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ISSC Vibrio Method Submission

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Method: *Vibrio parahaemolyticus* enumeration and detection through MPN and real-time PCR

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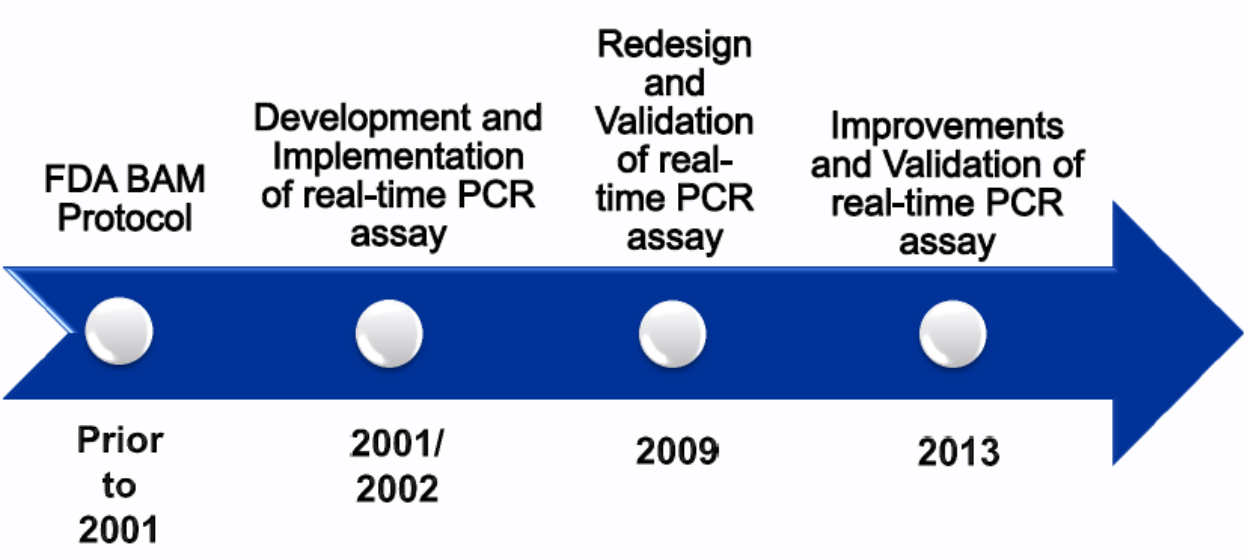
Purpose of method for use in NSSP

The purpose of this method is to provide laboratories supporting the NSSP the ability to rapidly quantify *Vibrio parahaemolyticus* (*Vp*) from oysters using a high throughput real-time PCR protocol.

History of Method



The Food and Shellfish Bacteriology Laboratory (FSBL) at the Washington State Public Health Laboratories (WAPHL) tests on average over 200 oyster samples per year for *Vibrio parahaemolyticus* (*Vp*). Culture based assays for the enumeration of *Vp* take four days or longer and require the Kanagawa test (media based) to detect pathogenicity. Due to the large number of samples and need for accurate and timely results, the FSBL at the WAPHL has tested Pacific oysters (*Crassostrea gigas*) for (*Vp*) using a MPN based real-time PCR assay for over 10 years. The real-time PCR assay utilized by the FSBL at the WAPHL has gone through redesigns and improvements by various scientists at the WAPHL based on new published literature, clinical *Vp* case data, experiences in WA State over the course of a season or seasons, and requests from the Office of Shellfish & Water Protection for enhanced detection of pathogenic *Vp* strains and additional surveillance capabilities.

The real-time PCR assay redesigned and implemented in 2009 and utilized through the 2013 *Vp* monitoring season (June – September) was designed to detect *Vp* using the species-specific thermolabile hemolysin gene (*tlh*) and virulent *Vp* using the thermostable direct hemolysin gene (*tdh*). This assay was designed for high throughput in a 384-well based format. Additionally, the *tlh* and *tdh* targets were redesigned yielding amplicons between 50-150 base pairs. This is optimal for real-time PCR and is known to produce consistent results[1]. Validation of the assay

and concept of a "molecular MPN" was conducted using FERN guidelines and was compared to the FDA BAM method. This assay served as the backbone for which further improvements and redesigns were made in 2013.

Current Assay Design & Improvements

(NOTE: Primer/ probe sequences are available upon request by the committee.)

This MPN based TaqMan probe real-time PCR assay was designed to provide quantitative results for total *Vibrio parahaemolyticus* (*Vp tlh*+), known pathogenic markers of *Vibrio parahaemolyticus* (*Vp tdh* + and *Vp trh*+), as well as some strains of potential pandemic Vibrio (*Vp* ORF8+). The assay is divided into 2 multiplex reactions utilizing FAM, VIC, NED dyes and run simultaneously under the same cycling parameters.

The assay continues using the *Vp* targets (*tlh* and *tdh)* designed for the 2009 assay. Multiple sequence alignments comparing target genes and existing published primers (i.e BAM, Nordstrom, Bej) were used along with ABI guidelines for primers and probes to aid in the design of the final primers and probes[2] [3] [4].

Final Amplicon Size ***tdh*-94bp | *tlh*-69bp**

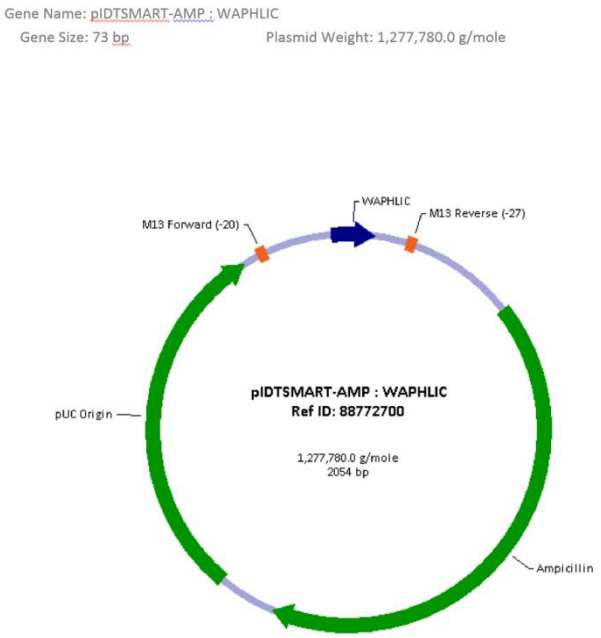
In addition, the current assay has two new *Vp* targets for the detection of the TDH-related hemolysin gene (*trh*) and ORF8 gene (*ORF8*). TRH has been shown to be an important virulence marker and is present in many of our clinical isolates [5] [6]. Due to reported sequence variation in the *trh* gene, multiple sequence alignments comparing *trh* gene sequences were performed as well as the position of existing published primers [7] [8]. Final *trh* primers for the assay consisted of: Degenerate forward primer redesigned based on the ward primer, two reverse primers, and the ward probe.

Strains of *Vibrio alginolyticus* have been described that contain a *trh* gene similar to that of *Vp* [9]. While these strains have not been previously described in WA State, during the course of the 2014 oyster season several strains of *Vibrio alginolyticus* were isolated from tubes that were negative for *tlh* using our assay. These strains were positive for urease and are currently under investigation to determine the presence of the *trh* gene. Please refer to our procedure manual for how we report these results.

ORF8 is present in pandemic *Vp* [10] [11] and although not routinely detected in Washington State, pandemic *Vp* was responsible for 6 illnesses in 2011. To alert public health officials to the potential presence of pandemic *Vp* serovar O3:K6, primers were designed and are included in the assay.

Final Amplicon Size ***trh*-104bp | *ORF8*-150bp**

The new assay also includes an exogenous non-naturally occurring internal control plasmid (WA IC) which is added to the PCR mastermix for the detection of matrix inhibition or other assay failures. The 73bp fragment is synthesized and cloned into a pIDTSMART-AMP plasmid by Intergrated DNA Technologies (IDT). See figure below.



The final addition to the new assay is the *Vibrio vulnificus (Vv)* cytolysin-hemolysin A gene (*vvha*). Published primers and probe are utilized in this assay which yields a 79bp amplicon [12] . *Vibrio vulnificus* is an important addition after the detection of several *Vv* positive samples during the 2013 season. While it is present in the assay, it is currently for investigational use only and the reporting of *vvha* during the 2014 season was for surveillance purposes only. All *Vp* targets were validated and are included in the attached validation data. *Vv* data will be available to share with the committee after validation of the *vvha* gene target scheduled to be completed by the end of 2015.

Current Method Overview

Matrix- Pacific oyster (*Crassostrea gigas*)

- Oyster tissue enriched (18-24 hours) in Alkaline Peptone Water in a 3-tube MPN series

DNA Isolation- Roche© MagnaPure LC using Roche© DNA Isolation Kit III

Instrument Platform- Applied Biosystems™ ViiA™ 7 (384-well format)

Mastermix- Bioline SensiFAST™ Probe Hi-ROX Kit (BIO-82020)

Real-time PCR targets- 2 multiplex reactions run under the same cycling parameters

- Multiplex 1: *tlh*, *vvha,* internal control (IC)
- Multiplex 2: *tdh, trh, orf8*

Control Strains-

- Target organism one (1)- *V. parahaemolyticus* ATCC BAA-240 (contains *tlh, tdh,* and *orf8* genes)
- Target organism two (2)-*V. parahaemolyticus* WA4647 Washington State Department of Health Reference Lab clinical isolate (contains *tlh* and *trh* genes)

Final Background Summary

This method in total has been optimized and developed for environmental conditions, staffing, available instrumentation, and acquired experiences in WA State with *Vp* surveillance. Although this assay has not been published yet, there are plans to publish the method in total upon completion of the validation of the *Vv* portion of the assay which is planned to be completed in 2015. Every effort was made to validate the *Vp* portions of the assay according to ISSC guidelines, due to resource limitations some departures were required. We feel that the validation that was conducted takes into account the methodology and nuances of a molecular MPN. It is our hope that the committee finds this body of work acceptable and that we can work together to ensure adoption of the assay as an approved method for states who would like to use a molecular method for Vibrio surveillance in oysters. Due to the number of targets in this assay the data generated is substantial. We have summarized the data and organized it for review. All of the raw data files and primer/probe/internal control sequences are available to the committee upon request if needed. If the committee requests this information it will be submitted on CDs due to the size of the files. Many people have worked or have been a part of the development of this method in total and we are grateful for the opportunity to share this culmination of work with the ISSC.

References

[1] TaqMan® Universal PCR Master Mix Protocol

[2] Kaysner, C. A., and A. DePaola. "Bacteriological analytical manual chapter 9: Vibrio." 2009-06-06]. http://www. fda. gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalytical ManualBA M/ucm070830. htm (2004).

