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**

DATA HANDLING

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

Legend

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 $\alpha = .05$, the level of significance of the test and $1 - \alpha = .95\%$, 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.

7. **<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 _____

$\sum X$ _____ $\sum Y$ _____

X_{avg} _____ Y_{avg} _____

Number of determinations, n = _____

Step 1. $\sum XY$ _____

Step 2. $(\sum X)(\sum Y)/n$ _____

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 $(n-2)s^2y$ (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 X^1 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.

8. **<u>Limit of Quantitation/Sensitivity</u>** – 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*.