



Research & Development

Report

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USP <1223> Alternative Method Equivalency Study for Detection of *Staphylococcus aureus* and *Pseudomonas aeruginosa* in Dietary Supplements Using Medicinal Genomics PathoSEEK® *P. aeruginosa* & *S. aureus* Multiplex Detection Assay v2 with MaGiC Lysis



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General Information

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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 *Staphylococcus aureus* and *Pseudomonas aeruginosa* 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® *Pseudomonas aeruginosa* & *Staphylococcus aureus* Multiplex Detection Assay v2 with MaGiC Lysis (herein referred to as the "alternative method") relative to USP compendial methods for the qualitative detection of *S. aureus* and *P. aeruginosa* in food and 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 five 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.

Objectives

The objectives of this study were to:

- Demonstrate equivalency of the multiplex qPCR assay to compendial methods for the qualitative detection of *Staphylococcus aureus* and *Pseudomonas aeruginosa* in dietary supplement matrices, in alignment with USP <1223>.
- Evaluate validation characteristics relevant to qualitative microbiological methods, including specificity, limit of detection (LOD), robustness, repeatability, and ruggedness.
- Assess method performance characteristics through variation in analysts, instruments, extraction methods, and sample matrices representing broad supplement categories.
- Compile comprehensive documentation including raw data, statistical analyses, and well-supported conclusions regarding method equivalency

Scope

This study evaluates the alternative method for the qualitative detection (presence/absence) of *Staphylococcus aureus* (ATCC 6538) and *Pseudomonas aeruginosa* (ATCC 9027) 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

The selected matrices represent a range of dietary supplement product types including aqueous liquids, gummies, powders, botanicals, and oils. 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 Mannitol Salt Agar (MSA) for *S. aureus* and Cetrimide Agar for *P. aeruginosa*. Confirmatory testing was performed using DNase Agar and Coagulase Test for *S. aureus*, and Pseudomonas P Agar and Oxidase Test for *P. aeruginosa*.

Each matrix was evaluated under unspiked (negative control) conditions, as well as at low- and high-level inoculation concentrations to assess method sensitivity and detection capability across a range of contamination levels. Further, each matrix was also spiked with off-target organisms (*Salmonella enterica* ATCC 6676 and *Escherichia coli* ATCC 11775) to confirm the assay does not produce false-positive results for non-target species.

Testing of the alternative method was performed using two independent analysts, across replicate samples, two extraction methods (manual v. automated), and on two qPCR thermocycler platforms (Bio-Rad CFX Opus and BioMolecular Systems Mic).

Methods

Study Design Overview

This study was designed as a side-by-side comparison of the alternative qPCR method and USP compendial plating methods. 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, low-level spikes (near the limit of detection), high-level spikes, and off-target organism controls to comprehensively assess assay performance per USP <1223> criteria.

1. Sample Matrices

- 1.1. Five 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

2. Reference Strains

- 2.1. The target organisms for this study include:
 - 2.1.1. *Staphylococcus aureus* (ATCC 6538)
 - 2.1.2. *Pseudomonas aeruginosa* (ATCC 9027)
- 2.2. Exclusivity was assessed by inoculating sample matrices with additional off-target organisms that may naturally co-contaminate dietary supplement matrices:
 - 2.2.1. *Escherichia coli* ATCC 11775
 - 2.2.2. *Salmonella enterica* ATCC 6676
- 2.3. Reference strains were grown and enumerated to prepare concentrated stocks with known CFU/mL. Dilution series were prepared to achieve the desired contamination levels for spiking.