[3] Nordstrom, Jessica L., et al. "Development of a multiplex real-time PCR assay with an internal amplification control for the detection of total and pathogenic Vibrio parahaemolyticus bacteria in oysters." Applied and Environmental Microbiology 73.18 (2007): 5840-5847.

[4] Bej, Asim K., et al. "Detection of total and hemolysin-producing Vibrio parahaemolyticus in shellfish using multiplex PCR amplification of tl, tdh and trh." Journal of Microbiological Methods 36.3 (1999): 215-225.

[5] Yeung, PS Marie, and Kathryn J. Boor. "Epidemiology, pathogenesis, and prevention of foodborne Vibrio parahaemolyticus infections." *Foodborne Pathogens & Disease* 1.2 (2004): 74-88.

[6] IIDA, TETSUYA, et al. "Evidence for genetic linkage between the ure and trh genes in Vibrio parahaemolyticus." *Journal of medical microbiology* 46.8 (1997): 639-645.

[7] Ward, Linda N., and Asim K. Bej. "Detection of Vibrio parahaemolyticus in shellfish by use of multiplexed real-time PCR with TaqMan fluorescent probes." *Applied and environmental microbiology* 72.3 (2006): 2031-2042.

[8] Kishishita, Masamichi, et al. "Sequence variation in the thermostable direct hemolysin-related hemolysin (trh) gene of Vibrio parahaemolyticus." *Applied and environmental microbiology* 58.8 (1992): 2449-2457.

[9] González-Escalona, Narjol, George M. Blackstone, and Angelo DePaola. "Characterization of a Vibrio alginolyticus strain, isolated from Alaskan oysters, carrying a hemolysin gene similar to the thermostable direct hemolysin-related hemolysin gene (trh) of Vibrio parahaemolyticus." Applied and environmental microbiology 72.12 (2006): 7925-7929.

[10] Myers, Michael L., Gitika Panicker, and Asim K. Bej. "PCR detection of a newly emerged pandemic Vibrio parahaemolyticus O3: K6 pathogen in pure cultures and seeded waters from the Gulf of Mexico." *Applied and environmental microbiology* 69.4 (2003): 2194-2200.

[11] Chen, Yuansha, et al. "Comparative genomic analysis of Vibrio parahaemolyticus: serotype conversion and virulence." *BMC genomics* 12.1 (2011): 294.

[12] Park, Jie Yeun, et al. "Multiplex Real-time Polymerase Chain Reaction Assays for Simultaneous Detection of< i> Vibrio cholerae, Vibrio parahaemolyticus, and Vibrio vulnificus." *Osong Public Health and Research Perspectives* 4.3 (2013): 133-139.

2014 Validation Data

Linear Range, Limit of Detection, Sensitivity

Purpose

To determine the range where the results are proportional to the concentration of the analyte present in the sample. To identify the minimum concentration at which the analyte can be identified and determine at what concentration detection occurs with an acceptable level of precision and accuracy.

Method

In order to determine the Limit of Detection and Linear Range of the assay, 5 oyster matrices were spiked with *Vp ATCC BAA-240* and *Vp WA4647*. In addition, the dilution series for each target without oyster tissue was also tested.

For each matrix 18 APW tubes were seeded with 1g of oyster tissue. The tubes were enriched overnight at 35°C for 18-24 hours. A dilution series of the 16-20 hour Vibrio enrichment was made in APW, the enriched APW tubes were then spiked with each dilution of target organism. The spiking level for *Vp ATCC BAA-240* ranged from 3.55×10^{-1} to 3.55×10^{7} CFU/mL and for *Vp WA4647* it ranged from 7.64×10^{-1} to 7.64×10^{7} CFU/mL. The spiked APW tubes and dilutions were then immediately lysed. DNA extraction was performed and then PCR was run in duplicate. Instrument detection limit was determined without enrichment of the spiked APW tubes. The method detection limit was determined using APW tubes enriched after spiking for 18-24 hours.

Results

Vp ATCC BAA-240 was used to determine the Linear Range and Limit of Detection (LOD) for *tlh*, *tdh*, and *ORF8. WA4647* was used to determine the Linear Range and LOD for *trh.* The Internal Control (IC) Ct averaged around 25 cycles and was not affected by low or high *Vp* spiking levels.

Instrument detection limit

The instrument detection limit for *tlh* and *tdh* is 100% at 3500 CFU/mL, 30% at 3.5 CFU/mL and 0% at .35 CFU/mL. *ORF8* has a detection limit of 90% at 3500 CFU/mL, 10% at 3.5 CFU/mL, and 0% at .35 CFU/mL. The instrument detection limit for *trh* is 100% at 7600 CFU/mL, 40% at 76 CFU/mL, and falls to 0% at 7.6 CFU/mL.

Method detection limit

The method detection limit for *tlh, tdh,* and *ORF8* is 100% at 5.8 cfu/g and falls to 33% at .58 cfu/g. The method detection limit for *trh* is 100% at .85 cfu/g and falls to 0% at .085 cfu/g.

Efficiency

See figures below

tlh = slope -3.09 = 111%

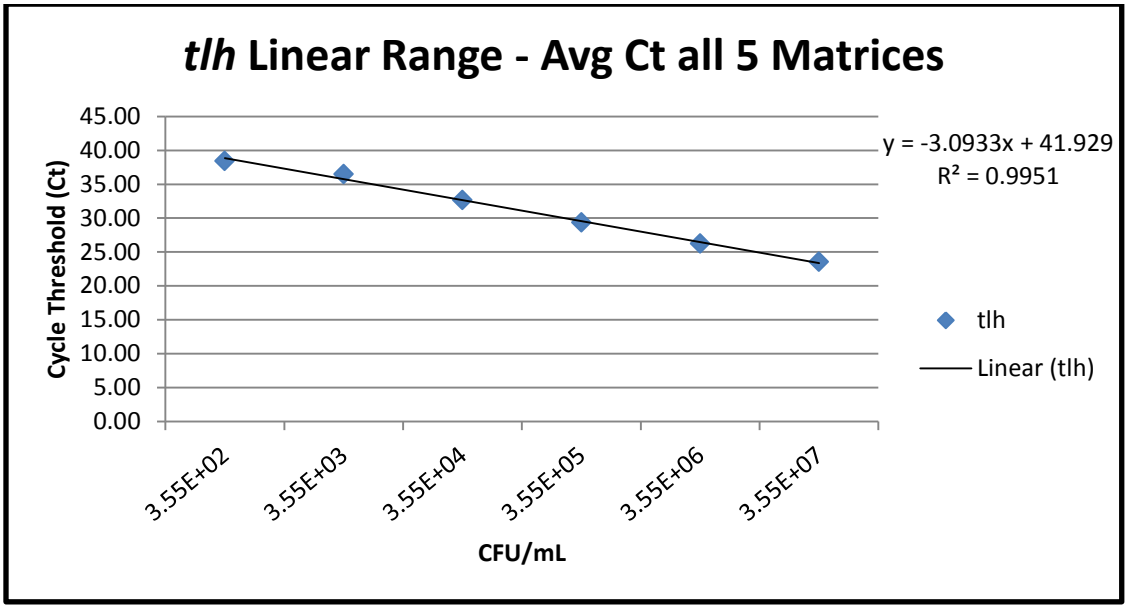
tdh = slope -3.23 = 104%

trh = slope -3.28 = 102%

ORF8 = slope -3.17 = 107%

Figure 1. Linear range graphs of (A) *tlh,(B) tdh, (C) trh,* and (D) *ORF8* along with slope equations used to calculate PCR efficiency and R^2 values. The data points were calculated by averaging all Ct values across all five spiked matrices.

A



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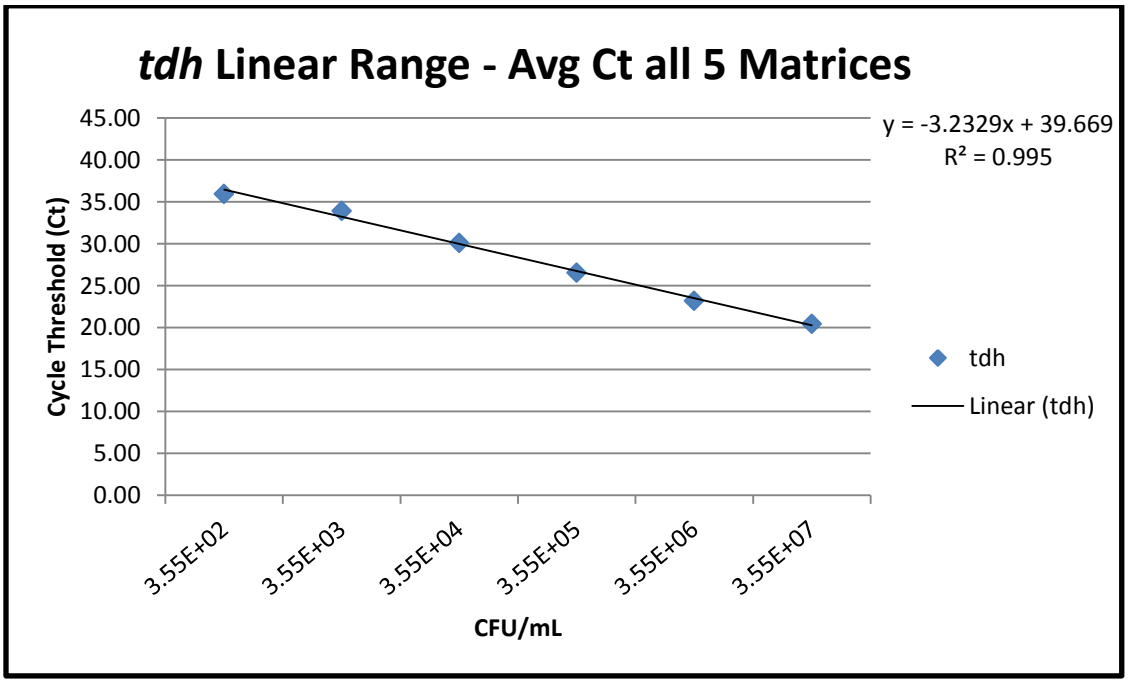

