized as the average and maximum RSD at each inoculation level (8 low, 8 intermediate, 2 high, and 4 uninoculated samples per matrix). The overall average includes all 22 samples per matrix.

Table 10. Repeatability Demonstrated by Average and Maximum RSD (%) of Log-Transformed Cq Values (HEX Internal Control, Both Instruments)

	Low (n=8)		Intermediate (n=8)		High (n=2)		Uninoc. (n=4)	Overall (n=22)
Matrix	***Avg RSD%***	***Max RSD%***	***Avg RSD%***	***Max RSD%***	***Avg RSD%***	***Max RSD%***	***Avg RSD%***	***Avg RSD%***
Energy Shot	0.33	0.86	0.61	2.83	0.18	0.33	0.55	0.46
Vitamin Gummy	5.00	6.09	4.05	5.27	3.93	4.01	2.10	4.03
Vitamin Powder	1.45	1.85	1.43	1.98	1.56	1.57	1.23	1.41
Botanical Powder	1.82	2.07	1.58	2.54	1.87	2.06	1.48	1.68
MCT Oil	1.86	2.79	2.05	2.32	2.01	2.21	0.79	1.75
Chocolate	1.49	1.76	1.41	1.77	1.67	1.80	1.08	1.40

Average RSDs ≤ 5.00%. RSD calculated for each sample from the paired $\log_{10}$ HEX Cq values on the Mic and Bio-Rad CFX Opus (standard deviation / mean × 100). Uninoculated = 2 unspiked blanks and 2 non-target-spiked negatives.

Discussion

Across all six matrices and inoculation levels, average HEX RSD values were ≤ 5.00% and no individual sample exceeded 6.09%. Uninoculated samples showed comparable precision (average ≤ 2.10%), confirming consistent internal control performance whether or not target organisms were present.

The Mic generally returned earlier Cq values than the Bio-Rad, with consistent, matrix-dependent offsets (typically about 2 cycles). Such offsets are expected between qPCR platforms with different optics and threshold-setting algorithms, did not vary with inoculation level, and had no effect on qualitative results, which were 100% concordant between instruments.

Conclusion

Qualitative results agreed across replicate samples in every matrix and inoculation level. Internal control (HEX) Cq precision between instruments is provided as supporting data; average RSDs were ≤ 5.00% and no individual value exceeded 6.09%.

Ruggedness

Evaluation Approach

USP <1223> defines ruggedness as the precision of results from the same samples under typical variations in test conditions, such as analysts and instruments. Ruggedness was evaluated as qualitative agreement across two qPCR instruments (Bio-Rad and Mic). Analyst and extraction method were not varied; all extractions used the Myra automated platform.

Discussion

Every DNA extract was analyzed on both the Bio-Rad CFX Opus and the BioMolecular Systems Mic, generating paired qualitative results for direct comparison (132 samples × 2 targets = 264 paired determinations). Qualitative concordance between instruments was 100% at every inoculation level, in every matrix, and for every target organism: each sample detected on one instrument was detected on the other, and each uninoculated sample was negative on both. Results on both instruments were also fully concordant with the compendial method.

Conclusion

The alternative method produced concordant qualitative results across both qPCR platforms for all matrices and targets.

Robustness

Evaluation Approach

Robustness is the capacity of the alternative method to remain unaffected by small but deliberate variations in method parameters and to provide reliable results under normal conditions of use. Deliberate parameter variation was not performed in this study; tolerance to matrix variation was evaluated by internal amplification control performance across six matrices.

Discussion

The six matrices tested in this study were selected to challenge the method across a broad range of dietary supplement product types:

Energy Shot: Aqueous liquid containing organic acids, vitamins, and caffeine

Vitamin Gummy: Semi-solid gelatin matrix with sugars, colorants, and vitamins

Vitamin Powder: Fine powder mix with mineral and vitamin components

Botanical Powder: Complex plant-based matrix containing polyphenols, tannins, and other potential PCR inhibitors

MCT Oil: Hydrophobic lipid matrix

Chocolate: High-fat confection containing cocoa polyphenols, methylxanthines, and sugars

The internal amplification control, detected on the HEX channel, monitors for PCR inhibition, DNA extraction failure, and instrument malfunction. Successful amplification of the HEX target is required for a valid test result.

