Proposal Subject	Laboratory Method Acceptance into NSSP
Specific NSSP Guide Reference	Constitution By-Laws and Procedures of the ISSC
Text of Proposal/ Requested Action	The Laboratory Methods Review Committee must review laboratory methods that are to be accepted into the ISSC program. The Laboratory Methods Review Committee must follow Procedure XVI found in the Constitution By-Laws and Procedures of the Interstate Shellfish Sanitation Conference. If a public health emergency should arise, the Executive Committee shall approve new laboratory procedures. Such emergency action shall involve consultation with a quorum of the Laboratory Methods Review Committee. The Laboratory Methods Review Committee at the next ISSC Meeting will review new methods, accompanied by their validation and collaborative studies data, which were allowed by the emergency action. Such review can result in the method being accepted as a particular type method or denied acceptance. During this time period, the committee will evaluate and review a laboratory checklist for the method submitted by the method's author(s). Since not all methods listed in the *Bacteriological Analytical Manual* (BAM) are collaboratively tested and approved, methods that appear in the BAM cannot be accepted into the program based solely on the method's inclusion in the BAM. At the time of method submission, the submitter(s) must also include a draft laboratory checklist that is to be used during laboratory evaluation by Laboratory Evaluation Officers.
Public Health Significance	Laboratory methods detecting the direct or indirect presence of human pathogens must be proven to consistently work at various laboratories throughout the country and participating MOU countries. Detailed review of scientific data (preferably from collaborative studies) by the Laboratory Methods Review Committee must be done.
Cost Information (if available)	None
Action by 2005 Task Force III	Recommended referral of Proposal 05-303 to the Executive Board to investigate ISSC approaches to adopting laboratory methods for use in the NSSP.
Action by 2005 General Assembly	Adopted recommendation of 2005 Task Force III.
Action by ISSC Executive Board August 19, 2005	Recommended appointment of a workgroup to determine what the role of the ISSC should be in adoption of laboratory methods. The workgroup is also directed to look at similar conferences' procedures regarding laboratory methods approval. The workgroup will report their findings to the Executive Board at the March 2006 meeting.
Action by USFDA	Concurred with Conference action.
Action by 2006 Laboratory Methods Validation Protocol Workgroup	Developed a protocol of Single Lab Validation for method approval within the NSSP and SOPs for *Vv* and *Vp* PCR Methods.
Action by Executive Board April 2006	Adopted as interim, the procedures developed by the Lab Methods Validation Protocol Workgroup.
Laboratory Methods Review Committee	Recommended adoption of the interim protocol.

Action by 2007 Task Force III — Recommended adoption of Single Lab Protocol which was adopted in 2006 as interim by the Executive Board.

Action by 2007 General Assembly — Adopted recommendation of 2007 Task Force III.

Action by USFDA — December 20, 2007
Concurred with Conference action.

Method Application and Single Lab Validation Checklist For Acceptance of a Method for Use in the NSSP

The purpose of single laboratory validation in the National Shellfish Sanitation Program (NSSP) is to ensure that the analytical method under consideration for adoption by the NSSP is fit for its intended use in the Program. A Checklist has been developed which explores and articulates the need for the method in the NSSP; provides an itemized list of method documentation requirements; and, sets forth the performance characteristics to be tested as part of the overall process of single laboratory validation. For ease in application, the performance characteristics listed under validation criteria on the Checklist have been defined and accompany the Checklist as part of the process of single laboratory validation. Further a generic protocol has been developed that provides the basic framework for integrating the requirements for the single laboratory validation of all analytical methods intended for adoption by the NSSP. Methods submitted to the Interstate Shellfish Sanitation Conference (ISSC) Laboratory Methods Review (LMR) Committee for acceptance will require, at a minimum, six (6) months for review from the date of submission.

Name of the New Method	
Name of the Method Developer	
Developer Contact Information	

Checklist	Y/N	Submitter Comments
A. Need for the New Method		
1. Clearly define the need for which the method has been developed.		
2. What is the intended purpose of the method?		
3. Is there an acknowledged need for this method in the NSSP?		
4. What type of method? i.e. chemical, molecular, culture, etc.		
B. Method Documentation		
1. Method documentation includes the following information:		
Method Title		
Method Scope		
References		
Principle		
Any Proprietary Aspects		
Equipment Required		
Reagents Required		
Sample Collection, Preservation and Storage Requirements		
Safety Requirements		
Clear and Easy to Follow Step-by-Step Procedure		
Quality Control Steps Specific for this		

Method		
C. Validation Criteria		
1. Accuracy / Trueness		
2. Measurement Uncertainty		
3. Precision Characteristics (repeatability and reproducibility)		
4. Recovery		
5. Specificity		
6. Working and Linear Ranges		
7. Limit of Detection		
8. Limit of Quantitation / Sensitivity		
9. Ruggedness		
10. Matrix Effects		
11. Comparability (if intended as a substitute for an established method accepted by the NSSP)		
D. Other Information		
1. Cost of the Method		
2. Special Technical Skills Required to Perform the Method		
3. Special Equipment Required and Associated Cost		
4. Abbreviations and Acronyms Defined		
5. Details of Turn Around Times (time involved to complete the method)		
6. Provide Brief Overview of the Quality Systems Used in the Lab		
Submitters Signature	Date:	
Submission of Validation Data and Draft Method to Committee	Date:	
Reviewing Members	Date:	
Accepted	Date:	
Recommendations for Further Work	Date:	
Comments:		

DEFINITIONS

1. Accuracy/Trueness - Closeness of agreement between a test result and the accepted reference value.
2. Analyte/measurand - The specific organism or chemical substance sought or determined in a sample.
3. Blank - Sample material containing no detectable level of the analyte or measurand of interest that is subjected to the analytical process and monitors contamination during analysis.