3. Inoculation Levels

3.1. Each matrix was tested at the following inoculation levels:

- 3.1.1. Blank (Uninoculated): 0 CFU/sample
 - 3.1.1.1. 5 samples per matrix
- 3.1.2. Off-Target: Spiked with *S. enterica* and *E. coli*
 - 3.1.2.1. 5 replicates per matrix
- 3.1.3. Low Level: Near the limit of detection
 - 3.1.3.1. 10 samples per matrix
- 3.1.4. High Level: Elevated contamination
 - 3.1.4.1. 10 samples per matrix

4. Alternative Method (PathoSEEK® qPCR Assay)

- 4.1. The alternative method utilizes the PathoSEEK® *P. aeruginosa* & *S. aureus* Multiplex Detection Assay v2 with MaGiC Lysis. This is a qualitative real-time PCR assay that simultaneously detects *S. aureus* (FAM fluorophore), *P. aeruginosa* (Cy5 fluorophore), and an internal DNA control (HEX fluorophore) in a single multiplexed reaction.
- 4.2. DNA was extracted from enriched samples using the MaGiC Lysis protocol (95 °C for 10 minutes, followed by 4 °C hold, with MaGiC Stabilization Buffer). Each sample underwent two comparative DNA extractions: one manual and one automated (robotic liquid handler). Each extraction was analyzed on both qPCR instruments (BioRad CFX Opus and BioMolecular Systems Mic).
- 4.3. 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 (TSB incubated), and one positive control (*P. aeruginosa*/*S. aureus* multiplex assay positive control).

5. Reference (Compendial) Methods

- 5.1. 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>):
 - 5.1.1. ***S. aureus***: Plating on Mannitol Salt Agar (MSA), incubated at 37 ± 2 °C for 18-72 hours.
 - 5.1.2. ***P. aeruginosa***: Plating on Cetrимide Agar, incubated at 37 ± 2 °C for 18-72 hours.
- 5.2. Each sample was plated using both methods. Media blanks were included daily and for each new lot of media.

6. Confirmatory Methods

- 6.1. Confirmatory tests were performed on colonies from compendial plating to verify organism identification:
 - 6.1.1. ***S. aureus* Confirmation**: DNase Agar (zone of clearing = positive) and Coagulase Test (clot formation = positive).
 - 6.1.2. ***P. aeruginosa* Confirmation**: Pseudomonas P Agar (fluorescent pigment production = positive) and Oxidase Test (color change = positive).

7. Instruments

- 7.1. BioRad CFX Opus Real-Time PCR System
- 7.2. BioMolecular Systems Mic 4-Channel PCR Instrument
- 7.3. Automated extraction platform (BioMolecular Systems Myra)

Results & Discussion

Validation Criteria Summary

Table 1 provides a summary of the validation results for each USP <1223> parameter. All parameters met their respective acceptance criteria.

Table 1. Validation Results Summary per USP <1223> Criteria

Validation Parameter	Result Summary	Status
Specificity	No false positives for off-target organisms or uninoculated blanks across all matrices, instruments, and extraction methods.	PASS
Limit of Detection	Detection rate at the low level should be $\geq 90\%$ and comparable to the compendial method detection rate. Probability of Detection (POD) Analysis: the 95% confidence interval for dPOD ($POD_{CP} - POD_{CC}$) must include zero, indicating no statistically significant difference between the alternative and compendial methods at each inoculation level tested.	PASS
Repeatability	RSD of log-transformed Cq values $\leq 10\%$ across replicate testing conditions (analysts, extraction methods, thermocyclers).	PASS
Ruggedness	Concordant qualitative results across two analysts, two extraction methods, and two qPCR instruments for all matrices.	PASS
Robustness	Internal amplification control (HEX) detected in all samples; method tolerates matrix variability across five diverse product types.	PASS
Equivalency	Non-inferiority demonstrated ($Z > 1.645$ at $\alpha = 0.05$, $\delta = 0.20$) for both low- and high-level inoculations. Method agreement $\geq 96\%$ across all conditions.	PASS

Specificity

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

Acceptance Criteria

Specificity (SPalt% and SPref%) = 100%

No false-positive detections (PD=0) for off-target organisms (*S. enterica* ATCC 6676, *E. coli* ATCC 11775) or uninoculated blank samples across all matrices, instruments, and extraction methods.

