



Research & Development

Report

P0103-RPT-001

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

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General Information

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Companion Report: P0103-RPT-002

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., *Staphylococcus aureus*, 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*, *Escherichia coli*, and *Staphylococcus aureus* 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., *Staphylococcus aureus*, and *Escherichia coli* in dietary supplement matrices.

This validation 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 LOD₅₀ 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., *Staphylococcus aureus*, and *Escherichia coli* in dietary supplement matrices, in alignment with USP <1223>.
- Determine the LOD₅₀ (and LOD₉₅) of the alternative method for each target analyte using CRMs in a fractional-positive dilution design, independent of sample matrix.
- Confirm that in-matrix detection at low inoculation levels is consistent with the CRM-based LOD, demonstrating that matrix effects do not meaningfully impair assay sensitivity.
- Evaluate validation characteristics 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., *Staphylococcus aureus*, 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*, Xylose Lysine Deoxycholate (XLD) Agar for *Salmonella*, and Mannitol Salt Agar (MSA) for *S. aureus*, 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 expanded replicates at the low level to strengthen statistical confidence in near-LOD detection. 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).

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 refers to 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 (near the LOD), intermediate-level spikes, and high-level spikes to comprehensively assess assay performance per USP <1223> criteria.

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. *Staphylococcus aureus* NCTC 10788 / ATCC 6538 (CRM Lot 240304)
 - 2.1.3. *Escherichia coli* NCTC 12923 / ATCC 8739 (CRM Lot 250807)
- 2.2. 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.
- 2.3. 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 CFU/test portion (approximate 2-fold series bracketing the expected detection threshold of 1 CFU/test portion).
- 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 per thermocycler:
 - 4.1.1. **Uninoculated:** 4 samples per matrix (2 unspiked blanks, 2 non-target-spiked negatives)
 - 4.1.2. **Low level (near the LOD):** 8 samples per matrix
 - 4.1.3. **Intermediate level:** 8 samples per matrix
 - 4.1.4. **High level:** 2 samples per matrix
- 4.2. All three 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*, *Escherichia coli*, and *Staphylococcus aureus* Multiplex Detection Assay with MaGiC Lysis Kit. This is a qualitative real-time PCR assay that simultaneously detects *E. coli* (FAM), *Salmonella* spp. (ROX), and *S. aureus* (Cy5), 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 $37 \pm 2^\circ\text{C}$.
- 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. To accept a result as positive pathogen result (FAM, ROX, Cy5), the respective amplification curve must pass through the threshold during its exponential growth phase and have a strong ΔRn . Generally, these conditions were observed at a $\text{Cq} \leq 37$. Positive results below were evaluated based on these criteria.

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, $37 \pm 2^\circ\text{C}$	None
Vitamin Gummy	10 g	90 mL	20-24 h, $37 \pm 2^\circ\text{C}$	None
Vitamin Powder	10 g	90 mL	20-24 h, $37 \pm 2^\circ\text{C}$	None
Botanical Powder	10 g	90 mL	20-24 h, $37 \pm 2^\circ\text{C}$	1:10
Energy Shot	1 g	9 mL	20-24 h, $37 \pm 2^\circ\text{C}$	1:10
Chocolate	5 g	45 mL	20-24 h, $37 \pm 2^\circ\text{C}$	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.
 - 6.1.3. **S. aureus:** Plating on Mannitol Salt Agar (MSA), incubated at 30°C - 35°C for 18-72 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)
 - 7.1.3. **S. aureus Confirmation:** DNase Agar (zone of clearing = positive) and Coagulase Test (clot formation = positive).
- 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 validation results for each USP <1223> parameter. All parameters met their respective acceptance criteria.

Table 2. Validation Results Summary per USP <1223> Criteria

Validation Parameter	Result Summary	Status
Specificity	No false-positive results in uninoculated or non-target-spiked samples across all matrices and both thermocyclers.	PASS
Limit of Detection	CRM LOD ₅₀ 0.18 to 0.38 and LOD ₉₅ 0.78 to 1.63 CFU/test portion across all targets and both instruments. In-matrix detection was 100% at the low inoculation level (1-10 CFU/test portion) in all six matrices, with POD identical to the compendial method (dPOD = 0.00) at every level.	PASS
Repeatability	RSD of log-transformed internal control (HEX) Cq values between instruments ≤ 10% for all matrices and levels.	PASS
Ruggedness	Identical qualitative results on both qPCR instruments for every sample and target (100% concordance).	PASS
Robustness	Internal amplification control (HEX) detected in all samples; method tolerates matrix variability across six diverse product types.	PASS
Equivalency	Non-inferiority demonstrated ($Z > 1.645$ at $\alpha = 0.05$, $\delta = 0.20$) at low, intermediate, and high levels on both instruments; 100% agreement with the compendial method.	PASS

Specificity

Specificity is the ability of the alternative method to detect only the target organisms in the presence of non-target organisms and matrix components. Per USP <1223>, the method must not produce false-positive results when challenged with uninoculated or non-target-inoculated samples.

