Section IV – Guidance Documents, Chapter III – Laboratory, @.02 Methods

.02 Analytical Method Verification

This guidance is provided to aid laboratories in verifying the performance of an NSSP Approved or Approved Limited Use Method of Analysis, as adopted, within their laboratory before implementation. Method performance verification is an NSSP Laboratory Quality Systems Checklist requirement. Specifically, Item 3.2.1 reflects, "*The laboratory will internally verify new methods to confirm with objective evidence that the intended protocols are demonstrated, and outcomes are fulfilled*." Further, NSSP Checklist Item 3.2.2 states, "*Methodologies do not deviate from the validated/verified method and the laboratory's internal verification remains on file in the laboratory.*" If a laboratory has reason to significantly modify/alter/deviate from a validated approved method, a full method validation is required.

When a laboratory intends to implement an NSSP method for the first time, the lab should contact the FDA Laboratory Evaluation Officer (LEO) Team to discuss the verification process, review their verification plan before implementation, and schedule the applicable Technical Laboratory Evaluation. The verification plan should include:

1. Type of verification and purpose of study.
2. Purpose of test and method description, including Standard Operating Procedure (SOP).
3. Details of study design, including:
    a. Number and type(s) of samples.
    b. Type of quality assurance (QA) and quality controls (QC) that will be used.
    c. Number of replicates, including how many days and how many analysts.
4. Performance characteristics that will be evaluated and the acceptance criteria.
5. Materials, equipment, and any other resources needed.
6. Safety considerations.
7. Expected timeline for completion.

After that, a series of experiments, based on the guidance provided here, should be conducted to verify proper method performance within the laboratory. Upon completion of those experiments, the full data package should be submitted to the FDA LEO Team for review. Once the verification data package has been approved by the LEO Team and the laboratory is conforming or provisionally conforming to the applicable NSSP Laboratory Evaluation Checklist requirements, the laboratory may begin to implement that method to support a State shellfish sanitation program or other interested parties intending to introduce bivalve mollusks into interstate commerce.

Each method verification is matrix specific. If the new laboratory method is utilized to test shellfish, these verification procedures must be repeated for each shellfish matrix category of interest. Shellfish matrix categories can be found in the table included in NSSP Guide for the Control of Molluscan Shellfish, Section IV – Guidance Documents, Chapter III: Laboratory, .03 – Laboratory Method Matrix Extensions. Verification experiments can be completed using a single species from each category of interest or can make use of a mix of species from within each category, as long as the total number of samples meets the criteria described in the guidelines below.

If a planned verification study deviates from these guidelines, the submitter is encouraged to consult with the FDA LEO Team to determine if the alternative procedures are acceptable prior to initiation of the study.

Any deviations from these suggested procedures should be highlighted in the final submission, along with a rationale for why an alternative procedure was used.

Method Verification Performance Characteristics

When verifying a method, the laboratory is required to demonstrate that they can achieve specific performance characteristics. These performance characteristics can vary by method, but may include some or all of the following:.

Accuracy/Trueness - The closeness of agreement between a test result and an accepted reference value. Good accuracy/trueness suggests the appropriateness of the method and the laboratory's performance of it for the intended work.

Recovery – The fraction or percentage of an analyte recovered following sample analysis. The recovery of the target analyte must be consistently good over the range of concentrations of importance to the application to be of benefit in the intended work.

Measurement Uncertainty – The possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. A narrow range of values indicates that the method as implemented by the laboratory produces reliable results.

Linear Range – The range within the working range where the results are proportional to the concentration of the analyte present in the sample.

Limit of Detection – The minimum concentration at which the analyte can be identified.

Limit of Quantitation – The minimum concentration of the analyte that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.

Precision (Repeatability) –The measure of agreement of replicate tests carried out on the same sample in the same laboratory by the same analyst within short intervals of time.

Microbiological Method Verification

Recommended Procedure: For shellfish meat samples, use samples free of the target organism(s). For each shellfish matrix of interest, use a minimum of 12 animals per sample (the quantity of meat and liquor should be sufficient to cover the blender blades, or additional shellfish should be used to ensure homogeneity) and prepare as a homogenate. For seawater or wastewater, locally collected samples autoclave- or filter-sterilized should be used for all aliquots.