4. Comparability – The acceptability of a new or modified method as a substitute for an established method in the NSSP. Comparability must be demonstrated for each substrate or tissue type by season and geographic area if applicable.
5. Fit for purpose – The analytical method is appropriate to the purpose for which the results are likely to be used.
6. HORRAT value – HORRAT values give a measure of the acceptability of the precision characteristics of a method.[4]
7. Limit of Detection – the minimum concentration at which the analyte or measurand can be identified. Limit of detection is matrix and analyte/measurand dependent.[4]
8. Limit of Quantitation/Sensitivity – the minimum concentration of the analyte or measurand that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.
9. Linear Range – the range within the working range where the results are proportional to the concentration of the analyte or measurand present in the sample.
10. Measurement Uncertainty – A single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including: overall precision of the complete method, the method and laboratory bias and matrix effects.
11. Matrix – The component or substrate of a test sample.
12. Method Validation – The process of verifying that a method is fit for purpose.[1]
13. Precision – the closeness of agreement between independent test results obtained under stipulated conditions.[1, 2] There are two components of precision:
    a. 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.
    b. Reproducibility – the measure of agreement between tests carried out in different laboratories. In single laboratory validation studies reproducibility is the closeness of agreement between results obtained with the same method on replicate analytical portions with different analysts or with the same analyst on different days.
14. Quality System - The laboratory's quality system is the process by which the laboratory conducts its activities so as to provide data of known and documented quality with which to demonstrate regulatory compliance and for other decision-making purposes. This system includes a process by which appropriate analytical methods are selected, their capability is evaluated, and their performance is documented. The quality system shall be documented in the laboratory's quality manual.
15. Recovery – The fraction or percentage of an analyte or measurand recovered following sample analysis.
16. Ruggedness – the ability of a particular method to withstand relatively minor changes in analytical technique, reagents, or environmental factors likely to arise in different test environments.[4]
17. Specificity – the ability of a method to measure only what it is intended to measure.[1]
18. Working Range – the range of analyte or measurand concentration over which the method is applied.

REFERENCES:

1. Eurachem Guide, 1998. The Fitness for Purpose of Analytical Methods. A Laboratory Guide to Method Validation and Related Topics. LGC Ltd. Teddington, Middlesex, United Kingdom.
2. IUPAC Technical Report, 2002. Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis, Pure Appl. Chem., Vol. 74, (5): 835-855.
3. Joint FAO/IAEA Expert Consultation, 1999. Guidelines for Single-Laboratory Validation of Anilytical Methods for Trace-Level Concentrations of Organic Chemicals.
4. MAF Food Assurance Authority, 2002. A Guide for the Validation and Approval of New Marine Biotoxin Test Methods. Wellington, New Zealand.
5. National Environmental Laboratory Accreditation. , 2003. Standards. June 5.
6. EPA. 2004. EPA Microbiological Alternate Procedure Test Procedure (ATP) Protocol for Drinking Water, Ambient Water, and Wastewater Monitoring Methods: Guidance. U.S. Environmental Protection Agency (EPA), Office of Water Engineering and Analysis Division, 1200 Pennsylvania Avenue, NW, (4303T), Washington, DC 20460. April.

SINGLE LABORATORY VALIDATION (SLV) PROTOCOL FOR SUBMISSIONTO THE INTERSTATE SHELLFISH SANITATION CONFERENCE (ISSC)

For Method Approval

Critical Information:

Applicants shall attach all procedures, with materials, methods, calibrations and interpretations of data with the request for review and potential approval by the ISSC. The ISSC also recommends that submitters include peer-reviewed articles of the procedure (or similar procedures from which the submitting procedure has been derived) published in technical journals with their submittals. Methods submitted to the ISSC LMR committee for acceptance will require, at a minimum, 6 months for review from the date of submission.

Note:
The applicant should provide all information and data identified above as well as the following material, if applicable:

Justification for New Method

- Name of the New Method.
- Specify the Type of Method (e.g., Chemical, Molecular, or Culture).
- Name of Method Developer.
- Developer Contact Information [e.g., Address and Phone Number(s)].
- Date of Submission.
- Purpose and Intended Use of the Method.
- Need for the New Method in the NSSP, Noting Any Relationships to Existing Methods.
- Method Limitations and Potential Indications of Cases Where the Method May Not Be Applicable to Specific Matrix Types.
- Other Comments.

Method Documentation

- Method Title.
- Method Scope.
- References.
- Principle.
- Analytes/Measurands.
- Proprietary Aspects.
- Equipment.
- Reagents.
- Media.
- Matrix or Matrices of Interest.
- Sample Collection, Preservation, Preparation, Storage, Cleanup, etc.
- Safety Requirements.
- Other Information (Cost of the Method, Special Technical Skills Required to Perform the Method, Special Equipment Required and Associated Cost, Abbreviations and Acronyms Defined and Details of Turn Around Times [Time Involved to Complete the Method]).
- Test Procedures, (Be Specific and Provide Easy-to-Follow Step-by-Step Procedures.).
- Quality Control (Provide Specific Steps.).
- Validation Criteria (Include Accuracy / Trueness, Measurement Uncertainty, Precision [Repeatability and Reproducibility], Recovery, Specificity, Working and Linear Ranges, Limit of Detection, Limit of Quantitation / Sensitivity, Ruggedness, Matrix Effects and Comparability (if intended as a substitute for an established method accepted by the NSSP).
- Data and Statistical Analyses Performed for Each Validation Criterion Tested (Be Specific and Provide Clear Easy-to-Follow Step-by-Step Procedures.).
- Calculations and Formulas Used for Each Validation Criterion Tested.
- Results for Each Validation Criterion Tested.
- Discussion of Each Validation Criterion Tested.
- Summary of Results.

Additional Requirement

If a laboratory method is found acceptable for use in the National Shellfish Sanitation Program and adopted by the Interstate Shellfish Sanitation Conference, the method submitter will draft a laboratory checklist that can be used to evaluate laboratories performing their procedure. The checklist will be submitted to the ISSC and reviewed by the Laboratory Quality Assurance Committee for Conference approval.