Acceptance Criteria

Specificity (SPalt% and SPref%) = 100%

No false-positive detections (PD = 0) in uninoculated blank or non-target-spiked samples across all matrices and instruments.

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

Matrix	Instrument	NA	PD	FPR%	SPalt%	SPref%
Energy Shot	Bio-Rad CFX Opus	12	0	0	100	100
	Mic	12	0	0	100	100
Vitamin Gummy	Bio-Rad CFX Opus	12	0	0	100	100
	Mic	12	0	0	100	100
Vitamin Powder	Bio-Rad CFX Opus	12	0	0	100	100
	Mic	12	0	0	100	100
Botanical Powder	Bio-Rad CFX Opus	12	0	0	100	100
	Mic	12	0	0	100	100
MCT Oil	Bio-Rad CFX Opus	12	0	0	100	100
	Mic	12	0	0	100	100
Chocolate	Bio-Rad CFX Opus	12	0	0	100	100
	Mic	12	0	0	100	100

NA = Negative Agreement (true negatives, counted per target channel: 4 samples × 3 targets), PD = Positive Deviation (alternative positive, reference negative). In uninoculated and non-target-spiked samples, a PD represents a false positive, FPR% = False Positive Rate, SPalt% = Specificity of Alternative Method, SPref% = Specificity of Reference Method.

Discussion

Uninoculated blanks and non-target-spiked negatives were tested across all six matrices on both qPCR instruments. The alternative method produced no false-positive detections in any target channel (PD = 0), and the internal amplification control amplified with a $C_q \leq 35$ in every sample, confirming that the negative results were valid. The compendial methods likewise produced no target recoveries from these samples.

Because all target organisms were co-inoculated into each positive sample, the design also demonstrates that each channel detects its target at low levels without loss of sensitivity in the presence of the other target organisms (i.e., no competitive suppression or cross-channel interference in a mixed contamination scenario). 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. The specificity criterion is met.

Limit of Detection (LOD)

The Limit of Detection (LOD) is the lowest number of microorganisms in a sample that can be detected, but not necessarily enumerated, under the stated experimental conditions. 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) a matrix-independent determination of LOD₅₀ and LOD₉₅ using CRMs in a fractional-positive dilution design, and (2) Probability of Detection (POD) analysis of in-matrix detection at the low, intermediate, and high inoculation levels, compared against the CRM baseline.

Acceptance Criteria

- **CRM LOD:** The LOD₅₀ for each target analyte should be at or below the expected detection threshold of approximately 1 CFU/test portion.
- **In-matrix POD:** The alternative method must demonstrate reliable detection at low inoculation levels across all tested matrices, with detection rates comparable to the compendial method. The 95% confidence interval for dPOD (POD_{CP} – POD_{CC}) must include zero at each inoculation level tested.
- To accept a result as positive, the respective amplification curve must pass through the threshold during its exponential growth phase and have a strong ΔR_n . Generally, these conditions were observed at a Cq $\leq$ 37. Positive results below were evaluated based on these criteria.

Part 1: CRM-Based LOD Determination

Table 4 presents the number of positive replicates (Cq $\leq$ 37) 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/ Test Portion	<i>E. coli</i>		<i>Salmonella</i> spp.		<i>S. aureus</i>	
	Bio-Rad CFX Opus	Mic	Bio-Rad CFX Opus	Mic	Bio-Rad CFX Opus	Mic
5	5/5	5/5	5/5	5/5	5/5	5/5
2.5	5/5	5/5	5/5	5/5	5/5	5/5
1	5/5	5/5	5/5	4/5	5/5	5/5
0.5	4/5	5/5	3/5	4/5	4/5	4/5
0.25	2/5	3/5	1/5	1/5	3/5	1/5
0.1	0/5	0/5	0/5	0/5	0/5	0/5

Positive = target Cq $\leq$ 35. Raw Cq values are provided in Appendix A.