1. For Vibrio methods, take a minimum of six (6) aliquots of the water or shellfish homogenate appropriately sized for the work, and spike five (5) of the aliquots with five (5) different concentrations of the target organism(s) spanning the desired working range. For MPN, Membrane Filtration, or ETCP Methods, take a minimum of four (4) aliquots of the water or shellfish homogenate appropriately sized for the work, and spike three (3) of the aliquots with three (3) different concentrations of the target organism spanning the desired working range. Vortex each aliquot after spiking.
2. Do not spike one aliquot, which will serve as the sample blank.

3. Determine the concentration of the target organism(s) used to spike each aliquot with an appropriate enumeration method (e.g., spread plating on the appropriate media in triplicate).
4. Process and analyze each spiked sample aliquot in triplicate, with only a single analysis for the sample blank.
5. Analyze each replicate according to the NSSP method, including the sample blank.
6. Repeat this process with a minimum of three samples collected from different growing areas, the same growing area harvested/collected on different days, or from different process lots (applicable to shellfish only). Target the same spike levels for each sample analyzed.

Microbiological Method Verification Data Handling and Analysis:

- ***For methods with existing SLV,*** utilizing the procedures described in the data handling sections of the appropriate SOPs here: https://www.issc.org/single-lab-validation-laboratory-methods, determine the values and ranges for each of the performance characteristics listed above.
    - Compare the method verification results for each applicable performance characteristic with the values obtained in the original SLV. It should be noted that acceptance criteria are method-specific, and because analytical methods are developed to be fit for purpose, there is no single universal tolerance that applies across all NSSP methods. Verification results do not need to perfectly replicate original SLV values to pass. However, any significant deviations must be statistically evaluated against protocol limits before the method is approved for routine use. The laboratory must demonstrate performance that satisfies established fit-for-purpose limits.
    - Within the Method Verification Report, the laboratory should determine if the method verification results are satisfactory and if the method has been successfully transferred. The FDA LEO Team will review the report and determine if they concur or do not concur with the laboratory's determination.
    - Data associated with those original SLVs can be accessed under the "Reference" column of the Laboratory Methods table located on the ISSC website at: https://www.issc.org/laboratory-method-references.
- ***For quantitative methods without documented SLV data***, utilize the results generated using the NSSP method as well as the results generated in step 3 of the "Recommended Procedure" above ("Confirmatory Test") to create a Bland-Altman Plot as described below.

  1) Create a data table as follows:

    a. X = mean of each pair of measurements (NSSP Method and Confirmatory Test Method)
    b. Y = difference between each pair of measurements

Example:

Sample	X	Y
1	19.9	1.2
2	19.8	1.1
3	23.4	-1.2
4	36.6	-0.3
5	21.1	0.8

2) Create a scatter plot using these X,Y coordinate pairs.
3) Calculate the overall average of all the differences and draw a horizontal line on the plot at this value (Bias Line).
4) Calculate the standard deviation (SD) of all the differences and draw horizontal lines at the Bias Line ± 1.96(SD) (Limits of Agreement).

5) The data points on the Bland-Altman Plot should be tightly clustered around the zero line, the bias line should be close to the zero line, and the separation between the limits of agreement should be small.

Marine Biotoxin Verification

Recommended Procedure: Use samples free of the target analyte(s). For each shellfish matrix of interest, use a minimum of 12 animals per sample (the quantity of meat and liquor should be sufficient to cover the blender blades, or additional shellfish should be used to ensure homogeneity) and prepare as a homogenate.

1. Take a minimum of six (6) aliquots of the homogenate appropriately sized for the work, and spike five (5) of those aliquots with five (5) different concentrations of the target analyte(s) spanning an appropriate range of values, based on total toxicity of all the target analytes in shellfish. Spiking levels should be appropriate for what is described in the associated Biotoxin Management Plan, and generally should include levels at half, at, and above the action level. In other situations, spikes focused on the lower part of the range may be necessary to determine linear range, limit of detection, and limit of quantitation. Vortex each aliquot after spiking.
2. Do not spike the sixth aliquot, as this will be used as the sample blank.
3. For marine biotoxin experiments, determine the concentration of the analytes(s) used to spike each aliquot.
4. Process and analyze each spiked sample aliquot in triplicate, with only a single analysis for the sample blank.
5. Analyze each replicate according to the NSSP method, including the sample blank.
6. Repeat this process with a minimum of three samples collected from different growing areas, the same growing area harvested/collected on different days, or from different process lots. Use the same spike levels for each sample analyzed.
7. For methods that need to be evaluated for matrix effects, matrix-matched standards (MMS) should be prepared by taking six (6) sub-aliquots of the extract of the sample blank and spiking the target analyte(s) into five (5) of the six sub-aliquots with concentrations matching those used in the experiments described above.
    a. Generate a calibration curve using the five (5) MMS for each sample and compare with that made from solvent standards. Calculate the matrix effects: Matrix Effects (%) = (Slope of MMS/Slope of Solvent Standards – 1) × 100. Adjust method parameters to have the three tested samples exhibit the same range of Matrix Effects if necessary. Determine MMS or solvent standard calibration curve is used for determination of toxins in samples based on the results of matrix effects. Test each aliquot including the replicates, along with the remaining sample blank, to determine the concentration of the target analyte(s) of interest. Repeat this process for each sample analyzed as part of this verification.