(For guidance: refer to the checklists in the National Shellfish Sanitation Program Guide for the Control of Molluscan Shellfish 2003, Guidance Documents, Chapter II – Growing Areas, .11 Evaluation of Laboratories by State Laboratory Evaluation Officers Including Laboratory Evaluation Checklists.)

Single Laboratory Validation (SLV) Protocol
For Submission to the Interstate Shellfish Sanitation Conference (ISSC)
For Method Approval

Critical Information: Applicants shall attach all procedures, with materials, methods, calibrations and interpretations of data with the request for review and potential approval by the ISSC. The ISSC also recommends that submitters include peer-reviewed articles of the procedure (or similar procedures from which the submitting procedure has been derived) published in technical journals with their submittals. Methods submitted to the ISSC LMR committee for acceptance will require, at a minimum, 6 months for review from the date of submission.

Note: The applicant should provide all information and data identified above as well as the following material, if applicable:

Justification for New Method

- Name of the New Method.
- Specify the Type of Method (e.g., Chemical, Molecular, or Culture).
- Name of Method Developer.
- Developer Contact Information [e.g., Address and Phone Number(s)].
- Date of Submission.
- Purpose and Intended Use of the Method.
- Need for the New Method in the NSSP, Noting Any Relationships to Existing Methods.
- Method Limitations and Potential Indications of Cases Where the Method May Not Be Applicable to Specific Matrix Types.
- Other Comments.

Method Documentation

- Method Title.
- Method Scope.
- References.
- Principle.
- Analytes/Measurands.
- Proprietary Aspects.
- Equipment.
- Reagents.
- Media.
- Matrix or Matrices of Interest.
- Sample Collection, Preservation, Preparation, Storage, Cleanup, etc.
- Safety Requirements.
- Other Information (Cost of the Method, Special Technical Skills Required to Perform the Method, Special Equipment Required and Associated Cost, Abbreviations and Acronyms Defined and Details of Turn Around Times [Time Involved to Complete the Method]).
- Test Procedures, (Be Specific and Provide Easy-to-Follow Step-by-Step Procedures.).
- Quality Control (Provide Specific Steps.).
- Validation Criteria (Include Accuracy / Trueness, Measurement Uncertainty, Precision [Repeatability and Reproducibility], Recovery, Specificity, Working and Linear Ranges, Limit of Detection, Limit of Quantitation / Sensitivity, Ruggedness, Matrix Effects and Comparability (if intended as a substitute for an established method accepted by the NSSP).
- Data and Statistical Analyses Performed for Each Validation Criterion Tested (Be Specific and Provide Clear Easy-to-Follow Step-by-Step Procedures.).
- Calculations and Formulas Used for Each Validation Criterion Tested.
- Results for Each Validation Criterion Tested.
- Discussion of Each Validation Criterion Tested.
- Summary of Results.

Additional Requirement

If a laboratory method is found acceptable for use in the National Shellfish Sanitation Program and adopted by the Interstate Shellfish Sanitation Conference, the method submitter will draft a laboratory checklist that can be used to evaluate laboratories performing their procedure. The checklist will be submitted to the ISSC and reviewed by the Laboratory Quality Assurance Committee for Conference approval.

(For guidance: refer to the checklists in the National Shellfish Sanitation Program Guide for the Control of Molluscan Shellfish 2003, Guidance Documents, Chapter II – Growing Areas, .11 Evaluation of Laboratories by State Laboratory Evaluation Officers Including Laboratory Evaluation Checklists.)

Summary Table for Validation Criteria Test and Data Handling

Validation Criteria	Test	Number of Tests	Statistical Test – Data Handling	Recommended Acceptable Criteria (if available)
1. Accuracy/Trueness	1.& 2. **For each shellfish of interest-** Spike with test analyte: Spiked sample Blank samples Range: Low, but detectable to 10^7 Run by QPCR & by plate	1. & 2. 20 samples 2 aliquots per sample: 1 spiked 1 blank Run by both plate and QPCR	1. Log transformation Avg QPCR MPN/Avg plate count X 100	
2. Measurement Uncertainty			2. Log transformation Find difference between plate and corresponding QPCR Find 95% confidence interval for differences	
3. Precision	3. & 4. **For each shellfish of interest:** Ten samples: Spike with three concentrations of analyte (low, medium & high). Analyze by plate method and QPCR	3. & 4. 10 Samples: 4 aliquots per sample Low, medium, high concentration + blank 2 replications of each concentration QPCR, 1 replication at each concentration plate method, 1 blank Run by plate and QPCR	3. Log transformation Nested ANOVA If F $\alpha 0.05$ is not significant, compare standard deviation of total over all concentrations to standard deviation of 3 tube decimal dilution (0.317) with one sided t test $\alpha 0.05$.	3. Calculated F < F $\alpha 0.05$ $S_L < 0.317$
4. Recovery			4. Log transformation Average QPCR replicates Subtract Avg QPCR from associated plate count Perform single ANOVA	4. Calculated F < F $\alpha 0.05$ If significant , use Tukey's HSD to compare recovery by concentration
5. Specificity	5. For each shellfish of interest: Spike with test organism (at low level)& interfering organism at moderate level Run by QPCR	5. 1 samples per interfering organism(io): 3 aliquots 1 spiked w analyte organism 1 spiked w analyte + io 1 blank Run: 5 replicates of spiked samples 1 repilcate of accompanying blank	5. Log transformation Specificity index (SI): SI = Analyte Spike / Analyte io Spike For SI > or < 1: Perform two sided t test	5. $[SI_{avg} - m_{0=1}] > u$

