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 $\mathbf{0.5487/n^{0.5}(D)}$ 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 ($\geq$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.