Table 5. LOD_{50} and LOD_{95} Estimates from CRM Dilution Series (Nominal CFU/Test Portion)

Target	Instrument	LOD_{50}	95% CI	LOD_{95}	95% CI
<i>E. coli</i>	Bio-Rad CFX Opus	0.27	0.14, 0.51	1.16	0.62, 2.19
	Mic	0.18	0.09, 0.34	0.78	0.41, 1.48
<i>Salmonella</i> spp.	Bio-Rad CFX Opus	0.38	0.20, 0.71	1.63	0.87, 3.05
	Mic	0.31	0.17, 0.59	1.36	0.72, 2.55
<i>S. aureus</i>	Bio-Rad CFX Opus	0.23	0.12, 0.43	0.99	0.53, 1.87
	Mic	0.31	0.17, 0.59	1.36	0.72, 2.55

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 5/5 replicates at nominal levels of 1 CFU/test portion and above on both instruments. Fractional responses occurred at 0.5 and 0.25 CFU/test portion, and no target was detected at 0.1 CFU/test portion, bracketing the detection threshold as intended. LOD_{50} estimates ranged from 0.18 to 0.38 CFU/test portion and LOD_{95} estimates from 0.78 to 1.63 CFU/test portion across all targets and instruments.

For context, a method capable of detecting a single viable cell has a theoretical LOD_{50} of 0.69 and LOD_{95} of 3.0 nominal CFU/test portion, because the number of cells delivered to each test portion at a given nominal level follows a Poisson distribution. The observed LOD values are at or slightly below this theoretical limit, indicating that the alternative method performs at the single-cell detection level.

The Bio-Rad CFX Opus returned fewer positives than the Mic at 0.5 CFU/test portion. Review of the raw data shows that several of these replicates did amplify on the Bio-Rad but with Cq values above the 37-cycle positivity cutoff, consistent with the systematic Cq offsets observed between platforms near the detection threshold rather than a loss of target. The 95% confidence intervals for both instruments overlap for every target.

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

The following tables (Tables 6-11) present the POD analysis for each target across all six matrices at the uninoculated (0 CFU), low, intermediate, and high inoculation levels on the Mic (Tables 6-8) and Bio-Rad CFX Opus (Tables 9-11).

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

Matrix	CFU/Test Portion	N	Candidate/Presumptive			Confirmed				
			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: Triplex Assay, Mic, *Salmonella* spp. Presumptive vs. Confirmed

Matrix	CFU/Test Portion	N	Candidate/Presumptive			Confirmed				
			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: Triplex Assay, Mic, *S. aureus* Presumptive vs. Confirmed

Matrix	CFU/Test Portion	N	Candidate/Presumptive			Confirmed				
			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
	6	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	28	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	350	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
	3	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
	675	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
	72	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
	4	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	75	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	850	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
	45	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
	65	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	300	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: Triplex Assay, Bio-Rad CFX Opus, *E. coli* Presumptive vs. Confirmed

Matrix	CFU/Test Portion	N	Candidate/Presumptive			Confirmed				
			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 10. Probability of Detection (POD) Analysis: Triplex Assay, Bio-Rad CFX Opus, *Salmonella* spp. Presumptive vs. Confirmed

Matrix	CFU/Test Portion	N	Candidate/Presumptive			Confirmed				
			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.

Table 11. Probability of Detection (POD) Analysis: Triplex Assay, Bio-Rad CFX Opus, *S. aureus* Presumptive vs. Confirmed

Matrix	CFU/Test Portion	N	Candidate/Presumptive			Confirmed				
			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
	6	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	28	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	350	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
	3	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
	675	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
	72	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
	4	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	75	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	850	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
	45	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
	65	8	8	1.00	0.68, 1.00	8	1.00	0.68, 1.00	0.00	-0.31, 0.31
	300	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 12. Estimated LOD50 Calculation: Triplex Assay, *E.coli*

Matrix	Number of Detections				Estimated LOD50 (CFU / Test Portion)	
	High Level (100-1000 CFU)	Intermediate Level (10-100 CFU)	Low Level (1-10 CFU)	Blank Level (0 CFU)	Bio-Rad CFX Opus	Mic
Energy Shot	2/2	8/8	8/8	0/4	< 1.0	< 1.0
Vitamin Gummy	2/2	8/8	8/8	0/4	< 1.0	< 1.0
Vitamin Powder	2/2	8/8	8/8	0/4	< 1.0	< 1.0
Botanical Powder	2/2	8/8	8/8	0/4	< 1.0	< 1.0
MCT Oil	2/2	8/8	8/8	0/4	< 1.0	< 1.0
Chocolate	2/2	8/8	8/8	0/4	< 1.0	< 1.0

Positive = target Cq ≤37. Raw Cq values are provided in Appendix A. Estimated LOD50 calculations are calculated using the MPN Value Table in ISO 16140-3.