Figure 1. Continued

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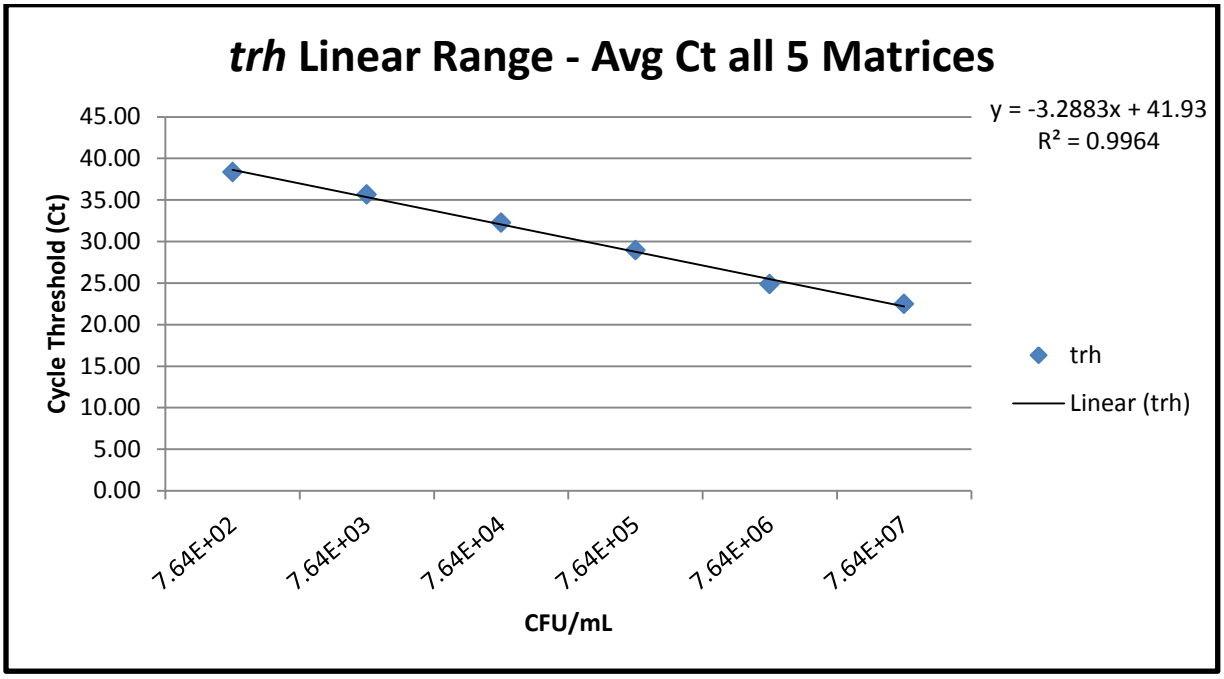

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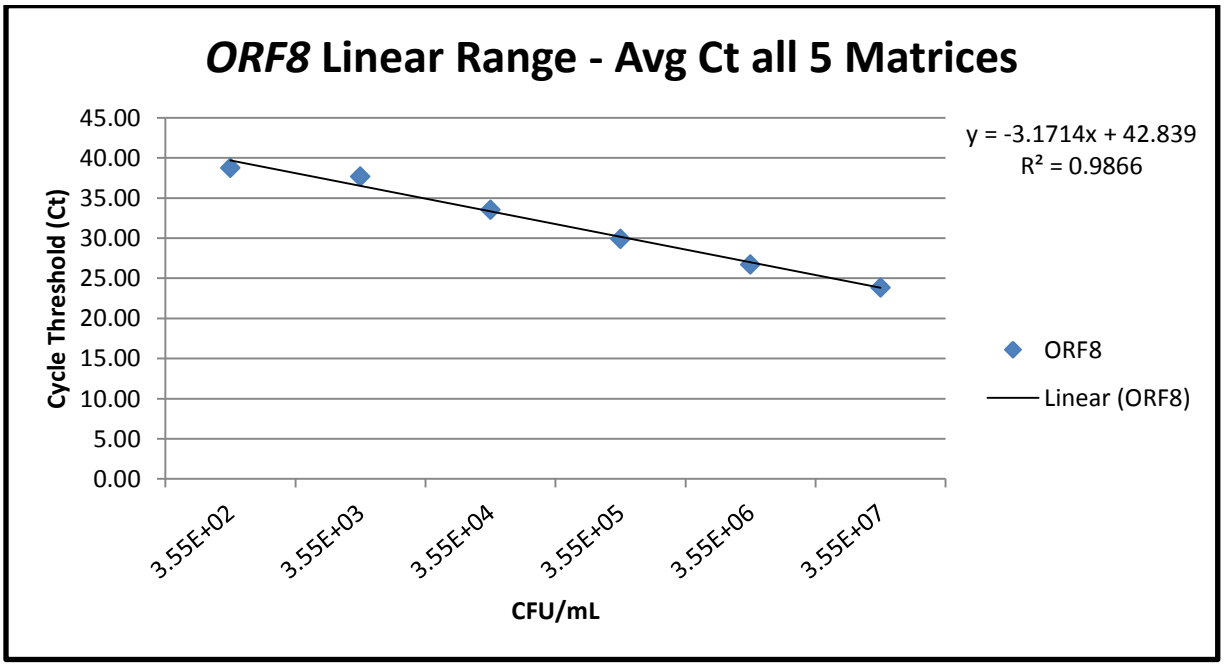
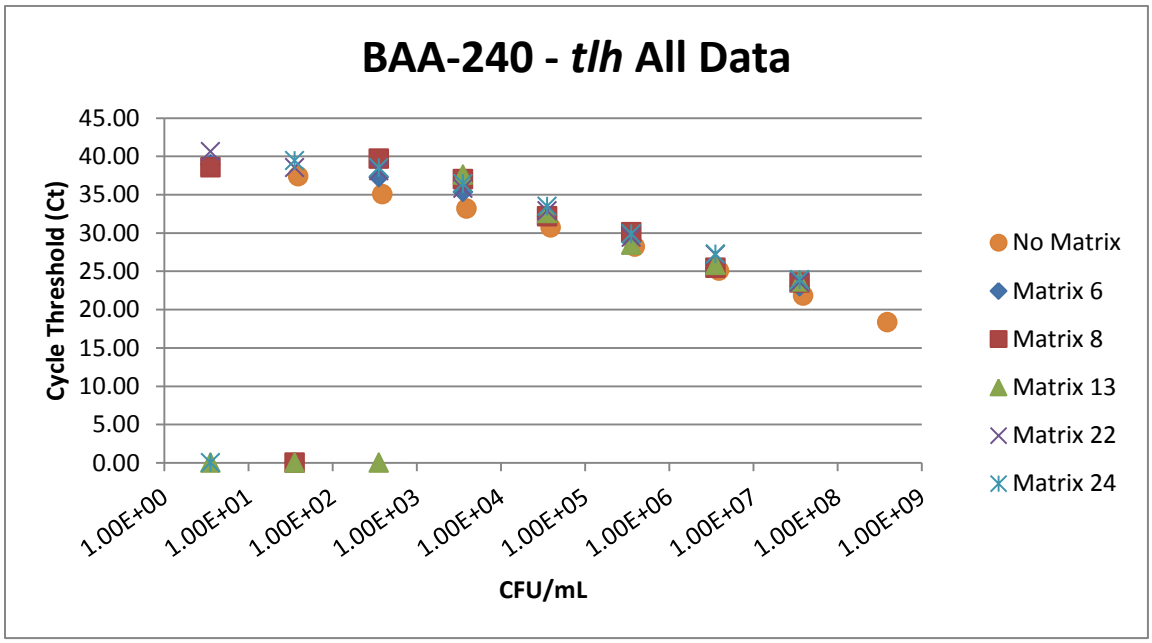


Figures 2. Graphs of the average Ct values for all data points within a matrix for (A) *tlh,(B) tdh,(C) trh,* and (D) *ORF8*.

A



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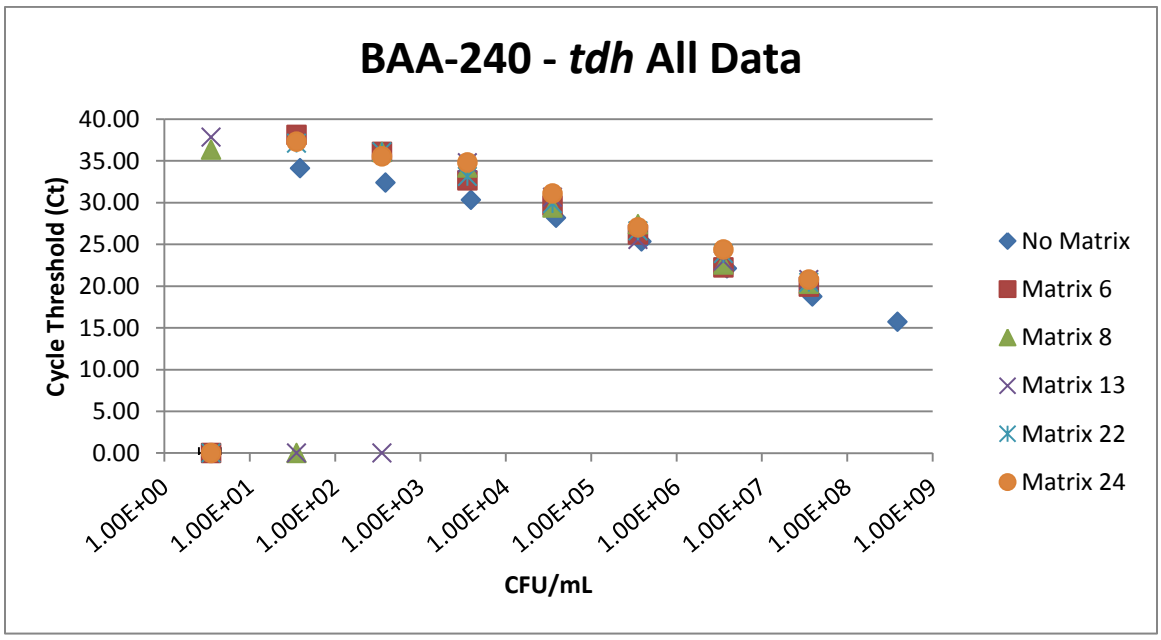

Figure 2. Continued

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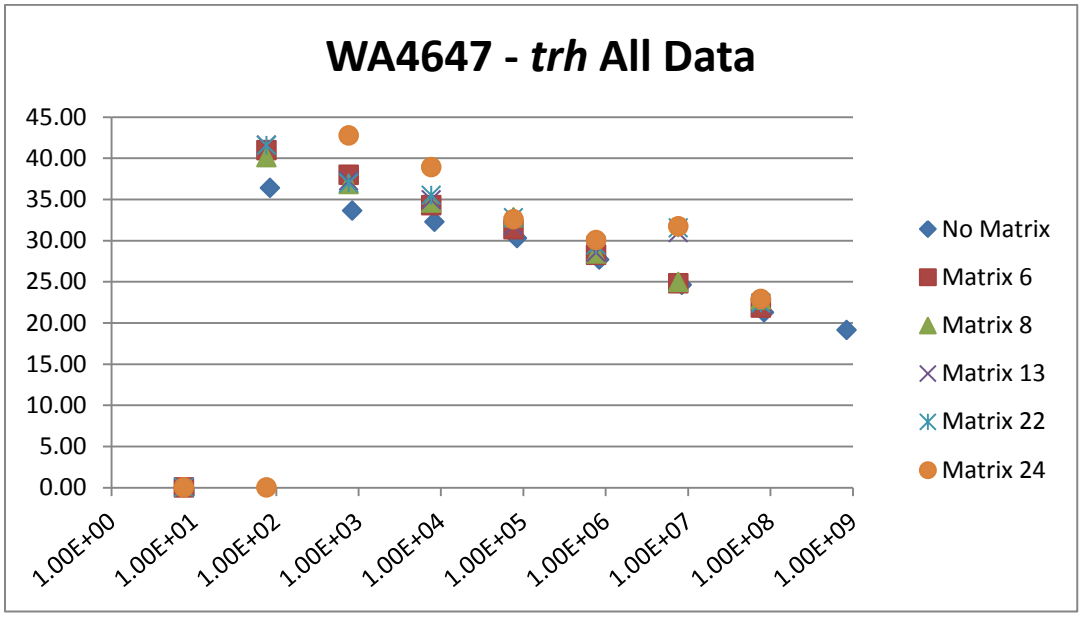