Validation Criteria	Test	Number of Tests	Statistical Test - Data Handling	Recommended Acceptable Criteria (if available)
6. Linear Range	6, 7, & 8. **For each shellfish of interest:** Each sample divide into 10 aliquots Spike with 9 concentrations $10^0, 10^1 \ldots 10^8$ Analyze by plate count and QPCR	6, 7, & 8. 5 Samples: 9 spike concentrations per sample: 2 replicates by QPCR 1 replicate by plate method	6. Plot Critical Threshold(Ct) verses Plate Counts (log transformed) Provide equation for the line Determine if linear relationship exists , compute correlation coefficient (Pearson's r)	6. Test r for significance
7. Limit of Detection			7. If linear, use y=mx+b Where: y = # cycles of PCR amplification Using slope and y-intercept from **6.** above, set y = to total number of PCR cycles and solve for x Take antilog of X	7. X = 1 If different from 1, determine if significantly different at 95% confidence interval.
8. Limit of Quantitation/ Sensitivity			8. If x = 1, LOQ based on 3 tube, 3 dilution ratio of 0.01, 0.001, 0.0001	
9. Ruggedness	9. **For each shellfish of interest:** Two aliquots Spike with analyte -range of concentrations Prepare two separate batches(or lots) of reagents Analyze by QPCR , 1 aliquot use set 1 reagents, second aliquot use set 2 reagents	9. 10 Samples Use set 1 and set 2 prepared reagents for all ten samples Samples should cover range of concentrations. Process samples over a period of days	9. Log transformation Perform two sided t-test at 95% Confidence interval	9. If $\lvert X_{A\,Avg} - X_{B\,Avg} \rvert < u$ Method sufficiently rugged

VALIDATION CRITERIA

1. Accuracy/Trueness is the closeness of agreement between test results and the accepted reference value. To determine method accuracy, the concentration of *Vibrio vulnificus* or *Vibrio parahaemolyticus* as measured by QPCR MPN is compared to a reference concentration.

2. Measurement Uncertainty is a single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including: overall precision of the complete method, the method and laboratory bias and matrix effects.

Procedure: For each shellfish type of interest use a minimum of 10-12 animals per sample. For each sample take two (2) aliquots of homogenate appropriately sized for your work and spike one(1) of the two (2) aliquots with a suitable concentration of either *Vibrio vulnificus* or *Vibrio parahaemolyticus* as appropriate. Do not spike the second aliquot. This is the sample blank. Determine the concentration of *Vibrio vulnificus* or *Vibrio parahaemolyticus* used to spike each sample by plating on appropriate agar. Process both aliquots of sample homogenate as usual to determine the QPCR MPN. Do twenty (20) samples for each shellfish tissue type of interest. **Use a range of concentrations (from low but detectable by QPCR MPN through 10^7) to spike sample homogenates**. Use samples from a variety of growing areas, the same growing area harvested on different days or from different process lots.

Data:

Sample	Plate count (CFU)	Sample blank, QPCR MPN	Spiked sample, QPCR MPN
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
17			
18			
19			
20			

DATA HANDLING

1. Accuracy/Trueness – Data handling

The accuracy/trueness of a method consists of two distinct components, the portion due to the method itself regardless of the laboratory performing it and the portion contributed by the laboratory's performance. In a single laboratory method validation, it is impossible to distinguish the contribution of each to the overall accuracy/trueness of the method. Consequently, what is being estimated is the accuracy/trueness of the method as implemented by the laboratory performing the analysis. Good accuracy/trueness suggests the appropriateness of the method and the laboratory's performance of it for the intended work. Poor accuracy/trueness on the other hand indicates the unsuitability of the method and/or the laboratory's performance of it for the intended work. Accuracy/trueness will be determined by calculating the closeness of agreement between the test results and an accepted reference value obtained by plate count.

Procedure: To determine the accuracy/trueness of the method as implemented by the laboratory over the range in concentrations important in post harvest processing operations or for implementation of the Interim Control Plan, the data is worked-up in the following manner.

1. If necessary use the sample blank to correct the QPCR MPN of the spiked sample for matrix effects.
2. Convert plate counts and QPCR MPNs to logs.
3. Calculate the average plate count of the data in logs.
4. Calculate the average QPCR MPN of the data in logs.
5. Divide the average QPCR MPN in logs by the average plate count in logs.

6. Multiply the quotient by 100. This provides an estimate in percent of the accuracy/trueness of the method as implemented by the laboratory over the range in concentrations of *Vibrio vulnificus* or *Vibrio parahaemolyticus* of importance in post harvest processing operations or for implementation of the Interim Control Plan.

2. Measurement uncertainty – Data handling
Measurement uncertainty can be determined by subtracting the QPCR MPN results for each sample from the accepted reference values for the samples as determined by the accompanying plate count and calculating the 95% confidence interval of these differences. The confidence interval of these differences represents the range in values within which the true measurement uncertainty lies. A narrow range in values indicates that the method as implemented by the laboratory produces reliable results. A wide range in values suggests that the method and/or the laboratory's implementation of it may not be suitable for the intended work.

Procedure: Use the log transformed data for both the plate count and the QPCR MPN results. Calculate the two-sided 95% confidence interval for the difference in log counts between the reference (plate count) and the QPCR MPN method.

1. Let $\alpha = .05$ and $1-\alpha = .95$ %, the confidence interval for the test.
2. Subtract the (corrected if necessary) log QPCR MPN for each sample tested from its accompanying log plate count value.
3. Calculate the average (X_{avg}) of these values. This is the average difference between the reference and test values.
4. Calculate the standard deviation (s) of these values. This is the standard deviation of the difference in counts between the reference and test method.
5. Look up $t = t_{1-\alpha/2}$ for n-1 degrees of freedom (n =19) in the Table of the distribution of *t*.
5. Calculate x_u and x_L:

$$x_u = X_{(avg)} + t(s/n^{0.5})$$
$$x_L = X_{(avg)} - t(s/n^{0.5})$$

6. Take the antilog of x_u and x_L.
7. The interval from x_L to x_u is the 95% confidence interval of the average difference between the reference and test method. This is the measurement uncertainty of the method as implemented.