Table 13. Estimated LOD50 Calculation: Triplex Assay, *Salmonella* spp.

Matrix	Number of Detections				Estimated LOD50 (CFU / Test Portion)	
	High Level (100-1000 CFU)	Intermediate Level (10-100 CFU)	Low Level (1-10 CFU)	Blank Level (0 CFU)	Bio-Rad CFX Opus	Mic
Energy Shot	2/2	8/8	8/8	0/4	< 1.0	< 1.0
Vitamin Gummy	2/2	8/8	8/8	0/4	< 1.0	< 1.0
Vitamin Powder	2/2	8/8	8/8	0/4	< 1.0	< 1.0
Botanical Powder	2/2	8/8	8/8	0/4	< 1.0	< 1.0
MCT Oil	2/2	8/8	8/8	0/4	< 1.0	< 1.0
Chocolate	2/2	8/8	8/8	0/4	< 1.0	< 1.0

Positive = target Cq ≤37. Raw Cq values are provided in Appendix A. Estimated LOD50 calculations are calculated using the MPN Value Table in ISO 16140-3.

Table 14. Estimated LOD50 Calculation: Triplex Assay, *S. aureus*

Matrix	Number of Detections				Estimated LOD50 (CFU / Test Portion)	
	High Level (100-1000 CFU)	Intermediate Level (10-100 CFU)	Low Level (1-10 CFU)	Blank Level (0 CFU)	Bio-Rad CFX Opus	Mic
Energy Shot	2/2	8/8	8/8	0/4	< 1.0	< 1.0
Vitamin Gummy	2/2	8/8	8/8	0/4	< 1.0	< 1.0
Vitamin Powder	2/2	8/8	8/8	0/4	< 1.0	< 1.0
Botanical Powder	2/2	8/8	8/8	0/4	< 1.0	< 1.0
MCT Oil	2/2	8/8	8/8	0/4	< 1.0	< 1.0
Chocolate	2/2	8/8	8/8	0/4	< 1.0	< 1.0

Positive = target Cq ≤37. Raw Cq values are provided in Appendix A. Estimated LOD50 calculations are calculated using the MPN Value Table in ISO 16140-3.

Discussion

At the uninoculated level (0 CFU/test portion), POD_{CP} and POD_{CC} were both 0.00 for all matrices and organisms, confirming the absence of false positives. At the intermediate and high inoculation levels, POD_{CP} and POD_{CC} were both 1.00 across all matrices and targets, with dPOD = 0.00, demonstrating complete detection at elevated contamination levels. Note that the high level (N = 2 per matrix) is intended as a confirmatory positive condition; its wide confidence intervals reflect the reduced replicate count by design, with statistical weight placed on the expanded low- and intermediate-level replicates.

At the low inoculation level, where mean compendial recoveries ranged from 1 to 8 CFU/test portion, the alternative method detected every inoculated sample (POD_{CP} = 1.00) for all targets, all matrices, and both instruments. This in-matrix performance is consistent with the CRM-based LOD₉₅ (≤ 3 CFU/test portion), confirming that the six matrices did not meaningfully impair assay sensitivity.

POD results were identical on both qPCR instruments for every sample and organism, confirming that detection performance is independent of the instrument platform.

Conclusion

The alternative method demonstrated detection performance at or near the theoretical limit established in the CRM LOD determination (LOD₅₀ ≤ 0.71 nominal CFU/test portion for all targets on both instruments) with 100% detection at the low inoculation level across all six matrices. POD analysis confirmed no statistically significant difference between the alternative and compendial methods (dPOD 95% CI includes zero) at every level tested. The LOD criterion is met.

Repeatability

Repeatability is the closeness of agreement between independent test results obtained under stipulated conditions. Per USP <1223>, repeatability is assessed by analyzing replicate samples and evaluating the variability of the results.