Marine Biotoxin Method Verification Data Handling and Analysis:

- Utilizing the procedures described in the data handling sections of the appropriate SOPs here: https://www.issc.org/single-lab-validation-laboratory-methods, determine the values and ranges for each of the performance characteristics listed above.
- For methods with existing SLVs, compare the method verification results for each performance characteristic with the values obtained in the original SLV. The acceptance criteria are determined in the SLV and are specific to the analytical method. When the method verification results are within the values determined during the SLV it can be concluded that the method has been successfully transferred.
    - Differences between the performance characteristics of the method verification data and SLV data may be observed. When direct comparison with SLV data is not possible, alternative comparisons may be acceptable instead. As an example, the values included in Reference 4 at the end of this document may be used to determine the acceptability of a method verification.
- Data associated with those original SLVs can be accessed under the "Reference" column of the Laboratory Methods table located on the ISSC website at: https://www.issc.org/laboratory-method-references.
- For methods without documented SLV data, determine the values and ranges for each performance characteristic using the same procedures as above. To evaluate for successful implementation, utilize established norms and refer to published guidelines such as those included in References 4, 5, and 6 at the end of this document. Discuss the acceptance criteria and comparison to reference standards in the laboratory's method verification report.

Method Verification Documentation

Method verification documentation should be submitted to the FDA LEO Team in a report that includes:

1. A written summary of the work performed, including how sample was prepared and analyzed, equipment used, etc.
2. Analytical testing data generated during the verification study, including raw data generated in appendices.
3. Statistical evaluation of verification data, noting any statistical programs used.
4. Determination of if the method verification results are satisfactory, and assessment of successful method transfer.
5. Plan for proficiency testing or other means of demonstrating continued laboratory analyst effectiveness
    a. This plan should describe a proficiency testing program, interlaboratory study, or other means of comparative analysis that the laboratory plans on participating in each year in order to demonstrate continued successful analyst performance of the method.

Visual Aides

Visual Aide 1: Examples of method verification bench sheet.