VALIDATION CRITERIA

3. Precision is the closeness of agreement between independent test results obtained under stipulated conditions.

4. Recovery is the fraction or percentage of an analyte or measurand recovered following sample analysis.

Procedure: For each shellfish type of interest use a minimum of 10-12 animals per sample. For each sample take four (4) aliquots of homogenate appropriately sized for your work. Spike one of the four aliquots with a low (but detectable by QPCR MPN) concentration of either *Vibrio vulnificus* or *Vibrio parahaemolyticus as* appropriate. Spike the second aliquot of homogenate with a medium concentration of either organism as appropriate. Spike the third aliquot of homogenate with a high (>10^6) concentration of either organism as appropriate. Do not spike the fourth aliquot of homogenate. This is the sample blank. Determine the concentration of *Vibrio vulnificus* or *Vibrio parahaemolyticus* used to spike each aliquot by plating on appropriate agar. Process each aliquot including the sample blank as usual to determine the QPCR MPN. Do two (2) replicates for each of the three (3) spiked aliquots. Replicate analysis is unnecessary for the sample blank. Do only one sample blank per sample. Do ten (10) samples for each shellfish tissue type of interest. Use the same spiking levels for each of the ten (10) samples analyzed in this exercise (i.e. 10^1, 10^4 and 10^7). Use samples from a variety of growing areas, the same growing area harvested on different days or from different process lots.

Data:

Sample	Plate count (CFU)	Spiked Sample QPCR, MPN
1L		$1L_a$
		$1L_b$
1M		$1M_a$
		$1M_b$
1H		$1H_a$
		$1H_b$
1B		1B
2L		$2L_a$
		$2\ L_b$
2M		$2M_a$
		$2M_b$
2H		$2H_a$
		$2H_b$
2B		2B

"	"
"	"
"	"
"	"
10L	$10L_a$
	$10L_b$
10M	$10M_a$
	$10M_b$
10H	$10H_a$
	$10H_b$
10B	

L, M and H refer to low, medium and high concentrations respectively. L_a, L_b, M_a, M_b, H_a and H_b refer to the replicate determinations of the sample aliquots spiked with low (L), medium (M) and high (H) concentrations of either *Vibrio vulnificus* or *Vibrio parahaemolyticus*. B refers to the sample blank.

DATA HANDLING

3. Precision – Data handling
The MPN provides the means through which these real time PCR methods become quantitative for application in the NSSP. As an MPN, they are limited in their maximum level of precision to that achievable by the number of tubes and the dilution ratio employed. These Vibrio methods use a 3-tube, decimal dilution MPN and are limited to the maximum precision described by the equation **$0.5487/n^{0.5}(D)$** where n is the number of tubes in each dilution and D is the log of the dilution ratio. In order for these real time PCR methods to be effective in monitoring post harvest processing operations or implementation of the Interim Control Plan, they cannot be significantly more variable than the 3-tube, decimal dilution MPN at their basis over the entire range of concentrations important for either operation.

Procedure: To determine the precision of the method as implemented by the laboratory over the range in concentrations important in post harvest processing operations or implementation of the Interim Control Plan, the data is manipulated in the following manner:

1. Calculate the precision of the 3-tube, decimal dilution MPN from the equation $0.5487/3^{0.5}(1) = 0.317$
2. If necessary use the sample blank to correct the QPCR MPNs of the spiked samples for matrix effects.
3. Convert plate counts and QPCR MPNs for the spiked samples to logs.
4. Perform a nested or hierarchical analysis of variance (ANOVA) with the following variance components:

Source of Variation	Degrees of freedom	Sum of Squares	Mean Square
Samples	9		
Concentrations in samples	20		
Determinations within concentrations	30		
Total	59		

5. Calculate the variance ratio (F) at the 95% confidence interval for the variance components, concentrations in samples/determinations within concentrations. If the variance ratio is significant this indicates that the precision of the method as implemented by the laboratory is not consistent over the range in concentrations important in post harvest processing operations or to implementation of the Interim Control Plan and may be unsuitable for the routine monitoring of such operations.

   If the variance ratio is not significant, compare the standard deviation (Mean Square$^{0.5}$) of the ANOVA variance component, total to the standard deviation for the 3-tube, decimal dilution MPN (0.317) by performing a one-sided t-test at the .05 significance level to determine if the variability of the QPCR MPN exceeds that of the 3-tube, decimal dilution MPN test.

Procedure: To determine if the variability of the QPCR MPN exceeds the variability of the 3-tube, decimal dilution MPN at the .05 significance level, the following procedure is used:

1. Let $\alpha = 0.05$, the significance level of the test.
2. Look up $A_{.05}$ for n -1 degrees of freedom from the Table of factors for calculating one-sided confidence limits for σ. n - 1 = the number of degrees of freedom for the ANOVA variance component, total.
3. Calculate the standard deviation s, s = (Mean Square)$^{0.5}$ of the ANOVA variance component, total.
4. Calculate s_L, $s_L = A_{.05}s$.
5. If $s_L > 0.317$, decide that the variability of the QPCR MPN exceeds the variability of the 3-tube, decimal dilution MPN test and that the method as implemented by the laboratory may be unsuitable for use in monitoring post harvest processing operations or the Interim Control Plan for *Vibrio parahaemolyticus*. If s_L

does not exceed 0.317 this indicates that the variability of the QPCR MPN is consistent with the variability of the MPN test used to make the real time PCR method quantitative and that the method as implemented by the laboratory may be of value in monitoring post harvest processing operations and the Interim Control Plan for *Vibrio parahaemolyticus*.