Across all six matrices, both instruments, and all inoculation levels, the internal control amplified successfully. HEX Cq values were consistent within each matrix, and no systematic inhibition patterns were observed.

The repeatability analysis (Table 10) further supports robustness, as RSD values for the HEX channel were comparable across matrices. The method tolerated the chemical and physical variability across all six product types using only the matrix-specific adjustments specified in the manufacturer's User Guide (pre-qPCR lysate dilutions, and a 1:5,000 internal control dilution for Chocolate). These adjustments are part of the method design and do not represent a robustness limitation.

Conclusion

The alternative method tolerated matrix variability across six diverse supplement matrices, and the internal amplification control confirmed successful PCR performance in all samples.

Equivalency

The United States Pharmacopeia (USP) General Notices 6.30 Alternative and Harmonized Methods and Procedures states that alternate methods may be used if they provide advantages in terms of accuracy, sensitivity, precision, selectivity, or adaptability to automation or computerized data reduction. The alternative method must be shown to produce equivalent or better results than the compendial method for any given quality attribute. When comparing two test procedures, to show equivalent or better performance, statistical evidence is assembled to show equivalence (non-inferiority).

Per USP <1223>, equivalency for qualitative methods can be demonstrated using Approach 1 (paired samples, non-inferiority hypothesis). Paired detect/non-detect outcomes are arranged in a 2×2 contingency table, and a one-sided Z-test is applied to determine whether the detection rate of the alternative method is not more than δ lower than that of the compendial method.

Acceptance Criteria (USP <1223> Approach 1)

Non-inferiority is demonstrated if $Z > z_{\alpha}$ where $\alpha = 0.05$ ($z_{0.05} = 1.645$) and $\delta = 0.20$. The hypothesis tested is:

H_0 (null): $p_A \leq p_C - \delta$ (alternative is inferior)

H_1 (alternative): $p_A > p_C - \delta$ (alternative is non-inferior)

where p_A is the proportion positive by the alternative method, p_C is the proportion positive by the compendial method, and $\delta = 0.20$ is the non-inferiority margin.

The alternative method is a duplex assay in which *Salmonella spp.* and *E. coli* are detected simultaneously from a single reaction. Both target organisms were co-inoculated into every test portion, and each portion was analyzed by the compendial procedure for each target (XLD for *Salmonella spp.*, MacConkey for *E. coli*).

Each test portion therefore yields a single paired duplex outcome rather than two independent comparisons. A sample was classified as discordant if the alternative and compendial results differed for any target organism, which is the conservative treatment for a multiplex result. No discordant samples were observed in this study. Each test portion was analyzed on two qPCR instruments (Bio-Rad CFX Opus and Mic). These are repeated measurements of the same test portion and are not statistically independent. Qualitative results were identical on both instruments for every sample and target (see Ruggedness). The non-inferiority analysis is therefore performed on independent test portions: n = 48 at the low and intermediate levels (8 portions per matrix across 6 matrices) and n = 12 at the high level (2 portions per matrix across 6 matrices).

For each inoculated test portion, the alternative method result (+/−) was paired with the compendial method result (+/−) to construct a 2×2 contingency table for each inoculation level.