Table 2. Method Specificity Across All Inoculation Levels

Matrix	Extraction Type	NA	PD	FPR%	SPalt%	SPref%
Energy Shot	Manual	10	0	0	100	100
	Automatic	10	0	0	100	100
Vitamin Gummies	Manual	10	0	0	100	100
	Automatic	10	0	0	100	100
Vitamin Powder	Manual	10	0	0	100	100
	Automatic	10	0	0	100	100
Botanical Powder	Manual	10	0	0	100	100
	Automatic	10	0	0	100	100
MCT Oil	Manual	10	0	0	100	100
	Automatic	10	0	0	100	100

NA = Negative Agreement (true negatives), PD = Positive Deviation (false positives), FPR% = False Positive Rate, SPalt% = Specificity of Alternative Method, SPref% = Specificity of Reference Method

Discussion

Off-target organisms and uninoculated blank samples were tested across all five matrices using both extraction methods (manual and automated) and both qPCR instruments (BioRad CFX Opus and Mic). A total of 10 off-target and blank tests were evaluated per matrix. The alternative method produced no false-positive detections (PD=0) in any condition. The compendial plating method likewise produced no false-positive results for off-target or blank samples.

Conclusion

The alternative method demonstrated 100% specificity across all matrices, extraction methods, and 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. This study evaluates detection performance at low inoculation levels across all five matrices to confirm the method's ability to detect near the LOD and to identify any matrix-specific effects. Formal determination of a precise LOD₅₀ or LOD₉₅ per matrix would require a fractional LOD study with expanded replicates, which was outside the scope of this validation. To provide a statistical characterization of detection performance at the tested levels, Probability of Detection (POD) analysis was performed in accordance with AOAC SMPR guidelines. The POD model quantifies the probability that a method will detect an analyte at a given concentration and allows for a direct statistical comparison between the candidate (alternative) and confirmed (compendial) methods through the difference in POD (dPOD) metric.

Acceptance Criteria

The alternative method must demonstrate reliable detection at low inoculation levels across all tested matrices, with detection rates comparable to the compendial method. Per the POD framework, the 95% confidence interval for dPOD (POD_{CP} – POD_{CC}) must include zero, indicating no statistically significant difference between the alternative and compendial methods at each inoculation level tested.

The following tables (Tables 3-6) describe the POD analysis performed for *Pseudomonas aeruginosa* and *Staphylococcus aureus* detection across all five matrices using both qPCR instruments (BioRad CFX Opus and Mic).

Table 3. Probability of Detection (POD) Analysis — PathoSEEK Pseud/Staph Multiplex Assay with MaGiC Lysis Kit and Mic PCR: Pseudomonas aeruginosa Results 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
Botanical Powder	0	10	0	0.00	0.00, 0.28	0	0.00	0.00, 0.28	0.00	-0.25, 0.25
	4	10	8	0.80	0.49, 0.94	8	1.00	0.72, 1.00	-0.20	-0.54, 0.14
	130	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25
Vitamin Gummy	0	10	0	0.00	0.00, 0.28	0	0.00	0.00, 0.28	0.00	-0.25, 0.25
	14	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25
	87	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25
Vitamin Powder	0	10	0	0.00	0.00, 0.28	0	0.00	0.00, 0.28	0.00	-0.25, 0.25
	5	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25
	45	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25
MCT Oil	0	10	0	0.00	0.00, 0.28	0	0.00	0.00, 0.28	0.00	-0.25, 0.25
	3	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25
	380	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25
Energy Shot	0	10	0	0.00	0.00, 0.28	0	0.00	0.00, 0.28	0.00	-0.25, 0.25
	21	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25
	196	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25