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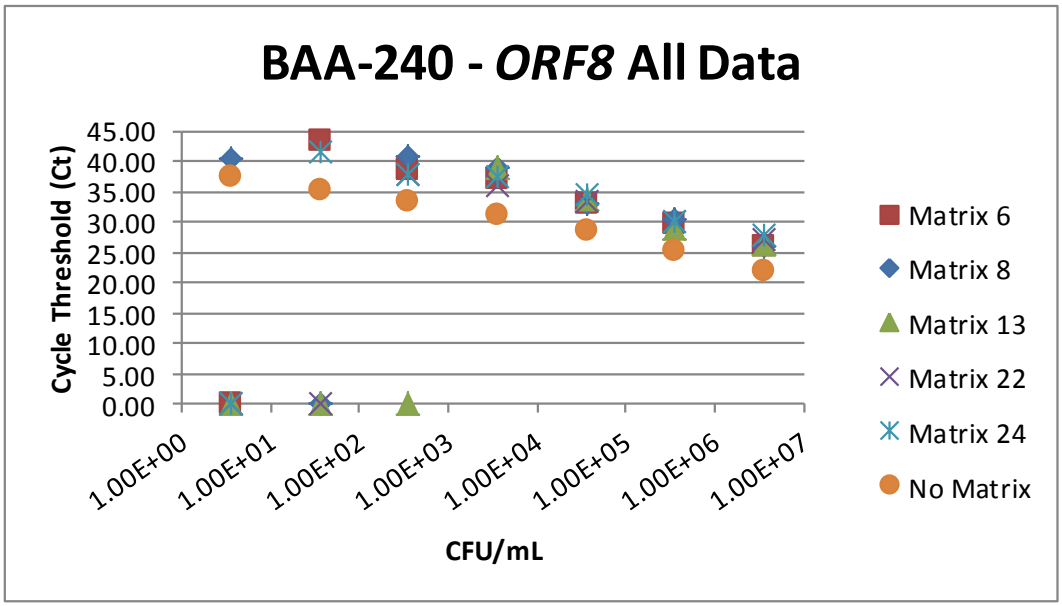
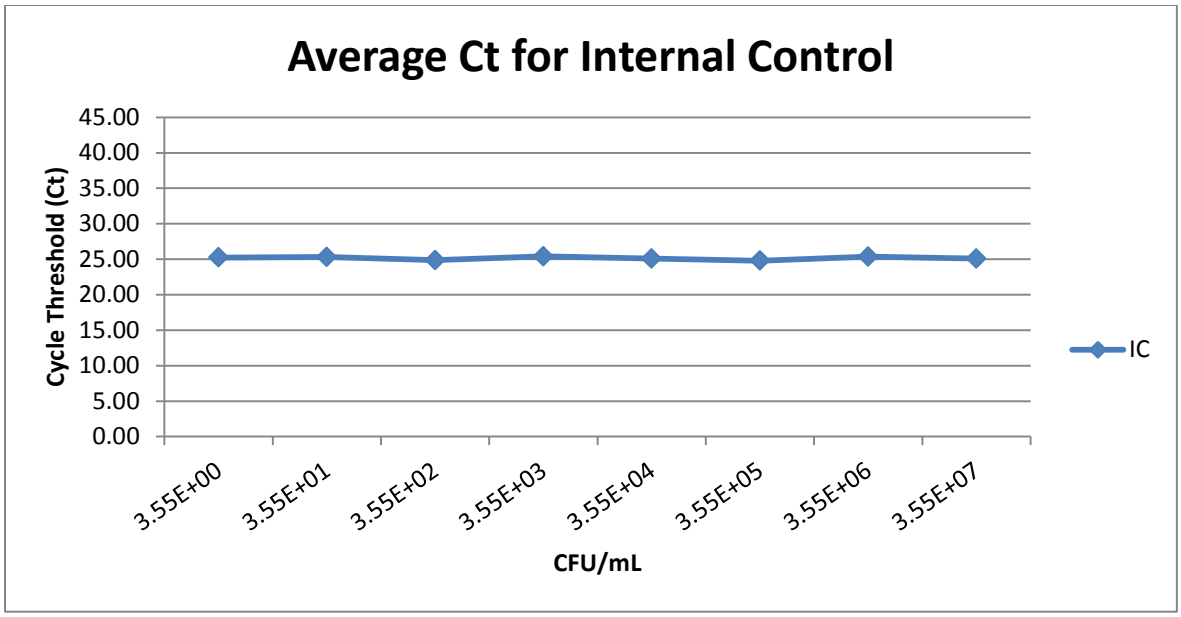


Figure 3. Graph of the average Ct for the Internal Control (IC) calculated from all matrices spiked with *Vp BAA-240.*



Inclusivity

Purpose

To assess the ability of the method to detect a wide range of target strains in various oyster tissues.

Method

Vibrio parahaemolyticus (*Vp*) strains were grown in APW for 18-24 hours. DNA was extracted using the MagNA Pure LC and was frozen at -20°C until PCR was performed in duplicate. The goal was at least 30 strains for each target. Confirmation was performed through a secondary assay. Since *tlh* is a species specific marker, a positive *Vp* identification through biochemicals or by ATCC paperwork was used as confirmation. For *tdh* some isolates were confirmed by NOAA and others were confirmed using *tdh* primers from Nordstrom et al. 2007. The *trh* marker was a little more problematic due to 2 variations of the *trh* gene. All isolates were confirmed using the the FDA BAM *trh* primer set and anything with discrepant results between our assay and the FDA assay we confirmed with a biochemical urease test. The ability of *Vp* to hydrolyze urea has been shown to be indicative of the presence of the *trh* gene (Iida et al paper 1997). The *ORF8* pandemic marker was confirmed using the primer set from Myers et al. 2003.

Results

See Table 1 for the breakdown of strains used and targets tested.

Primer / Probe Sensitivity

Sensitivity= (# of true positives/ (# of true positives + # of false negatives))
tlh sensitivity = 78/78 = **100% *tlh* Sensitivity**
tdh sensitivity =38/38 = **100% *tdh* Sensitivity**
trh sensitivity = 33/33 = **100% *trh* Sensitivity**
ORF8 senstivity = 29/29 = **100% *ORF8* Sensitivity**

Table 1. Inclusivity panel with results by target. Targets not tested are blocked out.

Strain	Source	tlh	tdh	trh	orf8
F5828	CDC	+	+		+
F5835	CDC	+	+		+
F5847	CDC	+	+		+
F6820	CDC	+	+		+
F7630	CDC	+	+		+
F7635	CDC	+	+		+
F7636	CDC	+	+		+
F7680	CDC	+	+		+
F8701	CDC	+	+		+
F8949	CDC	+	+		+
F9083	CDC	+	+		+
K0071	CDC	+	+		+
K0456	CDC	+	-	+	-
K1533	CDC	+	+		+
17803	ATCC	+	-	+	
27519	ATCC	+	-		
27969	ATCC	+			
33844	ATCC	+	+		
33845	ATCC	+	+		
33846	ATCC	+	+		
33847	ATCC	+	+		
35117	ATCC	+	-		
35118	ATCC	+	+		
43996	ATCC	+	+		
49398	ATCC	+	-		
AP14861	NOAA - NWFSC	+	+		+
BAA-238	ATCC	+	+		+
BAA-239	ATCC	+	+		+
BAA-240	ATCC	+	+		+
BAA-241	ATCC	+	+		+
BAA-242	ATCC	+	+		+
BAC03255	NOAA - NWFSC	+	+		+
BE98-2029	NOAA - NWFSC	+	+		+
NWF 261	NOAA - NWFSC	+	-	-	-
NWF 512	NOAA - NWFSC	+	-	-	-
NWF 586	NOAA - NWFSC	+	+	-	+
NWF 605	NOAA - NWFSC	+	+	-	+
NWF 609	NOAA - NWFSC	+	+	-	+
NWF 735	NOAA - NWFSC	+	+		+
NWF 782	NOAA - NWFSC	+	+	-	+

Strain	Source	tlh	tdh	trh	orf8
NWF 797	NOAA - NWFSC	+	-	-	
NWF 800	NOAA - NWFSC	+	-		
NWF 805	NOAA - NWFSC	+	-		
NWF 843	NOAA - NWFSC	+	-		
NWF 846	NOAA - NWFSC	+	+	+	
NWF 864	NOAA - NWFSC	+	+		+
NWF 930	NOAA - NWFSC	+	+	-	+
VPHY145	NOAA - NWFSC	+	+		+
5412	WA PHL	+		+	
5419	WA PHL	+		+	
5423	WA PHL	+	+	+	
5424	WA PHL	+		+	
5425	WA PHL	+	+	+	
5426	WA PHL	+		+	
5429	WA PHL	+		+	
5430	WA PHL	+		+	
5434	WA PHL	+		+	
5436	WA PHL	+		+	
5437	WA PHL	+		+	
5442	WA PHL	+		+	
5444	WA PHL	+		+	
5454	WA PHL	+		+	
5456	WA PHL	+		+	
5463	WA PHL	+		+	
5468	WA PHL	+		+	
5469	WA PHL	+		+	
5470	WA PHL	+		+	
5471	WA PHL	+		+	
5473	WA PHL	+		+	
5474	WA PHL	+		+	
5475	WA PHL	+		+	
5487	WA PHL	+		+	
5488	WA PHL	+		+	
5492	WA PHL	+		+	
5501	WA PHL	+		+	
5508	WA PHL	+		+	
5518	WA PHL	+		+	
5519	WA PHL	+		+	
Total Confirmed Isolated		78	38	33	29

Exclusivity

Purpose
To demonstrate the ability of the method to distinguish the targeted analyte from other potentially cross-reactive non-target strains that could possibly contaminate shellfish.

Method
All organisms were inoculated into APW and incubated for 18-24 hours. DNA was extracted using the MagNA Pure LC and extract was frozen at -20°C until PCR was performed in duplicate.