4. Recovery: Data handling
The recovery of *Vibrio vulnificus* or *Vibrio parahaemolyticus* as appropriate must be consistently good (≥95%) over the range of concentrations of importance in post harvest processing operations or to implementation of the Interim Control Plan to be of benefit in the intended work. To determine whether recovery by the method as implemented by the laboratory recovers consistently over the range in concentrations important in post harvest processing operations or to implementation of the Interim Control Plan, the data is manipulated in the following manner:

1. If necessary, use the sample blank to correct the QPCR MPNs of the spiked samples for matrix effects.
2. Convert plate counts and QPCR MPN data for the spiked samples to logs.
3. For each sample determine the average in logs of the replicate QPCR MPN counts at each concentration such that there is only one log value, the average of the two replicate counts at each concentration.
4. For each sample subtract the average QPCR MPN count in logs from its associated log plate count value at each concentration.
5. Perform a single classification analysis of variance (ANOVA) on the data formatted by sample concentration with the following variance components:

Source of variation	Degrees of freedom	Sum of Squares	Mean Square
Concentration	2		
Error	27		
Total	29		

6. Calculate the variance ratio (F) at the 95% confidence interval for the mean square for concentration divided by the mean square for error. If the variance ratio or F test is significant at the 95% confidence interval, Tukey's Honestly Significant Difference (HSD) may be used to compare recovery by concentration. A significant F test suggests that recovery of the method as implemented by the laboratory is not consistent over the range in concentrations important in post harvest processing operations or to implementation of the Interim Control Plan and may not be suitable for the work intended.

   If the variance ratio or F test is not significant at the 95% confidence interval, conclude that the recovery is consistent over the range in concentrations important in post harvest processing operations or to implementation of the Interim Control Plan and calculate the overall percent recovery of the method as implemented by the laboratory.

Procedure: To determine the percent recovery of the method as implemented by the laboratory, the data is manipulated in the following manner:

1. If necessary use the sample blank to correct the QPCR MPNs of the spiked samples for matrix effects.
2. Convert plate counts and QPCR MPN data for the spiked samples into logs.
3. Calculate the average plate count in logs by summing over concentrations and dividing by 30.
4. Calculate the average OPCR MPN in logs by summing over concentrations and replicates and dividing by 60.
5. Divide the average QPCR MPN in logs from step 4 by the average plate count in logs from step 3 and then multiply by 100. This is the percent recovery of the method as implemented by the laboratory and should be equal to or greater than 95% to be useful in post harvest processing operations or for implementation of the Interim Control Plan.

VALIDATION CRITERIA

5. Specificity is the ability of the method to measure only what it is intended to measure. To determine method specificity samples containing suspected interferences (i.e. interfering organisms) are analyzed in the presence of the analyte/measurand of interest (*Vibrio vulnificus*, *Vibrio parahaemolyticus*, etc).

Procedure: For each shellfish tissue type of interest use a minimum of 10-12 animals per sample. For each sample take three (3) aliquots of homogenate appropriately sized for your work and spike two (2) of the three (3) with a low but determinable level (by QPCR MPN) of *Vibrio vulnicus* or *Vibrio parahaemolyticus* as appropriate. Take one of these two (2) aliquots and spike it with a moderate to high level of a suspected interfering organism. Do not spike the third aliquot. This is the sample blank. Process each aliquot, the blank, the aliquot spiked with either *Vibrio vulnificus* or *Vibrio parahaemolyticus* and the aliquot spiked with *Vibrio vulnicus* or *Vibrio parahaemolyticus* plus the suspected interfering

organism as usual to determine the QPCR MPN. Do five (5) replicates for each aliquot excluding the sample blank. Do one sample blank per analysis. Repeat this process for all suspected interfering organisms.

Data:
Name of suspected interfering organism #1 ________________________________

Sample blank, QPCR MPN _____________

	Aliquot spiked with Vv or Vp, QPCR MPN	Aliquot spiked with Vv or Vp and interfering organism, QPCR MPN
Replicate 1		
2		
3		
4		
5		

Repeat for each suspected interfering organism tested.

DATA HANDLING

5. Specificity – Data handling
The **Specificity index** will be used to test the specificity of the method in the presence of suspected interfering organisms. The Specificity index (SI) is calculated as indicated below.

$$\text{Specificity index (SI)} = \frac{\text{Sample spiked with analyte}}{\text{Sample spiked with analyte and suspected interference}}$$

Samples spiked with analyte and analyte in the presence of suspected interferences may have to be corrected for matrix effects before determining the Specificity index (SI). A sample blank must accompany the analysis and is used for this purpose.

Rationale: The specificity index should equal one (1) in the absence of interferences. To test the significance of a specificity index other than one (1) for any suspected interfering organism, a two-sided t-test is used.

Procedure: For each suspected interfering organism calculate the Specificity index (SI) for each of the 5 replicates analyzed for each sample using the formula below.

$$SI = \frac{\text{Log QPCR MPN of sample spiked with analyte}}{\text{Log QPCR MPN of sample spiked with analyte and suspected interference}}$$

Perform the two-sided t-test to determine if the average specificity index (SI) obtained from the 5 replicates of each analysis differs from one (1).

1. Let the significance level, $\alpha = .05$
2. Look up $t_{1-\alpha/2}$ for n-1 degrees of freedom (n = 4) in the Table of the distribution of *t*.
3. Calculate the average Specificity index (SI_{avg}) from the data for each analysis.
4. Calculate the standard deviation (s).
5. Calculate u, $u = t_{1-\alpha/2}\, s/n^{0.5}$, n = # of replicates per analysis.
6. If $[SI_{avg} - m_o] > u$, where $m_o = 1$ decide that the average Specificity index (SI_{avg}) differs from one (1) and that the method may not be specific for the analyte (i.e. *Vibrio vulnificus* or *Vibrio parahaemolyticus*)

Repeat this analysis for all interfering organisms tested.

Data Summary:
Interfering organism #1_____________ SI_{avg} ______ Significant difference from 1 _____
Interfering organism #2_____________ SI_{avg} ______ Significant difference from 1 _____
Interfering organism #3 _____________ SI_{avg} ______ Significant difference from 1 _____
Interfering organism #n _____________ SI_{avg} ______ Significant difference from 1 _____

VALIDATION CRITERIA

6. Linear Range is the range within the working range where the results are proportional to the concentration of the analyte or measurand present in the sample.