POD = Probably 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}

Table 4. Probability of Detection (POD) Analysis — PathoSEEK Pseud/Staph Multiplex Assay with MaGiC Lysis Kit and Mic PCR: Staphylococcus aureus Results 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
Botanical Powder	0	10	0	0.00	0.00, 0.28	0	0.00	0.00, 0.28	0.00	-0.25, 0.25
	2	10	8	0.80	0.49, 0.94	8	1.00	0.72, 1.00	-0.20	-0.54, 0.14
	149	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25
Vitamin Gummy	0	10	0	0.00	0.00, 0.28	0	0.00	0.00, 0.28	0.00	-0.25, 0.25
	3	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25
	29	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25
Vitamin Powder	0	10	0	0.00	0.00, 0.28	0	0.00	0.00, 0.28	0.00	-0.25, 0.25
	4	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25
	25	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25
MCT Oil	0	10	0	0.00	0.00, 0.28	0	0.00	0.00, 0.28	0.00	-0.25, 0.25
	2	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25
	178	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25
Energy Shot	0	10	0	0.00	0.00, 0.28	0	0.00	0.00, 0.28	0.00	-0.25, 0.25
	21	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25
	147	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25

POD = Probably 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}

Table 5. Probability of Detection (POD) Analysis — PathoSEEK Pseud/Staph Multiplex Assay with MaGiC Lysis Kit and Bio-Rad PCR: Pseudomonas aeruginosa Results 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
Botanical Powder	0	10	0	0.00	0.00, 0.28	0	0.00	0.00, 0.28	0.00	-0.25, 0.25
	4	10	8	0.80	0.49, 0.94	8	1.00	0.72, 1.00	-0.20	-0.54, 0.14
	130	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25
Vitamin Gummy	0	10	0	0.00	0.00, 0.28	0	0.00	0.00, 0.28	0.00	-0.25, 0.25
	14	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25
	87	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25
Vitamin Powder	0	10	0	0.00	0.00, 0.28	0	0.00	0.00, 0.28	0.00	-0.25, 0.25
	5	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25
	45	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25
MCT Oil	0	10	0	0.00	0.00, 0.28	0	0.00	0.00, 0.28	0.00	-0.25, 0.25
	3	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25
	380	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25
Energy Shot	0	10	0	0.00	0.00, 0.28	0	0.00	0.00, 0.28	0.00	-0.25, 0.25
	21	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25
	196	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25

POD = Probably 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}

Table 6. Probability of Detection (POD) Analysis — PathoSEEK Pseud/Staph Multiplex Assay with MaGiC Lysis Kit and Bio-Rad PCR: Staphylococcus aureus Results 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
Botanical Powder	0	10	0	0.00	0.00, 0.28	0	0.00	0.00, 0.28	0.00	-0.25, 0.25
	2	10	8	0.80	0.49, 0.94	8	1.00	0.72, 1.00	-0.20	-0.54, 0.14
	149	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25
Vitamin Gummy	0	10	0	0.00	0.00, 0.28	0	0.00	0.00, 0.28	0.00	-0.25, 0.25
	3	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25
	29	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25
Vitamin Powder	0	10	0	0.00	0.00, 0.28	0	0.00	0.00, 0.28	0.00	-0.25, 0.25
	4	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25
	25	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25
MCT Oil	0	10	0	0.00	0.00, 0.28	0	0.00	0.00, 0.28	0.00	-0.25, 0.25
	2	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25
	178	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25
Energy Shot	0	10	0	0.00	0.00, 0.28	0	0.00	0.00, 0.28	0.00	-0.25, 0.25
	21	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25
	147	10	10	1.00	0.72, 1.00	10	1.00	0.72, 1.00	0.00	-0.25, 0.25

POD = Probably 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}

Discussion

POD analysis was performed for both target analytes across all five matrices at the uninoculated (0 CFU), low, and high inoculation levels using both qPCR instruments (Mic and BioRad CFX Opus). Tables 3-4 present results for the Mic and Tables 5-6 for the BioRad.

