IV. Summary Table for QPCR Methods

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 α0.05 is not significant, compare standard deviation of total over **a**ll concentrations to standard deviation of 3 tube decimal dilution (0.317) with one sided t test α0.05.	**3.** Calculated F < F α0.05 $S_L < 0.317$
4. **Recovery**			**4.** Log transformation Average QPCR replicates Subtract Avg QPCR from associated plate count Perform single ANOVA	**4.** Calculated F < F α0.05 If significant , use Tukey's HSD to compare recovery by concentration

<table>
<tr>
<td>5.
Specificity</td>
<td>5.
For each shellfish of interest:
Spike with test organism (at low level)& interfering organism at moderate level
Run by QPCR</td>
<td>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</td>
<td>5.
Log transformation
Specificity index (SI):

$SI = \frac{\text{Analyte Spike}}{\text{Analyte io Spike}}$

For SI > or < 1:
Perform two sided t test</td>
<td>5.
$[SI_{avg} – m_{0=1}] > u$</td>
</tr>
<tr>
<td>6.
Linear Range</td>
<td rowspan="3">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</td>
<td rowspan="3">6, 7, & 8.
5 Samples:
9 spike concentrations per sample:
2 replicates by QPCR
1 replicate by plate method</td>
<td>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)</td>
<td>Test r for significance</td>
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<tr>
<td>7.
Limit of Detection</td>
<td>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</td>
<td>X = 1
If different from 1, determine if significantly different at 95% confidence interval.</td>
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<tr>
<td>8.
Limit of Quantitation/ Sensitivity</td>
<td>8.
If x = 1, LOQ based on 3 tube, 3 dilution ratio of 0.01, 0.001, 0.0001</td>
<td></td>
</tr>
</table>

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 $\|X_{A\ Avg} - X_{B\ Avg}\| < u$ Method sufficiently rugged