VALIDATION CRITERIA

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.