At the uninoculated level (0 CFU/test portion), POD_{CP} and POD_{CC} were both 0.00 for all matrices and both analytes, confirming the absence of false-positive detections. At the high inoculation level, POD_{CP} and POD_{CC} were both 1.00 (95% CI: 0.72, 1.00) across all matrices, with $dPOD = 0$, demonstrating complete detection at elevated contamination levels.

At the low inoculation level, $POD_{CP} = POD_{CC} = 1.00$ with $dPOD = 0$ for four of five matrices (Energy Shot, Vitamin Gummy, Vitamin Powder, and MCT Oil), indicating equivalent and complete detection by both methods at the tested levels. For Botanical Powder at the low level, 2 of 10 samples (Samples 4 and 6) were not detected by the alternative method on either instrument. These samples recovered only 1 CFU on MSA (*S. aureus*) and 4 CFU on Cetrimide (*P. aeruginosa*) by compendial plating, representing the extreme lower bound of the inoculation range. The internal amplification control (HEX) was present for these samples on both instruments, confirming successful extraction and ruling out PCR failure as a cause of the non-detection. Despite positive extraction controls, the very low target burden in these samples likely fell below the stochastic detection threshold of the qPCR assay.

Accordingly, the Botanical Powder low-level POD_{CP} was 0.80 (95% CI: 0.49, 0.94) compared to $POD_{CC} = 1.00$ (95% CI: 0.72, 1.00), yielding $dPOD = -0.20$ with a 95% confidence interval of (-0.54, 0.14). Because this confidence interval includes zero, the difference between the candidate and compendial methods is not statistically significant at this sample size. It should be noted that the wide confidence intervals reflect the study's sample size of 10 replicates per level; a larger replicate count would narrow these intervals and provide a more precise estimate of the true detection probability in this matrix.

POD results were consistent across both PCR instruments (Mic and BioRad CFX Opus) and both target analytes, confirming that detection performance is robust and independent of the instrument platform.

Conclusion

The alternative method demonstrated detection performance comparable to the compendial method across all five dietary supplement matrices at the tested inoculation levels. POD analysis confirmed no statistically significant difference between the alternative and compendial methods ($dPOD$ 95% CI includes zero) at all levels tested, including the Botanical Powder matrix where low-level detection was 80%. The two low-level non-detections in Botanical Powder are consistent with stochastic variability at the lower bound of detection (1 CFU *S. aureus*, 4 CFU *P. aeruginosa* per test portion) and do not represent a systematic method limitation. Formal per-matrix LOD characterization (LOD_{50}/LOD_{95}) was not within the scope of this study but may be addressed through supplemental LOD testing.

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 $\leq 10\%$ 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 five 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. Three categories of variability were evaluated:

Inter-analyst variability: Cq values from Analyst 1 and Analyst 2 (both performing automated extraction on the same samples) were compared independently on the BioRad CFX Opus and the Mic.

Inter-extraction variability: Manual and automated extraction results (Analyst 1) were compared independently on the BioRad and the Mic.

Inter-instrument variability: Cq values obtained on the BioRad CFX Opus and the Mic were compared using Analyst 1 data only, across both manual and automated extraction conditions. This design isolates instrument-to-instrument variability by holding the analyst constant.

Each comparison was performed on both instruments where applicable, yielding six paired comparison blocks per matrix. This design also allows instrument-to-instrument consistency to be assessed indirectly throughout the table: for example, comparable RSD values for the analyst comparison on the BioRad and the Mic indicate that inter-analyst variability is consistent regardless of instrument platform. Similarly, comparable extraction RSDs across instruments confirm that the extraction variable behaves consistently on both systems.