Table 11. Low Inoculation Level (n = 48 paired test portions): Paired 2×2 Contingency Table

	Reference (+)	Reference (–)	Total
Alternative (+)	X_{11} = 48	X_{10} = 0	48
Alternative (–)	X_{01} = 0	X_{00} = 0	0
Total	48	0	48

Table 12. Intermediate Inoculation Level (n = 48 paired test portions): Paired 2×2 Contingency Table

	Reference (+)	Reference (–)	Total
Alternative (+)	X_{11} = 48	X_{10} = 0	48
Alternative (–)	X_{01} = 0	X_{00} = 0	0
Total	48	0	48

Table 13. High Inoculation Level (n = 12 paired test portions): Paired 2×2 Contingency Table

	Reference (+)	Reference (–)	Total
Alternative (+)	X_{11} = 12	X_{10} = 0	12
Alternative (–)	X_{01} = 0	X_{00} = 0	0
Total	12	0	12

Calculations (Low Level):

$p_A = (X_{11} + X_{10}) / n = (48 + 0) / 48 = 1.000$

$p_C = (X_{11} + X_{01}) / n = (48 + 0) / 48 = 1.000$

Difference ($p_A - p_C$) = 0.000

With perfect agreement and no discordant pairs, $Z \to \infty$. **Non-inferiority is demonstrated**.

The intermediate (n = 48) and high (n = 12) levels likewise showed perfect agreement ($p_A = p_C = 1.000$), so $Z \to \infty$ at every level.

Table 14. Non-Inferiority Test Results (δ = 0.20, α = 0.05)

Level	N	p_A	p_C	Z-statistic	z_α (0.05)	Result
Low	48	1.000	1.000	∞	1.645	**PASS**
Intermediate	48	1.000	1.000	∞	1.645	**PASS**
High	12	1.000	1.000	∞	1.645	**PASS**

Equivalency of the alternative method to the compendial method was demonstrated by non-inferiority testing per USP <1223> Approach 1. At the low, intermediate, and high inoculation levels, the alternative and compendial methods were in complete agreement, so the detection rate of the alternative method was not more than 0.20 less than that of the compendial method. Method agreement metrics (Tables 15-16) further support equivalency. The equivalency criterion is met.

USP <1223> recommends evaluating non-inferiority at an inoculation level where 50 to 75% of samples are positive, with at least 75 samples per procedure. This study tested 48 samples per level at the low and intermediate levels, at levels producing complete detection by both methods. Non-inferiority is therefore demonstrated at the levels tested, but relative sensitivity near the detection threshold was not characterized.

Method Agreement Summary

Method agreement was assessed by calculating Positive Agreement (PA), Negative Agreement (NA), Positive Deviation (PD), Negative Deviation (ND), Sensitivity of the Alternative Method (SEalt%), Sensitivity of the Reference Method (SEref%), Relative Trueness (RT%), and False Positive Rate (FPR%) across all matrices and conditions, counted per sample and target organism.

Counts in this table are measurement-level: each test portion and target is counted once per instrument. Statistical determinations in the Equivalency section are made on independent test portions (n = 48 per level).

Table 15. Method Agreement by Matrix and Instrument

Matrix	Instrument	PA	NA	PD	ND	SEalt%	SEref%	RT%	FPR%
Energy Shot	Bio-Rad CFX Opus	36	8	0	0	100	100	100	0
	Mic	36	8	0	0	100	100	100	0
Vitamin Gummy	Bio-Rad CFX Opus	36	8	0	0	100	100	100	0
	Mic	36	8	0	0	100	100	100	0
Vitamin Powder	Bio-Rad CFX Opus	36	8	0	0	100	100	100	0
	Mic	36	8	0	0	100	100	100	0
Botanical Powder	Bio-Rad CFX Opus	36	8	0	0	100	100	100	0
	Mic	36	8	0	0	100	100	100	0
MCT Oil	Bio-Rad CFX Opus	36	8	0	0	100	100	100	0
	Mic	36	8	0	0	100	100	100	0
Chocolate	Bio-Rad CFX Opus	36	8	0	0	100	100	100	0
	Mic	36	8	0	0	100	100	100	0

PA = Positive Agreement, NA = Negative Agreement, PD = Positive Deviation, ND = Negative Deviation, SEalt% = Sensitivity of Alternative, SEref% = Sensitivity of Reference, RT%=Relative Trueness, FPR% = False Positive Rate