7. Limit of Detection is the minimum concentration at which the analyte or measurand can be identified.

8. Limit of Quantitation/Sensitivity is the minimum concentration of the analyte or measurand that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.

Procedure: For each shellfish type of interest use a minimum of 10-12 animals per sample. For each sample take ten (10) aliquots of homogenate appropriately sized for your work and spike the first aliquot with 10^8 *Vibrio vulnificus* or *Vibrio parahaemolyticus* organisms as appropriate. Spike the second, third, fourth, fifth, sixth, seventh, eighth and ninth aliquots with 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 and 10^0 *Vibrio vulnificus* or *Vibrio parahaemolyticus* organisms as appropriate. Do not spike the tenth aliquot of each sample. This is the sample blank. Determine the concentration of *Vibrio vulnificus* or *Vibrio parahaemolyticus* used to spike each aliquot of each sample by plating on appropriate agar. Process each aliquot including the sample blank for QPCR. Do two (2) replicates for each aliquot including the sample blank. Do five (5) samples for each shellfish tissue type of interest. Use samples from a variety of growing areas, the same growing area harvested on different days or from different process lots.

Data:
Plot the standard curve for the Critical Threshold (Ct) Value for the QPCR of the samples analyzed (on the y-axis) versus the plate count in logs for either *Vibrio vulnificus* or *Vibrio parahaemolyticus* as appropriate (on the x-axis). Provide the equation of the line produced. Tabulate the data in the following manner.

Spike level	0**	10^0	10^1	10^2	10^3	10^4	10^5	10^6	10^7	10^8
Sample 1										
Plate count (cfu)*										
Ct value, replicate 1										
Ct value, replicate 2										
Sample 2										
Plate count (cfu)*										
Ct value, replicate 1										
Ct value, replicate 2										
Sample 3										
Plate count (cfu)*										
Ct value, replicate 1										
Ct value, replicate 2										

Spike level	0**	10^0	10^1	10^2	10^3	10^4	10^5	10^6	10^7	10^8
Sample 4										
Plate count (cfu)*										
Ct value, replicate 1										
Ct value, replicate 2										
Sample 5										
Plate count (cfu)*										
Ct value, replicate 1										
Ct value, replicate 2										

*Plate count converted to logs
**Unspiked sample blank

Linear Range – Data handling
In an MPN the more target bacteria present the greater the number of tubes and dilutions expected to show positives. The more positive tubes in each dilution, the higher the MPN count will be. Thus, a linear relationship must exist between the number of target organisms and the method of detecting their presence. In this case a linear relationship must exist between the number of *Vibrios* and the means of detecting their presence, the number of PCR cycles required for the fluorescent signal to cross the threshold referred to as the critical threshold or Ct value.

Procedure: To determine if a linear relationship exists between the number of *Vibrios* and the critical threshold or Ct value generated by the method as implemented by the laboratory, the data is tabulated for ease in calculation as follows:

X	Y		∑Y	$(\sum Y)^2$	$\sum Y^2$	n_i	n_iX	n_iX^2	∑XY	$(\sum Y)^2/n_i$
	R_1	R_2								
		T_1		T_2	n	T_3	T_4	T_5	T_6	

<u>Legend</u>

X is the number of *Vibrios* from the plate count in logs.

Y is the corresponding Critical Threshold or Ct value for each spike.

R_1 and R_2 are replicate Ct values for each spike.

T_1, T_2, n, T_3, T_4, T_5 and T_6 are column totals.

n_i is the number of replicate Ct values for each spike. In this case n_i is 2.

k = n/2

1. Let α = .05, the level of significance of the test and 1 – α = .95%, the confidence level of the test.
2. Calculate $Y_{avg} = T_1/n$ and $X_{avg} = T_3/n$
3. Calculate $S_1 = T_6 - (T_1)^2/n$.
4. Calculate $b = T_5 - (T_3T_1/n)/T_4 - (T_3)^2/n$.
5. Calculate $S_2 = b[T_5 - (T_3T_1/n)]$.
6. Calculate $S_3 = T_2 - (T_1)^2/n$.
7. Look up $F_{1-\alpha}$ for (k-2, n-k) degrees of freedom in the Table of the *F* distribution.
8. Calculate $F = (S_1\text{-}S_2/S_3\text{-}S_1)\ (n - k/k - 2)$.
9. If $F > F_{1-\alpha}$ decide that the relationship between the number of *Vibrios* and the Ct value is not linear and that the method as implemented may not be appropriate for its intended use.

<u>Limit of Detection</u> – Data handling

In an MPN test, one organism should be capable of producing a positive test. Consequently, one *Vibrio* cell should be the limit of detection of this MPN based real time PCR procedure.

Procedure: Assuming that the relationship between the number of *Vibrios* present and the Ct value is linear, the equation of the line describing this relationship can be used to determine the limit of detection of the method as it is implemented.

$y = mx + b$

where: y is the number of cycles of amplification of the analysis.
m is the slope of the line describing the relationship between the number of *Vibrios* present and the Ct value.
x is the number of *Vibrios* in logs.
b is the y-intercept of the line.

In order to determine the limit of detection of the method as implemented, set y in the above equation equal to the total number of PCR amplification cycles used and solve the equation for x. Take the antilog of x and this value is the limit of detection of the method as implemented. If the limit of detection as implemented is a value other than one (1), it must be determined whether this value is significantly different than one (1). To do this, the 95% confidence interval estimate for the y-intercept, the Critical Threshold (Ct) value must be determined at a density of one (1) *Vibrio* cell. If this confidence interval estimate encompasses the y-intercept derived from the data of the line, then it can be concluded that the limit of detection of the method as implemented is one cell consistent with the MPN requirement that a single cell should be able to produce a positive test. The data is manipulated as indicated in the worksheet below. Use the log values for bacterial counts.