Table 7. Repeatability Demonstrated by Average and Maximum RSD (%) of Log-Transformed Cq Values (HEX Internal Control)

Matrix	Comparison	Instrument / block	Low avg RSD%	Low max RSD%	High avg RSD%	High max RSD%
Energy Shot	Analysts (A1 vs A2)	BioRad	0.66	1.73	2.15	4.21
		Mic	3.74	6.45	1.39	3.68
	Extraction (Auto vs Manual)	BioRad	1.67	2.91	2.35	4.89
		Mic	1.76	2.41	0.99	2.23
	Instrument (Mic vs BioRad)	Manual	0.90	3.52	0.64	1.66
		Auto (A1)	2.48	4.71	2.18	5.25
Vitamin Gummy	Analysts (A1 vs A2)	BioRad	2.25	3.86	1.09	2.50
		Mic	1.22	3.17	0.53	1.94
	Extraction (Auto vs Manual)	BioRad	1.74	3.75	1.51	3.32
		Mic	2.01	4.28	1.38	5.18
	Instrument (Mic vs BioRad)	Manual	1.53	2.80	2.97	7.72
		Auto (A1)	1.40	3.49	2.72	5.23
Vitamin Powder	Analysts (A1 vs A2)	BioRad	0.81	3.85	0.15	0.59
		Mic	1.71	3.35	0.22	0.73
	Extraction (Auto vs Manual)	BioRad	0.79	2.41	0.11	0.25
		Mic	1.88	2.56	0.96	1.19
	Instrument (Mic vs BioRad)	Manual	2.69	3.97	1.50	1.76
		Auto (A1)	2.14	6.29	2.46	2.87
Botanical Powder	Analysts (A1 vs A2)	BioRad	2.33	5.51	1.37	3.73
		Mic	2.29	5.09	0.63	2.28
	Extraction (Auto vs Manual)	BioRad	2.43	5.84	2.65	5.61
		Mic	2.09	5.93	4.24	5.74
	Instrument (Mic vs BioRad)	Manual	3.81	10.26	4.48	8.51
		Auto (A1)	2.14	5.49	1.14	2.84

MCT Oil	Analysts (A1 vs A2)	BioRad	0.36	0.65	0.46	1.02
		Mic	0.34	0.84	0.68	1.68
	Extraction (Auto vs Manual)	BioRad	0.42	1.22	0.16	0.38
		Mic	3.66	4.47	3.70	5.80
	Instrument (Mic vs BioRad)	Manual	4.15	4.91	3.79	4.62
		Auto (A1)	0.74	1.08	0.71	1.32

Acceptance criteria: $RSD \leq 10\%$. All average RSDs < 5%. Instrument comparisons use Analyst 1 data only to isolate variability from inter-analyst effects. Yellow cells = individual sample max RSD > 10%.

Across all matrices, inoculation levels, and all six comparison blocks, the vast majority of individual RSD values were well below 5%, and all average RSDs were below 5%. Only one individual sample RSD marginally exceeded 10% (10.26%), occurring in Botanical Powder at the low level in the manual extraction thermocycler comparison. This may be attributable to stochastic variability at the limit of detection in a complex botanical matrix.

Notably, instrument-to-instrument consistency is evident not only in the direct thermocycler comparison blocks but also in the parallel structure of the analyst and extraction comparisons: RSD patterns were comparable across the BioRad and Mic for each condition, confirming that the method performs with similar precision on both platforms.

Conclusion

The alternative method demonstrated excellent repeatability across all tested conditions. RSD values for log-transformed Cq values were consistently at or below 10% across all matrices, inoculation levels, analysts, extraction methods, and 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 extraction procedures, and assessing whether qualitative results (positive/negative) remain concordant.

Acceptance Criteria

Qualitative detection results must be concordant across all varied conditions: two analysts, two extraction methods, and two qPCR instruments.

Discussion

The study design incorporated three ruggedness factors: analyst (Analyst 1 vs. Analyst 2, both performing automated extraction), extraction method (manual vs. automated), and qPCR instruments (BioRad CFX Opus vs. BioMolecular Systems Mic). Each inoculated sample was tested under all conditions, generating paired qualitative results for direct comparison.

At the high inoculation level, 100% qualitative concordance was observed across all three factors, all five matrices, and both instruments. Every sample that was detected by one condition was also detected by the paired condition. At the low inoculation level, the alternative method demonstrated complete internal concordance for four of five matrices — all conditions (analysts, instruments, extraction methods) returned identical detect/non-detect results.