Table 16. Method Agreement by Overall Performance Across All Matrices, Organism Targets, and Instruments (Inoculated Samples)

Metric	Low Level	Intermediate Level	High Level	Overall
SEalt% (Alt. Sensitivity)	100%	100%	100%	100%
SEref% (Ref. Sensitivity)	100%	100%	100%	100%
RT% (Rate of Agreement)	100%	100%	100%	100%
FPR% (False Positive Rate)	0%	0%	0%	0%

Conclusion

Method Equivalency Study Summary

Non-inferiority of the PathoSEEK® *Salmonella* and *Escherichia coli* Multiplex Detection Assay with MaGiC Lysis Kit to compendial plating was demonstrated per USP <1223> Approach 1 at the inoculation levels tested in Energy Shot, Vitamin Gummy, Vitamin Powder, Botanical Powder, MCT Oil, and Chocolate matrices. The alternative method offers a faster and more efficient alternative to traditional culture-based testing. Per USP <1223>, laboratories adopting the method should verify method suitability for each product tested. Table 17 provides a summary of the results for the six USP <1223> parameters evaluated in this study; equivalency met the USP <1223> non-inferiority criterion.

Table 17. Summary of USP <1223> Validation Parameters

Validation Parameter	Result Summary	Status
Specificity	No false positives for off-target organisms or uninoculated blanks across all matrices and both instruments.	**Reported**
Limit of Detection	CRM LOD_{50} 0.27 to 0.38 and LOD_{95} 1.16 to 1.63 nominal CFU (CRM dilution series, direct lysis). In-matrix detection 100% at the low level in all six matrices.	**Reported**
Repeatability	Qualitative results agreed across all replicate samples; average IC Cq RSD ≤ 5.00%	**Reported**
Ruggedness	Identical qualitative results on both qPCR instruments for every sample and target (100% concordance).	**Reported**
Robustness	Internal amplification control (HEX) detected in all samples; method tolerates matrix variability across six diverse product types.	**Reported**
Equivalency	Non-inferiority demonstrated ($Z > 1.645$ at $\alpha = 0.05$, $\delta = 0.20$) at all levels; 100% agreement with the compendial method.	**Met**

Method Feedback & Recommendations

The botanical powder initially selected for testing had been autoclaved (121 °C for 15 minutes) to reduce its native bacterial load. When processed using the standard lysis and extraction protocol, the autoclaved material produced invalid results for pathogen-targeting channels (FAM and ROX). Non-autoclaved botanical powder from the same lot performed as expected under the same protocol.

Autoclaving would be expected to kill the native microbial population while leaving behind a high concentration of damaged, cell-free DNA. Although this DNA does not represent viable organisms, it remains available to the qPCR reaction. A large excess of non-target DNA can bind polymerase and primers non-specifically, competing with the target DNA and suppressing amplification. This was considered the most likely cause of the observed results.

To test this hypothesis, the affected autoclaved samples were re-extracted using the optional Grim Reefer free DNA removal step described in the User Guide. All results were valid, with no inhibition observed after this free DNA removal step. Thus, it is worth exploring the inclusion of a Grim Reefer free-DNA removal step for matrices that have been heat- or irradiation-treated and may contain high concentrations of residual DNA.

This extraction process was repeated on a new botanical powder lot that had not been autoclaved, and all samples performed as expected without additional treatment. Results reported in this study for botanical powder are from this repeated run on the new, non-autoclaved material.

Recommendations

- For botanical and other matrices likely to contain high levels of non-viable or cell-free DNA (for example, heat-treated, autoclaved, or irradiated materials), consider including the Grim Reefer free DNA removal step or an equivalent DNA cleanup before lysis.
- Laboratories should verify method performance on such matrices, including detection at low inoculation levels, before routine use.