The data used to determine the *G. hollisae* threshold cutoff was produced from two clinical strains of *G. hollisae* that were enriched in APW at 35°C for 18-24 hours in 15 replicates (total 30 tubes). Each replicate was processed as described above and PCR was performed.

Results
For the exclusivity panel 59 non-*Vibrio parahaemolyticus* organisms were used (Table 2). None of the exclusivity panel had detection of *tlh*, *trh*, or *ORF8*. *Grimontia hollisae*, ATCC 33564, did show amplification of *tdh*.

Primer / Probe Specificity
Specificity = (# of true negative/ (# of true negatives + # of false positives))
tlh specificity= 59/59= **100% *tlh* Specificity**
tdh specificity = 58/59= **98% *tdh* Specificity**
trh specificity = 59/59= **100% *trh* Specificity**
ORF8 specificity = 59/59= **100% *ORF8* Specificity**

Known limitations and interferences

The specificity of *tdh* is a known issue with *G. hollisae*, formerly known as *V. hollisae*, it has been shown to have greater than 93% homology with *Vp tdh* (Yamasaki et al. 1991).

Since the primers/probe for *tdh* have not been redesigned since the last method validation, the same principal to identify possible cross-reactivity with *G. hollisae* will be employed.
After analysis of the ΔRn values for all positive *Vp tdh* readings, we are able to identify a ΔRn threshold setting to exclude *G. hollisae tdh* amplification. An amplification curve will only be considered positive if it has a ΔRn above 104,000 in a linear analysis of the amplification plot (Figure 1). This threshold setting is 25% above the highest *G. hollisae tdh* amplification curve and 25% below the lowest *Vp tdh* amplification curve (Table 1).

Table 1. ΔRn values for *tdh* positive *Vp* and *tdh* positive *G. hollisae*.

Organism	Average ΔRn	Low ΔRn	High ΔRn
tdh Positive *Vp*	236,777	138,688	390,110
tdh Positive *G. hollisae*	64,769	39,711	83,130
tdh Negative wells	-12,766	-22,752	-3,331

Figure 1. Linear amplification plot showing true *Vp* positive *tdh* amplification and *G. hollisae tdh* amplification.

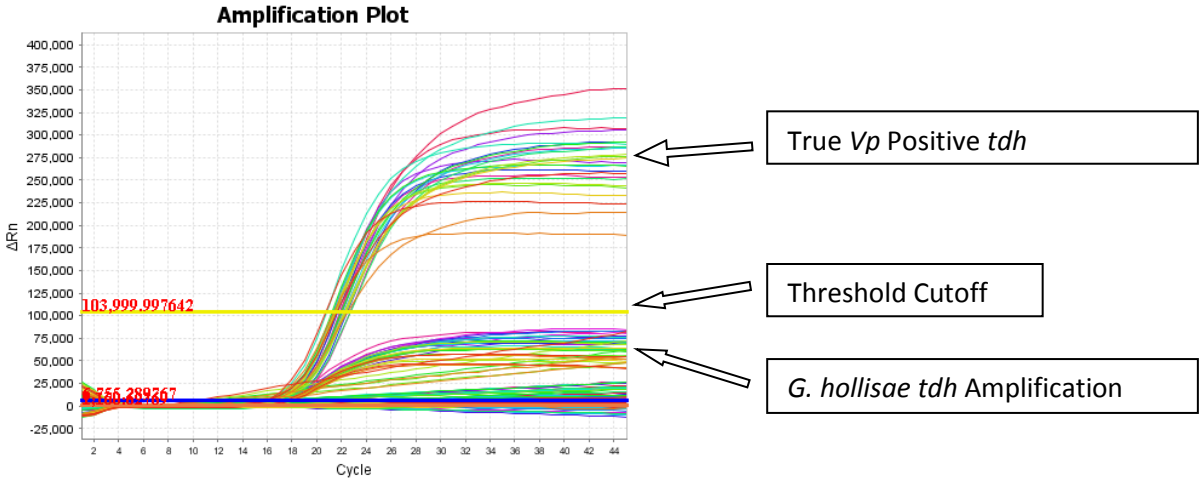


Table 2. All Exclusivity strains tested and results by target. All strains were purchased from ATCC, unless noted in parentheses.

Organism	ATCC #	*tlh*	*tdh*	*trh*	*orf8*
E. coli	25922	-	-	-	-
G. hollisae	33564	-	+	-	-
K. pneumoniae	33495	-	-	-	-
P. aeruginosa	33495	-	-	-	-
S. aureus	10145	-	-	-	-
S. sonnei	25925	-	-	-	-
S. typhimurium	9290	-	-	-	-
V. aestuarians	35048	-	-	-	-
V. alginolyticus	17749	-	-	-	-
V. algosus	14390	-	-	-	-
V. campbellii	25920	-	-	-	-
V. cholerae	39050	-	-	-	-
V. cinncinatiensis	35912	-	-	-	-
V. furnissii	33813	-	-	-	-
V. marinagilis	14398	-	-	-	-
V. marinofulvus	14395	-	-	-	-
V. marinovulgaris	14394	-	-	-	-
V. metschnikovii	700040	-	-	-	-
V. mimicus	33653	-	-	-	-
V. natriegens	14048	-	-	-	-
V. nereis	25917	-	-	-	-
V. nigripulchritudo	27043	-	-	-	-
V. ponticus	14391	-	-	-	-
V. proteolyticus	15338	-	-	-	-
V. splendidus	33789	-	-	-	-
V. tubiashii	19106	-	-	-	-

Table 1. Exclusivity Data (continued).

Organism	ATCC #	*tlh*	*tdh*	*trh*	*orf8*
V. vulnificus	06-2410 (CDC)	-	-	-	-
V. vulnificus	06-2450 (CDC)	-	-	-	-
V. vulnificus	07-2405 (CDC)	-	-	-	-
V. vulnificus	08-2468 (CDC)	-	-	-	-
V. vulnificus	08-2470 (CDC)	-	-	-	-
V. vulnificus	08-2472 (CDC)	-	-	-	-
V. vulnificus	08-2485 (CDC)	-	-	-	-
V. vulnificus	1831-81 (CDC)	-	-	-	-
V. vulnificus	2009V-1002 (CDC)	-	-	-	-
V. vulnificus	2009V-1055 (CDC)	-	-	-	-
V. vulnificus	2010V-1021 (CDC)	-	-	-	-
V. vulnificus	2011V-1065 (CDC)	-	-	-	-
V. vulnificus	2011V-1162 (CDC)	-	-	-	-
V. vulnificus	2012V-1089 (CDC)	-	-	-	-
V. vulnificus	2013V-1091 (CDC)	-	-	-	-
V. vulnificus	209V-1035 (CDC)	-	-	-	-
V. vulnificus	2409-05 (CDC)	-	-	-	-
V. vulnificus	2431-01 (CDC)	-	-	-	-
V. vulnificus	2431-04 (CDC)	-	-	-	-
V. vulnificus	2473-85 (CDC)	-	-	-	-
V. vulnificus	2492-88 (CDC)	-	-	-	-
V. vulnificus	2809-78 (CDC)	-	-	-	-
V. vulnificus	430-79 (CDC)	-	-	-	-
V. vulnificus	774-83 (CDC)	-	-	-	-
V. vulnificus	AM38622 (CDC)	-	-	-	-
V. vulnificus	AM38623 (CDC)	-	-	-	-
V. vulnificus	AM38625 (CDC)	-	-	-	-
V. vulnificus	27562	-	-	-	-
V. vulnificus	29307	-	-	-	-
A. trota	2013V-1197 (CDC)	-	-	-	-
A. punctata	N/A (CDC)	-	-	-	-
A. veronii	N/A (CDC)	-	-	-	-
Total Strains	59				

Accuracy/Trueness

Purpose
To assess the ability of the method to produce test results which are in agreement with the accepted reference value.

Method
Five (5) oyster samples were spiked with varying concentrations of *Vp ATCC BAA-240* and *Vp WA4647.* In PBS, serial dilutions were prepared from 18-24 hour growth of the target organisms. A 3-tube, 6-dilution MPN set was inoculated from the spiked oyster homogenate. The MPN set was incubated at 35°C for 18-24 hours, each individual tube was read for turbidity, DNA extraction was performed on the MagNA Pure LC, and PCR was performed on all selected tubes from positive turbidity readings.

For each matrix, 7 MPN sets (3 MPN replicates of *BAA-240*, 3 MPN replicates of *WA4647*, 1 MPN set uninoculated) were spiked with target organism. Concentrations of the target organism(s) were calculated by spread plate counts on T1N3 agar. Actual spiking levels achieved were 10^{-1}, 10^{0}, 10^{1}, 10^{2}, and 10^{4}.

Results
The actual spiking level was compared to the generated MPN value and the 95% confidence intervals associated with the MPN value. In all matrices the spiked CFU/g of *BAA-240* (*tlh*, *tdh*, and *ORF8*) and *WA4647* (*tlh*, *tdh*, *trh*) were evaluated to determine if the value was within the acceptable range of the lower and upper 95% confidence interval. For all matrices and targets there was only one instance of the generated MPN value falling outside the acceptable range. At the 10^0 spiking level (1.2 CFU/g), the *tdh* MPN of *BAA-240* Replicate 3 was 7.5 MPN/g. The positive pcr reaction was in the C1 tube and had a late tdh Ct of 37.15. During our routine surveillance a result such as this would be repeated twice to ensure it was not cross-contamination on the 384-well pcr plate. While this MPN is outside the confidence intervals the MPN generated for *tlh* was 4.3 MPN/g. Since the 4.3 MPN/g is acceptable and our reporting algorithm prevents reporting a positive *tdh* in the absence of *tlh* this data point was disregarded.

The matrix 24 blank did have late amplification of *tlh, tdh* and *ORF8* in the C2 tube, this matrix has previously been confirmed as negative and has been run several times resulting in no detection of *Vp*. We suspect a cross-contamination issue in the MagNA Pure cartridge; especially since it was *ORF8* positive it is very unlikely it was present in the oyster matrix prior to processing. This result was not used to adjust the overall MPN result of the spiked matrix 24 samples.

The FDA BAM 3-tube MPN chart has been copied as Figure 1.

Table 1. Tables display spiking level and calculated MPN along with the allowable range for associated MPN values. For each matrix 3 replicated were performed per target (R1, R2, and R3). (A) Matrix 6 – *ATCC BAA-240*, Matrix 7 – *WA4647* (B) Matrix 8 – *ATCC BAA-240* & *WA4647* (C) Matrix 13 - *ATCC BAA-240* & *WA4647* (D) Matrix 22 - *ATCC BAA-240* & *WA4647* (E) Matrix 24 - *ATCC BAA-240* & WA4647.