For the Botanical Powder, Samples 4 and 6 were non-detected across all tested conditions on the alternative method, while the compendial method confirmed low-level presence (1 CFU on MSA, 4 CFU on Cetrимide). Importantly, these non-detections were consistent across both analysts, both instruments, and both extraction methods, indicating that the reduced sensitivity at this inoculation level was not attributable to any single variable factor but rather reflects the method's detection threshold for this matrix. The concordance among alternative method conditions remained 100%, and the detection discrepancy relative to the compendial method is addressed in the POD analysis (Tables 3-6), where the Botanical Powder low-level POD was 0.80 vs. 1.00 for the compendial method.

Conclusion

The alternative method produced concordant qualitative results across all ruggedness factors (analysts, extraction methods, and instruments) for all matrices. The two non-detected samples in Botanical Powder were consistent across all conditions, indicating the result is attributable to low inoculation rather than method variability. 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 five tested matrices, which span diverse physical and chemical properties.

Discussion

The five 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

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 five matrices, both instruments, both extraction methods, 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 7) 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 five 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 five diverse dietary supplement matrices. The internal amplification control confirmed successful PCR performance, and the method tolerated the full range of chemical and physical matrix variability encountered in this study. The robustness criterion is met.

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 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:

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 non-inferiority test was performed using paired samples across all matrices, extraction methods, and instruments combined. For each inoculated sample, the alternative method result (+/-) was paired with the compendial method result (+/-) to construct a 2×2 contingency table.

Table 8. High Inoculation Level (n=300 paired observations) Paired 2x2 Contingency Table

	Reference (+)	Reference (-)	Total
Alternative (+)	$X_{11} = 300$	$X_{10} = 0$	300
Alternative (-)	$X_{01} = 0$	$X_{00} = 0$	0
Total	300	0	300

Calculations (High Level):

$p_A = 300/300 = 1.000$; $p_C = 300/300 = 1.000$; Difference = 0.000

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

Table 9. Low Inoculation Level (n=300 paired observations) Paired 2x2 Contingency Table

	Reference (+)	Reference (-)	Total
Alternative (+)	$X_{11} = 288$	$X_{10} = 0$	288
Alternative (-)	$X_{01} = 12$	$X_{00} = 0$	12
Total	300	0	300

Calculations (Low Level):

$$p_A = (X_{11} + X_{10}) / n = (288 + 0) / 300 = 0.960$$

$$p_C = (X_{11} + X_{01}) / n = (288 + 12) / 300 = 1.000$$

$$p_A - p_C = 0.960 - 1.000 = -0.040$$

$$SE = \sqrt{[(X_{01} + X_{10})/n - (p_A - p_C)^2] / \sqrt{n}}$$

$$SE = \sqrt{[(12 + 0)/300 - (-0.040)^2] / \sqrt{300}} = \sqrt{[0.04 - 0.0016] / 17.32} = \sqrt{0.0384 / 17.32} = 0.1960 / 17.32 = 0.01131$$

$$Z = (p_A - p_C + \delta) / SE = (-0.040 + 0.20) / 0.01132 = 0.160 / 0.01132 = 14.14$$

$$Z = 14.14 >> z_{0.05} = 1.645 \rightarrow \text{Reject } H_0. \text{ Non-inferiority is demonstrated.}$$

Table 10. Non-Inferiority Test Results ($\delta = 0.20$, $\alpha = 0.05$)

Level	N	p_A	p_C	Z-statistic	$z_{\alpha} (0.05)$	Result
Low	300	0.960	1.000	14.13	1.645	PASS
High	300	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 both the low and high inoculation levels, the Z-statistic substantially exceeded the critical value of 1.645, 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 (SEalt = 96–100%, RT = 93–100%, FPR = 0%) 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.