References

1. USP <1223> Validation of Alternative Microbiological Methods. United States Pharmacopeia.

2. USP <2022> Microbiological Procedures for Absence of Specified Microorganisms — Nutritional and Dietary Supplements. United States Pharmacopeia.

3. USP <62> Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms. United States Pharmacopeia.

4. PathoSEEK® *Salmonella* and *Escherichia coli* Multiplex Detection Assay with MaGiC Lysis Kit: User Guide, v1 (June 2026, Limited Release). Medicinal Genomics.

5. P0103-PRO-001-03. USP <1223> Alternative Method Equivalency Study for Detection of *Salmonella* spp., *Staphylococcus aureus*, and *E. coli* in Dietary Supplements: Proposal. Cambium Analytica.

6. P0211-PRO-001. Change Order (+ Chocolate Matrix), Amendment to P0103-PRO-001-03. Cambium Analytica.

7. ISO 16140-2:2016. Microbiology of the food chain – Method validation – Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method.

8. ISO 16140-3:2021. Microbiology of the food chain: Method validation, Part 3: Protocol for the verification of reference methods and validated alternative methods in a single laboratory.

9. FDA Bacteriological Analytical Manual (BAM). U.S. Food and Drug Administration.

10. AOAC Official Methods of Analysis (2016) Guidelines for Standard Method Performance Requirements Appendix F. AOAC International.

List of Appendices

Appendix A — Raw Data Compiled Workbook & Calculations

Appendix B — Certified Reference Materials (CRM) Certificates of Analysis (COAs)

Revision History

Version	Date	Description
1	02 Oct 2026	Initial Document Creation

2	15 Oct 2026	Revisions made following statistical peer review. Study conclusions are unchanged.
		• Report framing: Language revised throughout to describe the work as an equivalency study rather than a full method validation. “Acceptance Criteria” sections replaced with “Evaluation Approach.” Status for parameters without criteria pre-specified in the study proposal changed from “PASS/Met” to “Reported” (Tables 2 and 17). Equivalency retains the USP <1223> Approach 1 criterion (“Met”).
		• Methods: Low inoculation level defined as 1 to 10 CFU/test portion, not adjusted to produce fractional results (4.1.2); Positivity and internal control criteria clarified (5.5-5.6).
		• Specificity: Negative agreement counts now reported per sample rather than per target channel (Table 3).
		• Limit of Detection: CRM LOD values now reported in nominal CFU from the direct-lysis dilution series; In-matrix estimated LOD_{50} tables removed; low-level in-matrix detection is now reported descriptively.
		  • LOD estimates for E. coli (Bio-Rad CFX Opus) recalculated to reflect positivity calls after curve review: LOD_{50} 0.23 to 0.27, LOD_{95} 0.99 to 1.16 nominal CFU (Table 5).
		  • Summary ranges updated to LOD_{50} 0.27 to 0.38 and LOD_{95} 1.16 to 1.63 nominal CFU.
		• Repeatability: Internal control (HEX) Cq precision is now presented as supporting data rather than against a 10% RSD criterion.
		• Ruggedness and Robustness: Robustness is described as tolerance to matrix variability.
		• Equivalency: Non-inferiority recalculated on independent test portions (n = 48 at the low and intermediate levels) rather than on pooled per-target, per-instrument observations. Results from the two instruments are noted as repeated measurements of the same test portion. Study design noted relative to USP <1223> recommendations.
		  • Conclusion: Revised to state non-inferiority at the inoculation levels tested and the user’s responsibility for verifying method suitability.
		• Editorial: Table numbering corrected. Table titles corrected from “Triplex Assay” to “Duplex Assay.”

3	29 Oct 2026	Revised the robustness statement to specify all matrix-specific adjustments made per the manufacturer's User Guide. The statement previously noted no changes "beyond the pre-qPCR dilutions" and now reads "only the matrix-specific adjustments specified in the manufacturer's User Guide (pre-qPCR lysate dilutions, and a 1:5,000 internal control dilution for Chocolate)." No change to data or conclusions.