A

Spiking Level	Organism	Actual CFU/g	Target	MPN/g R1	MPN/g R2	MPN/g R3
10^-1	ATCC BAA-240	0.17	tlh	0.36	0.92	0.36
			tdh	0.36	0.92	0.36
			orf8	0.36	0.92	0.36
	WA4647	0.63	tlh	0.36	0.92	0.92
			tdh	0.36	0.92	0.92
			trh	0.36	0.92	0.92

Allowable Range		
BAM MPN/g	Lower CI	Upper CI
0.36	0.017	1.8
0.92	0.14	3.8

B

Spiking Level	Organism	Actual CFU/g	Target	MPN/g R1	MPN/g R2	MPN/g R3
10^0	ATCC BAA-240	1.2	tlh	4.3	3.8	4.3
			tdh	4.3	3.8	7.5*
			orf8	2.3	3.8	4.3
	WA4647	6.3	tlh	9.3	23	21
			tdh	9.3	23	21
			trh	2.3	3.8	4.3

Allowable Range		
BAM MPN/g	Lower CI	Upper CI
2.3	0.46	9.4
3.8	0.87	11
4.3	0.9	18
7.5	1.7	20
21	4	43
23	4.6	94

*late Ct

C

Spiking Level	Organism	Actual CFU/g	Target	MPN/g R1	MPN/g R2	MPN/g R3
10^1	ATCC BAA-240	14	tlh	43	43	43
			tdh	43	43	43
			orf8	43	43	43
	WA4647	84	tlh	75	93	120
			tdh	75	150	120
			trh	75	93	120

Allowable Range		
BAM MPN/g	Lower CI	Upper CI
43	9	180
75	17	200
93	18	420
120	37	420
150	37	420

Table 1. Continued

D

Spiking Level	Organism	Actual CFU/g	Target	MPN/g R1	R2	R3
10^2	ATCC BAA-240	460	tlh	430	240	930
			tdh	430	240	2400
			orf8	430	240	930
	WA4647	630	tlh	430	930	430
			tdh	430	930	430
			trh	430	930	430

Allowable Range		
BAM MPN/g	Lower CI	Upper CI
240	42	1000
430	90	1800
930	180	4200
2400	420	10000

E

Spiking Level	Organism	Actual CFU/g	Target	MPN/g R1	R2	R3
10^4	ATCC BAA-240	58,000	tlh	46,000	46,000	46,000
			tdh	46,000	46,000	46,000
			orf8	46,000	46,000	46,000
	WA4647	58,000	tlh	24,000	24,000	46,000
			tdh	24,000	24,000	46,000
			trh	24,000	24,000	46,000

Allowable Range		
BAM MPN/g	Lower CI	Upper CI
24,000	4,200	100,000
46,000	9,000	200,000

Figure 1. FDA BAM 3-tube MPN chart.

Table 1. For 3 tubes each at 0.1, 0.01, and 0.001 g inocula, the MPNs per gram and 95 percent confidence intervals.

Pos. tubes			MPN/g	Conf. lim.		Pos. tubes			MPN/g	Conf. lim.	
0.10	0.01	0.001		Low	High	0.10	0.01	0.001		Low	High
0	0	0	<3.0	–	9.5	2	2	0	21	4.5	42
0	0	1	3.0	0.15	9.6	2	2	1	28	8.7	94
0	1	0	3.0	0.15	11	2	2	2	35	8.7	94
0	1	1	6.1	1.2	18	2	3	0	29	8.7	94
0	2	0	6.2	1.2	18	2	3	1	36	8.7	94
0	3	0	9.4	3.6	38	3	0	0	23	4.6	94
1	0	0	3.6	0.17	18	3	0	1	38	8.7	110
1	0	1	7.2	1.3	18	3	0	2	64	17	180
1	0	2	11	3.6	38	3	1	0	43	9	180
1	1	0	7.4	1.3	20	3	1	1	75	17	200
1	1	1	11	3.6	38	3	1	2	120	37	420
1	2	0	11	3.6	42	3	1	3	160	40	420
1	2	1	15	4.5	42	3	2	0	93	18	420
1	3	0	16	4.5	42	3	2	1	150	37	420
2	0	0	9.2	1.4	38	3	2	2	210	40	430
2	0	1	14	3.6	42	3	2	3	290	90	1,000
2	0	2	20	4.5	42	3	3	0	240	42	1,000
2	1	0	15	3.7	42	3	3	1	460	90	2,000
2	1	1	20	4.5	42	3	3	2	1100	180	4,100
2	1	2	27	8.7	94	3	3	3	>1100	420	–

Precision/Recovery

Purpose

To assess the closeness of agreement between independent rest results obtained under stipulated conditions and the percentage of an analyte recovered following sample analysis.

Method

The data generated in the accuracy/trueness phase was used to evaluate precision/recovery. The variation in the total number of tubes tested is determined by the turbidity in the MPN series and can vary between replicates without affecting overall results.

Results

The precision of the assay is difficult to demonstrate due to the nature of enumerating through MPN dilution sets. However, by showing the total number of positive tubes/target the precision of the assay is demonstrated between all targets tested at a specific spiking level.

Table 1. MPN results for all matrices along with the total number of positive tubes in the MPN dilution series/total number of tubes tested (determined by turbidity). (A) Matrix 6 – *ATCC BAA-240*, Matrix 7 – *WA4647* (B) Matrix 8 – *ATCC BAA-240* & *WA4647* (C) Matrix 13 - *ATCC BAA-240* & *WA4647* (D) Matrix 22 - *ATCC BAA-240* & *WA4647* (E) Matrix 24 - *ATCC BAA-240* & WA4647.

A

Spiking Level	Organism	Actual CFU/g	Target	Data					
				R1		R2		R3	
M#6&7				MPN/g	tubes	MPN/g	tubes	MPN/g	tubes
10^-1	ATCC BAA-240	0.17	tlh	0.36	1/12	0.92	2/12	0.36	1/12
			tdh	0.36	1/12	0.92	2/12	0.36	1/12
			orf8	0.36	1/12	0.92	2/12	0.36	1/12
	WA4647	0.63	tlh	0.36	1/12	0.92	2/15	0.92	2/12
			tdh	0.36	1/12	0.92	2/15	0.92	2/12
			trh	0.36	1/12	0.92	2/15	0.92	2/12

B

Spiking Level	Organism	Actual CFU/g	Target	Data					
				R1		R2		R3	
M#8				MPN/g	tubes	MPN/g	tubes	MPN/g	tubes
10^0	ATCC BAA-240	1.2	tlh	4.3	4/12	3.8	4/15	4.3	4/12
			tdh	4.3	4/12	3.8	4/15	7.5*	5/12
			orf8	2.3	3/12	3.8	4/15	4.3	4/12
	WA4647	6.3	tlh	9.3	5/12	23	6/12	21	7/12
			tdh	9.3	5/12	23	6/12	21	7/12
			trh	2.3	5/12	3.8	6/12	4.3	7/12

*late Ct – see Accuracy/Trueness data for explanation.

Table 1. Continued

C

Spiking Level	Organism	Actual CFU/g	Target	Data					
				R1		R2		R3	
M#13				MPN/g	tubes	MPN/g	tubes	MPN/g	tubes
10^1	ATCC BAA-240	14	tlh	43	7/12	43	7/12	43	7/12
			tdh	43	7/12	43	7/12	43	7/12
			orf8	43	7/12	43	7/12	43	7/12
	WA4647	84	tlh	75	8/12	93	8/12	120	9/15
			tdh	75	8/12	150	9/12	120	9/15
			trh	75	8/12	93	8/12	120	9/15

D

Spiking Level	Organism	Actual CFU/g	Target	Data					
				R1		R2		R3	
M#22				MPN/g	tubes	MPN/g	tubes	MPN/g	tubes
10^2	ATCC BAA-240	460	tlh	430	10/15	240	9/12	930	11/15
			tdh	430	10/15	240	9/12	2400	12/15
			orf8	430	10/15	240	9/12	930	11/15
	WA4647	630	tlh	430	10/15	930	11/15	430	10/15
			tdh	430	10/15	930	11/15	430	10/15
			trh	430	10/15	930	11/15	430	10/15

E

Spiking Level	Organism	Actual CFU/g	Target	Data					
				R1		R2		R3	
M#24				MPN/g	tubes	MPN/g	tubes	MPN/g	tubes
10^4	ATCC BAA-240	58000	tlh	46000	16/18	46000	16/18	46000	16/18
			tdh	46000	16/18	46000	16/18	46000	16/18
			orf8	46000	16/18	46000	16/18	46000	16/18
	WA4647	58000	tlh	24000	15/18	24000	15/18	46000	16/18
			tdh	24000	15/18	24000	15/18	46000	16/18
			trh	24000	15/18	24000	15/18	46000	16/18

Specificity/Competitor Strain

Purpose
To assess the ability of the method to measure only what it is intended to measure.

Method
Two (2) oyster samples, both from Matrix #13, were spiked with 10^1 CFU/g of *Vp ATCC BAA-240* and *Vp WA4647.* The target organisms were prepared in PBS serial dilutions from 18-24 hour growth. Along with the target organisms the matrices were also spiked with *V. alginolyticus* at one log higher (10^2). A 3-tube, 6-dilution MPN set was inoculated from the spiked oyster homogenate. The MPN set was incubated at 35°C for 18-24 hours, each individual tube was read for turbidity, DNA extraction was performed on the MagNA Pure LC, and PCR was performed on all selected tubes from turbidity readings.

Concentrations of the target organism(s) were calculated by spread plate counts on T1N3 agar.

Results

There was no issue detecting the target organism in the presence of *V. alginolyticus*. All MPN values generated were within the acceptable ranges.

Table 1. *Vp WA4647* spiked at 84 cfu/g (2520 cells); Competitor *V. alginolyticus* spiked at 380 cfu/g (11400 cells).

	tlh	MPN/g	Lower CI	Upper CI	**tdh**	MPN/g	Lower CI	Upper CI	**trh**	MPN/g	Lower CI	Upper CI
R1	**3-2-0 x10**	**93**	18	420	**3-2-0 x10**	**93**	18	420	**3-2-0 x10**	**93**	18	420
R2	**3-2-0 x10**	**93**	18	420	**3-2-1* x10**	**150**	37	420	**3-2-0 x10**	**93**	18	420

*Late Ct

Table 2. *Vp ATCC BAA-240* spiked at 14 cfu/g (420 cells); Competitor *V. alginolyticus* spiked at 130 cfu/g (3900 cells)

	tlh	MPN/g	Lower CI	Upper CI	**tdh**	MPN/g	Lower CI	Upper CI	**orf8**	MPN/g	Lower CI	Upper CI
R1	**3-0-0 x10**	**23**	**4.6**	**94**	**3-0-0 x10**	**23**	**4.6**	**94**	**3-0-0 x10**	**23**	**4.6**	**94**
R2	**3-1-0 x10**	**43**	**9**	**180**	**3-1-0 x10**	**43**	**9**	**180**	**3-1-0 x10**	**43**	**9**	**180**

*The extra positive tube in the *tdh* MPN level for *Vp WA4647* R2 resulted from a very late tdh only amplification (Ct = 39.87). Usually, when this situation occurs it is due to cross-contamination on the 384-well PCR plate and the extract will be re-run twice to ensure proper reporting.