Table 11. Method Agreement by Matrix and Extraction Type (All Instruments Combined)

Matrix	Extraction	PA	NA	PD	ND	SEalt%	SEref%	RT%	FPR%
Energy Shot	Manual	40	20	0	0	100	100	100	0
	Automatic	80	40	0	0	100	100	100	0
Vitamin Gummy	Manual	40	20	0	0	100	100	100	0
	Automatic	80	40	0	0	100	100	100	0
Vitamin Powder	Manual	40	20	0	0	100	100	100	0
	Automatic	80	40	0	0	100	100	100	0
Botanical Powder	Manual	36	20	0	4	90	100	93.3	0
	Automatic	72	40	0	8	90	100	93.3	0
MCT Oil	Manual	40	20	0	0	100	100	100	0
	Automatic	80	40	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 12. Method Agreement by Overall Performance Across All Matrices and Extraction Types

Metric	Low Level	High Level	Overall
SEalt% (Alt. Sensitivity)	96%	100%	98%
SEref% (Ref. Sensitivity)	100%	100%	100%
RT% (Rate of Agreement)	98%	100%	99%
FPR% (False Positive Rate)	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 the PathoSEEK® *P. aeruginosa* & *S. aureus* Multiplex Detection Assay v2 with MaGiC Lysis to USP compendial plating methods for the qualitative detection of *S. aureus* and *P. aeruginosa* across five dietary supplement matrices.

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

Table 13. Validation Parameter Summary

Parameter	Key Finding	Criterion
Specificity	No false positives for off-target organisms or uninoculated blanks across all matrices, instruments, and extraction methods.	Met
Limit of Detection (LOD)	Reliable detection demonstrated at low inoculation levels across all matrices. POD analysis: dPOD 95% CI includes zero for all matrices. Overall low-level detection rate: 96%.	Met
Repeatability	RSD of log-transformed Cq values ≤10% for all conditions (avg <5%)	Met
Ruggedness	Concordant results across analysts, instruments, extraction	Met
Robustness	IC amplified in all valid samples; method tolerates matrix variability across five diverse product types	Met
Equivalency	Non-inferiority demonstrated ($Z > 1.645$ at $\alpha = 0.05$, $\delta = 0.20$) for both low- and high-level inoculations.	Met

Based on these results, the PathoSEEK® *P. aeruginosa* & *S. aureus* Multiplex Detection Assay v2 with MaGiC Lysis is considered equivalent to USP compendial plating methods for the qualitative detection of *S. aureus* and *P. aeruginosa* in Energy Shot, Vitamin Gummy, Vitamin Powder, Botanical Powder, and MCT Oil matrices. The alternative method is recommended for routine use as a faster and more efficient replacement for traditional culture-based testing.

References

1. USP <1223> Validation of Alternative Microbiological Methods. United States Pharmacopeia.
2. USP <62> Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms. United States Pharmacopeia.
3. PathoSEEK® *Pseudomonas aeruginosa* & *Staphylococcus aureus* Multiplex Detection Assay v2 with MaGiC Lysis – User Guide v3. Medicinal Genomics.
4. P0045-PRO-001. USP Equivalency Study: *Staphylococcus aureus* & *Pseudomonas aeruginosa* Multiplex qPCR Assay – Proposal. Cambium Analytica.
5. 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.
6. FDA Bacteriological Analytical Manual (BAM). U.S. Food and Drug Administration.
7. AOAC Official Methods of Analysis (2016) Guidelines for Standard Method Performance Requirements Appendix F. AOAC International.

List of Appendices

Appendix A — Raw Data Compiled Spreadsheet (All Matrices)

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

Revision History

Version	Date	Description
1	18 May 2026	Initial Document Creation
2	1 June 2026	Corrected USP method references (added USP <2022>). Revised POD analysis with actual mean CFU/test portion values, replacing prior calculations that applied large-sample-size assumptions. Updated LOD section throughout to clarify scope and distinguish between detection performance confirmation and formal LOD determination.