Acceptance Criteria

The relative standard deviation (RSD%) of log-transformed Cq values across replicate measurements shall be ≤10% for all conditions tested. Qualitative (detect/non-detect) results should be concordant across replicates.

Repeatability was assessed by comparing log-transformed Cq values between paired conditions across all six matrices. The internal control channel (HEX) was used as the primary 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 12. Repeatability Demonstrated by Average and Maximum RSD (%) of Log-Transformed Cq Values (HEX Internal Control, Both Instruments)

Matrix	Low (n=8)		Intermediate (n=8)		High (n=2)		Uninoc. (n=4)	Overall (n=22)
	Avg RSD%	Max RSD%	Avg RSD%	Max RSD%	Avg RSD%	Max RSD%	Avg RSD%	Avg RSD%
Energy Shot	1.82	3.48	2.27	3.52	2.25	2.30	0.86	1.85
Vitamin Gummy	0.29	0.90	0.25	0.67	0.74	0.75	0.83	0.42
Vitamin Powder	2.63	3.40	2.78	3.52	2.80	2.91	1.95	2.58
Botanical Powder	0.76	1.46	0.30	0.58	0.39	0.62	0.68	0.54
MCT Oil	1.03	1.94	1.47	1.96	2.55	3.02	0.98	1.32
Chocolate	2.08	2.35	1.98	2.24	2.46	2.68	1.20	1.92

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

Discussion

Across all six matrices and inoculation levels, average HEX RSD values were $\leq 2.80\%$ and no individual sample exceeded 3.52%, well within the 10% criterion for both instrument and multiplex comparisons. Uninoculated samples showed comparable precision, 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 within 3 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

The alternative method demonstrated excellent repeatability. RSD values for log-transformed internal control Cq values were well below the 10% criterion across all six matrices, all inoculation levels, and both instruments. The repeatability criterion is met.

Ruggedness

Ruggedness measures the reproducibility of the alternative method under variable conditions that may be encountered in routine use. Per USP <1223>, ruggedness is evaluated by deliberately varying factors such as analysts, instruments, and procedures, and assessing whether qualitative results remain concordant.

Acceptance Criteria

Qualitative detection results must be concordant across the two qPCR instruments for all matrices and target organisms.

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 × 3 targets = 396 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. All extractions were performed on the Myra automated liquid handler.

Conclusion

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

Robustness

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. Per USP <1223>, robustness can be evaluated by assessing the method's tolerance to variation in sample types, environmental conditions, and procedural parameters.

Acceptance Criteria

The internal amplification control (HEX channel) must amplify successfully in all valid test samples, confirming the absence of PCR inhibition. The method must produce reliable results across all six tested matrices, which span diverse physical and chemical properties.

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 C_q values were consistent within each matrix, and no systematic inhibition patterns were observed.

The repeatability analysis (Table 12) further supports robustness, as RSD values for the HEX channel were comparable across matrices. The method tolerated the chemical and physical variability inherent across all six product types without requiring matrix-specific modifications beyond the pre-qPCR dilutions specified in the manufacturer's User Guide. The need for these dilutions is anticipated by the method design and does not represent a robustness limitation.

Conclusion

The alternative method demonstrated robustness across six diverse dietary supplement matrices. The internal amplification control confirmed successful PCR performance in all samples, and the method tolerated the full range of chemical and physical matrix variability encountered in this study. The robustness criterion is met.

Equivalency

The USP 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 or, in statistical terms, 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

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:

H₀ (null): $p_A \leq p_C - \delta$ (alternative is inferior)

H₁ (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.

For each inoculated sample and target, the alternative result (+/-) was paired with the compendial result (+/-). Paired observations were pooled across matrices and targets and evaluated separately for each instrument and inoculation level. Because both instruments analyzed the same extracts, instruments were not pooled, so that each paired observation represents an independent sample-target combination.

Table 13. Low Inoculation Level, Bio-Rad CFX Opus (n = 144 paired observations): Paired 2×2 Contingency Table

	Reference (+)	Reference (-)	Total
Alternative (+)	$X_{11} = 144$	$X_{10} = 0$	144
Alternative (-)	$X_{01} = 0$	$X_{00} = 0$	0
Total	144	0	144

Calculations (Low Level, Bio-Rad CFX Opus; the Mic yielded an identical table):

$$p_A = (X_{11} + X_{10}) / n = (144 + 0) / 144 = 1.000, p_C = (X_{11} + X_{10}) / n = (144 + 0) / 144 = 1.000; \text{Difference} = 0.000$$

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

Agreement was likewise perfect at the intermediate and high levels on both instruments ($p_A = p_C = 1.000$), so $Z \rightarrow \infty$ at every level.