Ruggedness

Purpose
To assess the ability of the method to withstand relatively minor changes in analytical technique, reagents or environmental factors likely to arise in different test environments.

Method
Using 10 oyster samples, the ruggedness of the assay was challenged with different media/reagents, variability in oyster tissue concentration, and other environmental factors inherent in testing over a period of time.

For each matrix, 36 APW tubes (18 from each of the two lots of media) were spiked with oyster tissue (1g oyster to .000001g oyster) – 6 of each dilution. Tubes were designated as Lot A or Lot B and then processed through the entire assay with Lot A or Lot B media/reagents. Five different spiking levels were tested using *Vp ATCC BAA-240* and *Vp WA4647*; each level was spiked into two oyster matrices. The testing was performed over the course of two weeks on three separate days.

Results
While there were fluctuations between Ct values from Lot A to Lot B (Table 1), all samples were expected to show amplification and did so within a relatively small range. The average Ct value and standard deviation across all matrix dilutions and between Lot A and Lot B were calculated (Table 2 and Table 3). This is a qualitative assay and as such amplification at a certain Ct level is not necessary and there is no cycle cutoff to determine a positive or negative sample.

Table 1. Comparison between Lot A and Lot B of observed positive results and the expected positive results. The average Ct is calculated from all 10 matrices tested for each target.

		Lot A	Lot B
IC	Observed/ Expected	120/120	120/120
	Ave Ct	23.78	24.71
tlh	Observed*/ Expected	118/120	118/120
	Ave Ct	16.98	17.44
tdh	Observed*/ Expected	118/120	118/120
	Ave Ct	16.79	17.13
trh	Observed*/ Expected	58/60	58/60
	Ave Ct	17.82	18.21
ORF8	Observed/ Expected	60/60	60/60
	Ave Ct	20.56	22.02

***** Two dilutions of data points were negative in the WA4647 data set. Due to the consistency of the data in dilutions on either side, the most likely explanation is the tubes were inadvertently skipped during the spiking experiment.

Table 2. Low and High Ct values across all matrices and oyster concentrations.

		IC Low	IC High	*tlh* Low	*tlh* High	*tdh* Low	*tdh* High	*trh* Low	*trh* High	*ORF8* Low	*ORF8* High
ATCC BAA-240	Lot A	21.16	25.07	16.23	17.94	15.29	17.31	n/a	n/a	19.06	21.34
	Lot B	19.05	26.12	16.00	20.48	15.38	19.54	n/a	n/a	19.74	24.41
WA4647	Lot A	20.73	24.96	15.68	19.97	15.73	20.40	16.35	21.27	n/a	n/a
	Lot B	19.35	27.89	15.85	18.67	15.97	19.02	16.74	20.52	n/a	n/a

Table 3. Average Ct and Standard Deviation across all oyster concentrations and reagent/media lots for all samples spiked with *ATCC BAA-240*.

ATCC BAA-240										
Matrix Samples	Spiking Level (cfu/ml)	IC Avg Ct	IC StDev	*tlh* Avg Ct	*tlh* StDev	*tdh* Avg Ct	*tdh* StDev	*ORF8* Avg Ct	*ORF8* StDev	*trh*
2,5	6.50E+00	24.45	1.50	17.45	0.54	16.83	0.50	21.09	0.74	n/a
6,8	6.50E+01	24.47	1.67	17.32	0.44	16.71	0.40	20.96	0.66	n/a
13,15	4.40E+02	23.70	1.31	17.17	0.37	16.37	0.33	20.85	0.48	n/a
17,22	1.15E+04	24.23	1.49	18.00	0.89	17.30	0.80	21.80	1.19	n/a
24,27	1.15E+06	24.21	1.07	18.00	1.48	17.30	1.35	21.75	1.77	n/a

Table 4. Average Ct and Standard Deviation across all oyster concentrations and reagent/media lots for all samples spiked with *WA4647*

WA4647										
Matrix Samples	Spiking Level (cfu/ml)	IC Avg Ct	IC StDev	*tlh* Avg Ct	*tlh* StDev	*tdh* Avg Ct	*tdh* StDev	*trh* Avg Ct	*trh* StDev	*ORF8*
2,5	3.90E+00	24.60	1.94	17.07	0.91	17.12	1.00	18.13	0.99	n/a
6,8	3.90E+01	23.67	1.29	16.83	0.75	16.98	0.78	17.95	0.87	n/a
13,15	9.05E+02	23.97	1.72	16.67	0.55	16.88	0.60	17.87	0.74	n/a
17,22	9.90E+04	24.54	1.33	17.04	1.14	17.32	1.21	18.35	1.28	n/a
24,27	9.90E+06	24.61	1.46	16.60	0.56	16.84	0.60	17.81	0.79	n/a

Screenshots from 2014 Vibrio Season

Run from July 17, 2014

Sample Numbers: S14-095, S14-096, S14-097, S14-098, S14-099, S14-100

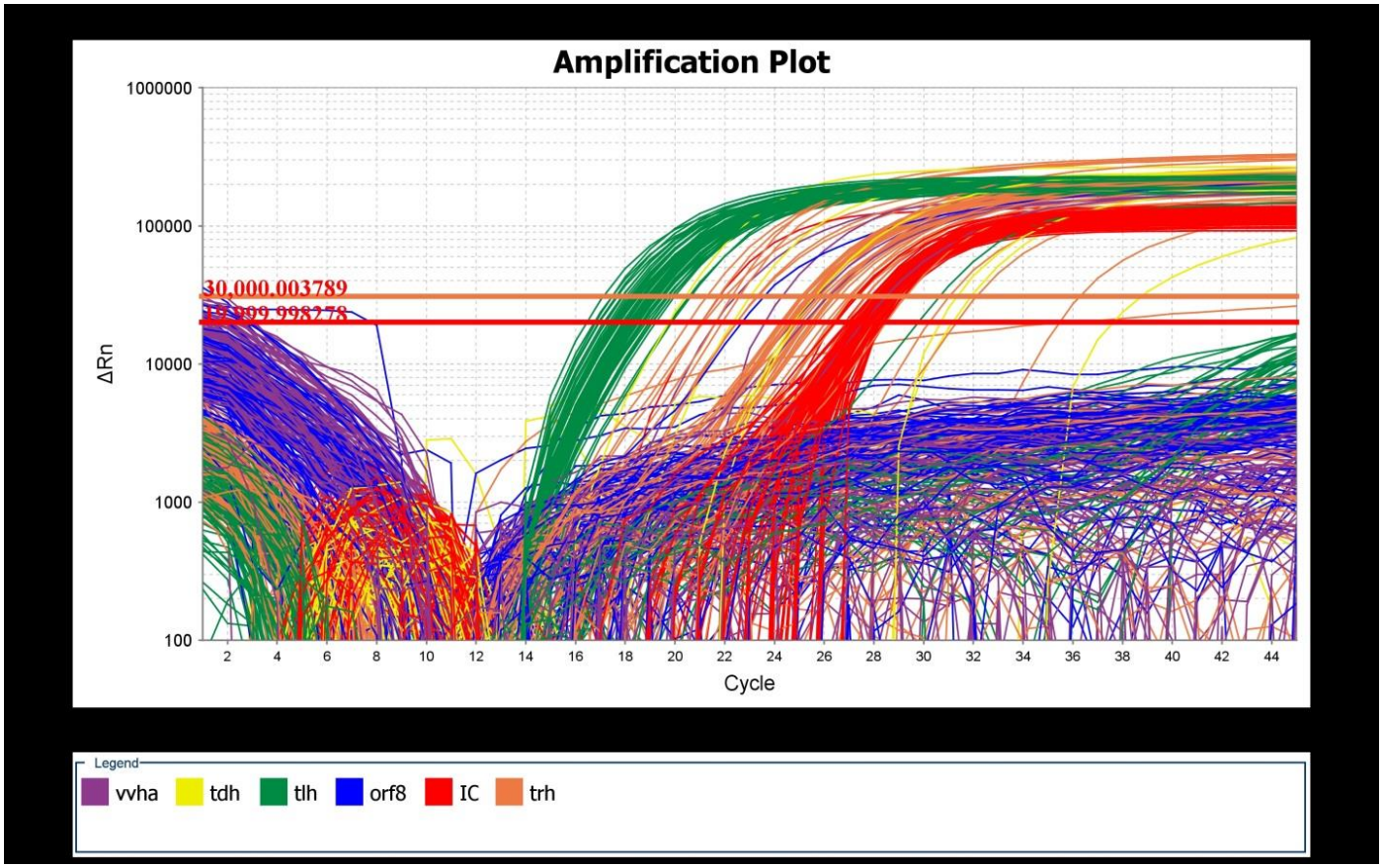


Figure 1. Screenshot showing all targets amplified over 45 cycles. Targets color-coded with legend at bottom of screenshot. For each multiplex 109 wells (including QC) were included. Samples ranged from 12 to 18 wells (per multiplex).