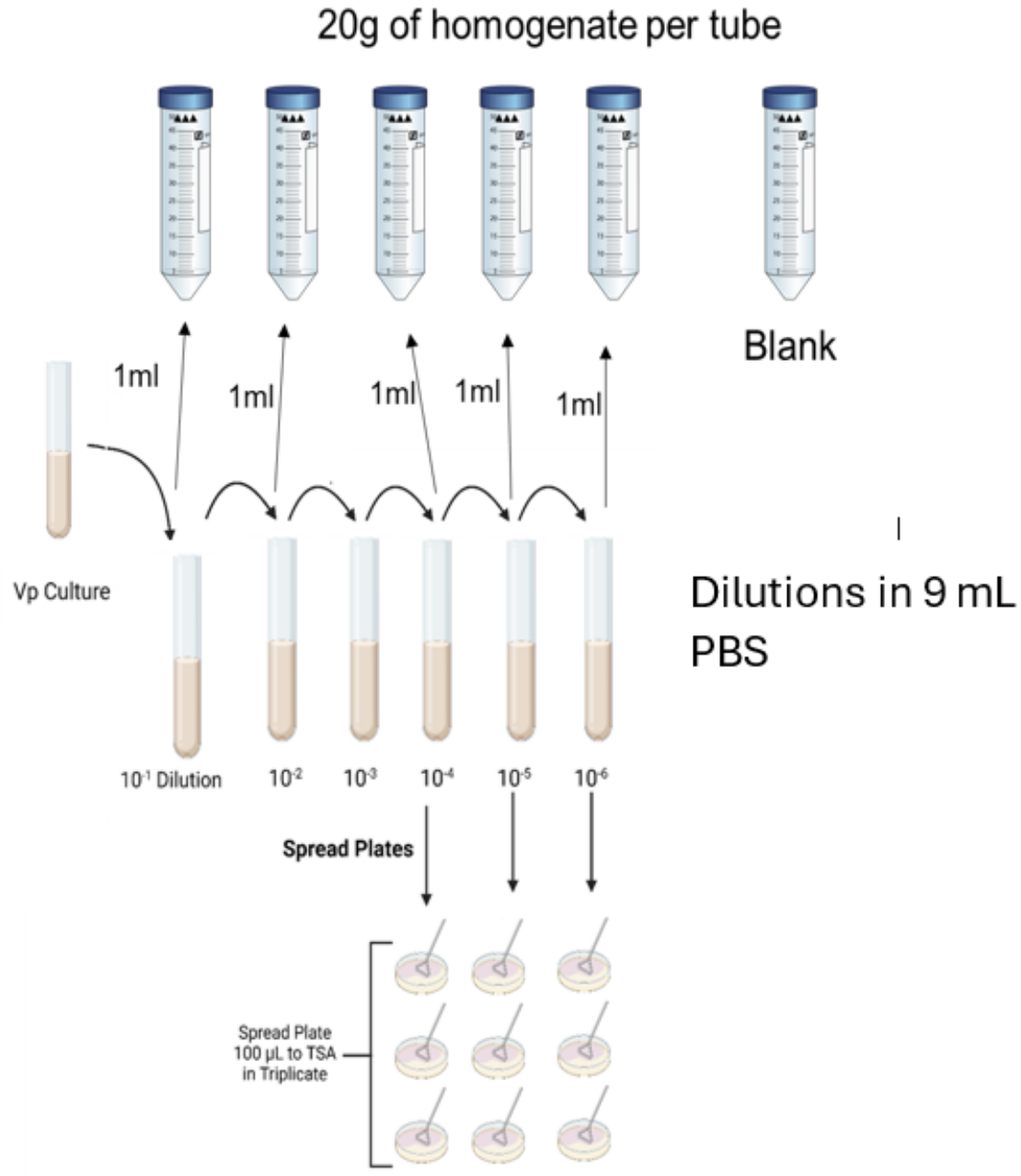
	Spike #1)			Spike #2			Spike #3			Spike #4			Spike #5			
	1A	1B	1C	2A	2B	2C	3A	3B	3C	4A	4B	4C	5A	5B	5C	Blank
Sample 1																
Sample 2																
Sample 3																

		Sample 1	Sample 2	Sample 3	Analyst Initials	Comments
Spike 1	1A					
	1B					
	1C					
Spike 2	2A					
	2B					
	2C					
Spike 3	3A					
	3B					
	3C					
Spike 4	4A					
	4B					
	4C					
Spike 5	5A					
	5B					
	5C					
Blank						

Standard series & matrix	Calibration curve slope	Matrix effects %
Sample 1 MMS		
Solvent standards		
Sample 2 MMS		
Sample 3 MMS		

Visual Aide 2: Suggested method verification set up for MPN real-time PCR for detection of Vibrio.

Suggested Method Verification Set Up for MPN real-time PCR for detection of Vibrio



References

1. The Analytical Laboratory Accreditation Criteria Committee, How to Meet ISO 17025 Requirements for Method Verification, AOAC International, 2007 - https://www.aoac.org/wp-content/uploads/2019/09/ALACC-method-verification.pdf

2. A2LA, G108 – Guidelines for Estimating Uncertainty for Microbiological Counting Methods - https://a2la.qualtraxcloud.com/ShowDocument.aspx?ID=10938

3. United States Food and Drug Administration (FDA), ORA Lab Manual Vol. II – Methods, Method Verification and Validation - https://www.fda.gov/media/73920/download

4. United States Food and Drug Administration, Guidelines for the Validation of Chemical Methods in Food, Feed, Cosmetics, and Veterinary Products, 3rd Edition – Table on Page 23: https://www.fda.gov/media/81810/download

5. European Union Reference Laboratory for Marine Biotoxins, EU-Harmonized Standard Operating Procedure for Determination of Domoic Acid in Shellfish and Finfish by RP-HPLC Using UV Detection, Annex A - https://www.aesan.gob.es/AECOSAN/docs/documentos/laboratorios/LNRBM/archivo4_EU-Harmonised-SOP-ASP-HPLC-UV_Version1.pdf

6. Codex Alimentarius – Recommended Methods of Analysis and Sampling, CXS 234-1999, pages 23-24.