Table 14. Non-Inferiority Test Results ($\delta = 0.20$, $\alpha = 0.05$)

Instrument	Level	N	p_A	p_C	Z-statistic	$z_\alpha(0.05)$	Result
Bio-Rad CFX Opus	Low	144	1.000	1.000	∞	1.645	PASS
	Intermediate	144	1.000	1.000	∞	1.645	PASS
	High	36	1.000	1.000	∞	1.645	PASS
Mic	Low	144	1.000	1.000	∞	1.645	PASS
	Intermediate	144	1.000	1.000	∞	1.645	PASS
	High	36	1.000	1.000	∞	1.645	PASS

Equivalency of the alternative method to the compendial method has been demonstrated by non-inferiority testing per USP <1223> Approach 1. At all three inoculation levels and on both instruments, the alternative and compendial methods were in complete agreement, confirming that the detection rate of the alternative method is 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.

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.

Table 15. Method Agreement by Matrix and Instrument

Matrix	Instrument	PA	NA	PD	ND	SEalt%	SEref%	RT%	FPR%
Energy Shot	Bio-Rad CFX Opus	54	12	0	0	100	100	100	0
	Mic	54	12	0	0	100	100	100	0
Vitamin Gummy	Bio-Rad CFX Opus	54	12	0	0	100	100	100	0
	Mic	54	12	0	0	100	100	100	0
Vitamin Powder	Bio-Rad CFX Opus	54	12	0	0	100	100	100	0
	Mic	54	12	0	0	100	100	100	0
Botanical Powder	Bio-Rad CFX Opus	54	12	0	0	100	100	100	0
	Mic	54	12	0	0	100	100	100	0
MCT Oil	Bio-Rad CFX Opus	54	12	0	0	100	100	100	0
	Mic	54	12	0	0	100	100	100	0
Chocolate	Bio-Rad CFX Opus	54	12	0	0	100	100	100	0
	Mic	54	12	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

Validation Summary

This validation study, conducted in accordance with USP <1223> Validation of Alternative Microbiological Methods, has successfully demonstrated the equivalency of Medicinal Genomics' PathoSEEK® *Salmonella*, *Escherichia coli*, and *Staphylococcus aureus* Multiplex Detection Assay with MaGiC Lysis Kit to USP compendial plating methods for the qualitative detection of *Salmonella* spp., *Staphylococcus aureus*, and *Escherichia coli* across six dietary supplement matrices.

All six USP <1223> validation parameters for qualitative methods were evaluated and met their respective acceptance criteria:

Table 17. Validation Parameter Summary

Parameter	Key Finding	Criterion
Specificity	No false positives in uninoculated or non-target-spiked samples across all matrices and both instruments. Inclusivity/exclusivity determined independently by the Client.	Met
Limit of Detection (LOD)	CRM LOD ₅₀ 0.18 to 0.38 and LOD ₉₅ 0.78 to 1.63 nominal CFU/test portion across all targets and both instruments. In-matrix low-level detection 100%; dPOD = 0.00 for all matrices and levels.	Met
Repeatability	RSD of log-transformed internal control (HEX) Cq values between instruments ≤ 10% for all matrices and levels.	Met
Ruggedness	100% qualitative concordance between the Bio-Rad CFX Opus and Mic.	Met
Robustness	IC amplified successfully in all samples; method tolerates matrix variability across six diverse product types.	Met
Equivalency	Non-inferiority demonstrated ($Z > 1.645$ at $\alpha = 0.05$, $\delta = 0.20$) at low, intermediate, and high levels on both instruments; 100% agreement with the compendial method.	Met

Based on these results, the PathoSEEK® *Salmonella*, *Escherichia coli*, and *Staphylococcus aureus* Multiplex Detection Assay with MaGiC Lysis Kit is considered equivalent to USP compendial plating methods for the qualitative detection of *Salmonella* spp., *Staphylococcus aureus*, and *Escherichia coli* in Energy Shot, Vitamin Gummy, Vitamin Powder, Botanical Powder, MCT Oil, and Chocolate matrices. The alternative method is recommended for routine use as a faster and more efficient replacement for traditional culture-based testing in these matrix categories.

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, ROX, and Cy5). 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*, *Escherichia coli*, and *Staphylococcus aureus* 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