Worksheet

X is the bacterial counts in logs ______ Y is the Critical Threshold (Ct) Value ______

∑X ________ ∑Y ________

X_{avg} ________ Y_{avg} ________

Number of determinations, n = ________

Step 1. ∑XY ________

Step 2. (∑X)(∑Y)/n ________

Step 3. S_{xy} (Step 1 - Step 2) ________

Step 4. $\sum X^2$ ________

Step 5. $(\sum X)^2/n$ ________
Step 6. S_{xx} (Step 4 - Step 5) ________
Step 7. $\sum Y^2$ __________
Step 8. $(\sum Y)^2/n$ __________
Step 9. S_{yy} (Step 7 - Step 8) ________
Step 10 $(S_{xy})^2/S_{xx}$
Step 11 $(n\text{-}2)s^2y$ (Step 9 – Step 10) _______
Step 12. s^2y (Step 11 /n-2) _________
Step 13 s_y (Step $12^{0.5}$) __________

14. Let $_{1-\alpha}$ = the 95% confidence interval. Look up $t_{1-\alpha}$ for n -2 degrees of freedom
15. S_y from Worksheet above ____________
16. Set X¹ to 0, the y-intercept
17. Calculate $W_2 = t_{1-\alpha}s_y[1/n + (X^1 - X_{avg})^2/s_{xx}]^{0.5}$ ____________
18. Calculate $Y_c = Y_{avg} + m(X^1 - X_{avg})$
19. The 95% confidence interval estimate for the Critical Threshold (Ct) Value at a bacterial concentration of one (1) cell is given by $Y_c \pm W_2$. If this confidence interval estimate encompasses the y-intercept (Ct value) derived from the data of the line, it can be concluded that the method as implemented is capable of determining one (1) cell and is consistent with the requirement that one (1) cell should produce a positive test in the MPN procedure.

Limit of Quantitation/Sensitivity – Data handling
If the method as implemented by the laboratory is capable of detecting one (1) cell, then the limit of quantitation/sensitivity is easily calculated. Because the QPCR procedure is MPN based, the bacterial concentration that can be quantified with an acceptable level of precision and accuracy depends on the number of tubes used for each dilution and the dilution ratio employed. For *Vibrio vulnificus* and *Vibrio parahaemolyticus* assuming the method as implemented is capable of a limit of detection of a single cell, use of a 3-tube MPN and a dilution ratio of 0.01, 0.001 and 0.0001 will result in a limit of quantitation/sensitivity of 30 cfu/gram or 3 cfu/0.1gram which is consistent with the action levels for both organisms in post harvest processing operations and to meet the requirements of the Interim Control Plan for *Vibrio parahaemolyticus*.

VALIDATION CRTITERIA

9. Ruggedness is the ability of a particular method to withstand relatively minor changes in analytical technique, reagents or environmental factors likely to arise in different test environments.

Procedure: For each shellfish type of interest use a minimum of 10 – 12 animals. For each sample take two (2) aliquots of homogenate appropriately sized for your work. Spike both aliquots with a suitable concentration of either *Vibrio vulnificus* or *Vibrio parahaemolyticus* as appropriate. Process both aliquots of the sample as usual to determine the QPCR MPN. For the second aliquot of each sample, however, use a different batch or lot of PCR buffer, dNTPs, enhancer solution, the fluorescent dye, primers and DNA polymerase to process this aliquot. Do ten (10) samples for each shellfish tissue type of interest using the same two sets of solutions to process each sample such that "set 1" is used to process "aliquot 1" of each sample and "set 2" is used to process "aliquot 2" of each sample. Use a range of concentrations (from low but detectable by QPCR MPN through 10^8) to spike sample homogenates. Use samples from a variety of growing areas, the same growing area harvested on different days or from different process lots. Process samples over a period of several days if possible.

Data:

Sample	QPCR MPN "Set 1 Reagents"	QPCR MPN "Set 2 Reagents"
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		

DATA HANDLING

9. Ruggedness – Data handling
In the day to day operations of the laboratory there will be changes in the batches/lots of reagents used to process samples by these PCR methods. Environmental factors are also likely to change over time. None of these factors,

however, should adversely impact test results if the method as implemented is to be used routinely as a tool for monitoring post harvest processing operations or the Interim Control Plan for *Vibrio parahaemolyticus*.

Procedure: To determine whether the method as implemented is sufficiently rugged to withstand the types of changes anticipated to occur in routine use, a two-sided t-test at a significance level (α) of .05 will be used to ascertain if the results obtained using different reagent lots/batches under slightly varying environmental conditions are significantly affected by such minor changes.

1. Convert the QPCR MPNs to logs.
2. Let $\alpha = .05$, the significance level of the test.
3. Look up $t_{1-\alpha/2}$ for $(n_A + n_B - 2)$ degrees of freedom in the Table of the *t* distribution.
4. Calculate the average QPCR MPN count (X_{Aavg}) in logs of the samples treated with "Set 1 Reagents."
5. Calculate the standard deviation (s_A) in logs of the samples treated with "Set 1 Reagents." Square it (s^2_A).
6. Calculate the average QPCR MPN count (X_{Bavg}) in logs of the samples treated with "Set 2 Reagents."
7. Calculate the standard deviation (s_B) in logs of the samples treated with "Set 2 Reagents." Square it (s^2_B).
8. Calculate $s_p = [(n_A - 1)s^2_A + (n_B - 1)s^2_B/n_A + n_B - 2]^{0.5}$
9. Calculate $u = t_{1-\alpha/2}s_p[n_A + n_B/n_An_B]^{0.5}$
10. If $| X_{Aavg} - X_{Bavg}| > u$, decide that the method as implemented is not sufficiently rugged to withstand minor changes.
11. If $| X_{Aavg} - X_{Bavg}|$ is not $> u$, conclude that the method as implemented is sufficiently rugged to withstand the minor changes anticipated to occur in routine use.