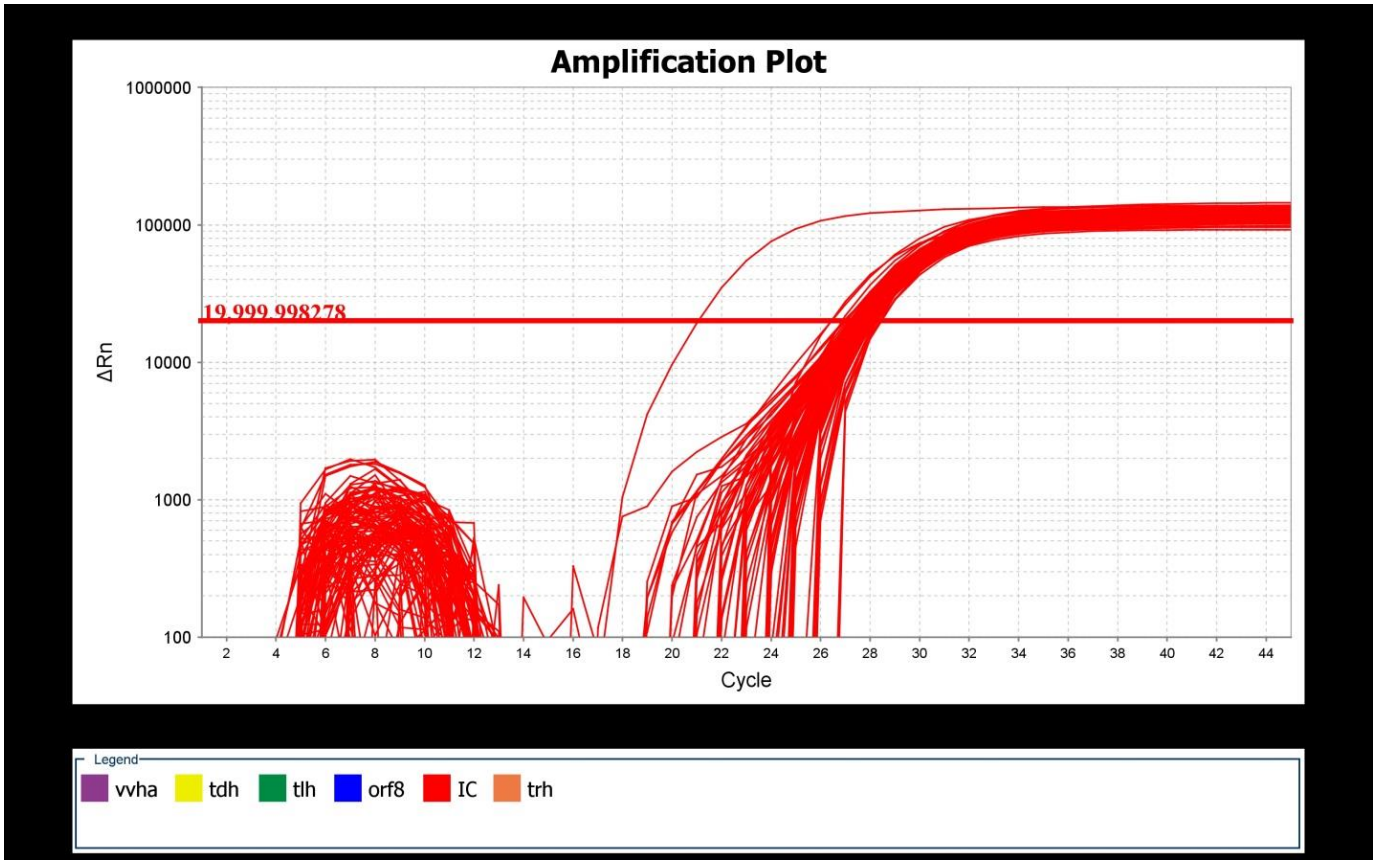


Figure 2. Internal control (IC) amplification plot. All wells in this run generated a positive result; however in the presence of a positive marker the IC may be negative. Most Ct values of our internal control range from 24 to 28, in this run a .00001g series MPN dilution tube generated an earlier Ct than usual. Since all quantitation is done with the MPN values generated from positive and negative tubes, PCR is presence/absence only so close scrutiny of Ct values is not necessary.

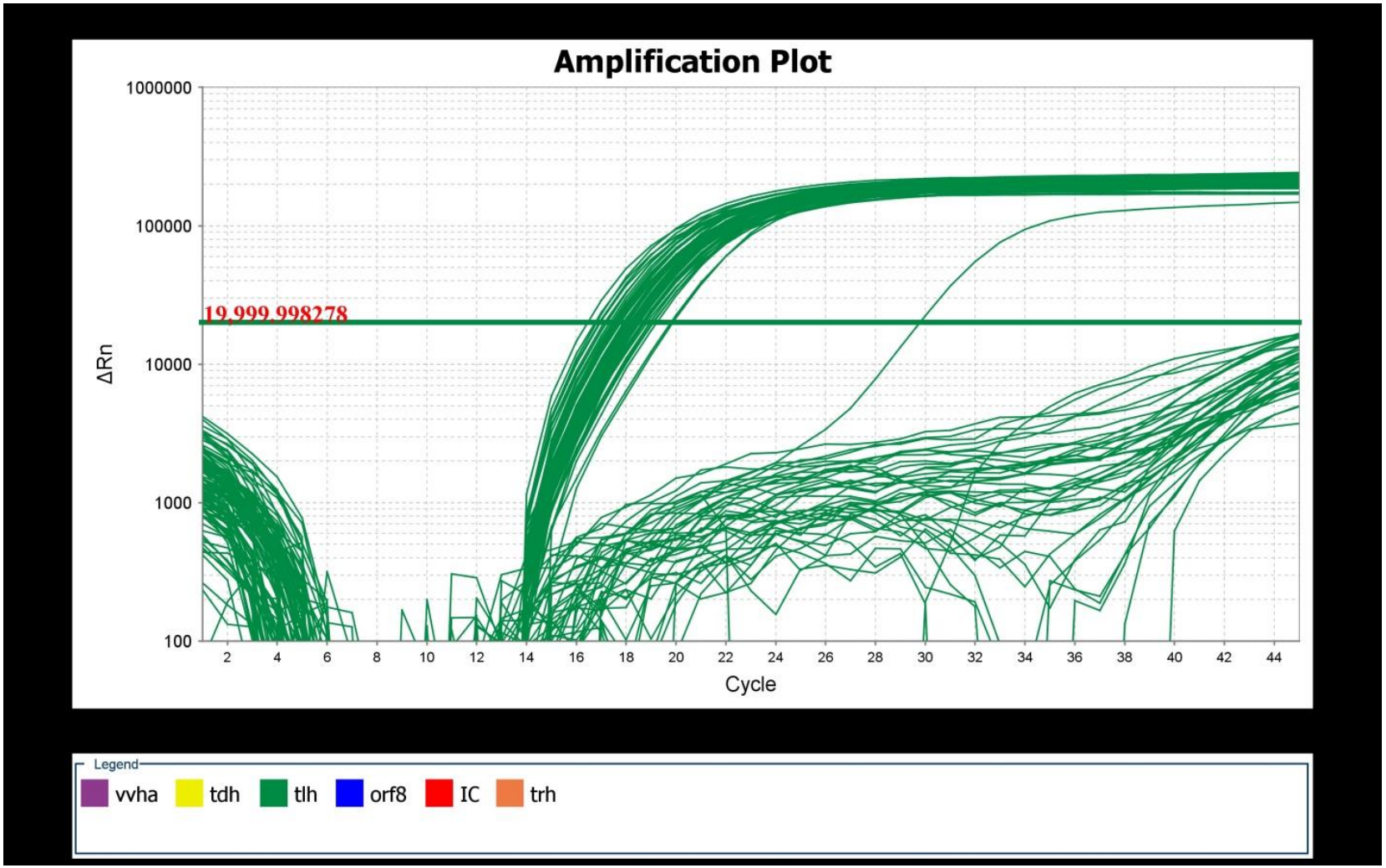


Figure 3. Thermolabile Hemolysin (*tlh*) amplification plot. Threshold set at approximately 20,000 ΔRn. One reaction with a significantly later Ct value is from a .01g dilution tube. No cutoffs have been assigned for this assay (other than *tdh*), this late amplification curve is considered positive.

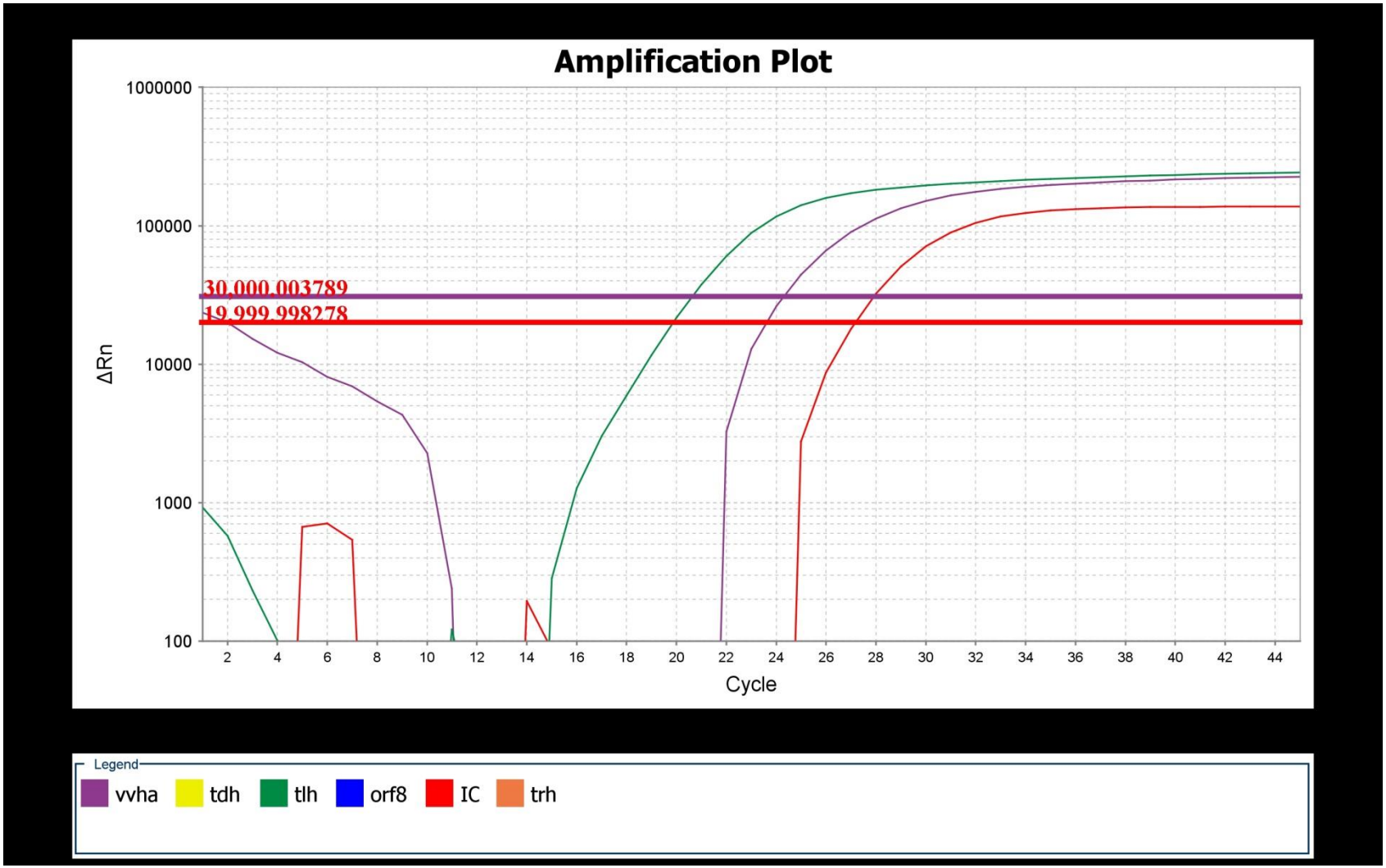


Figure 4. Positive QC for Multiplex 1. Shows amplification for IC (red), *tlh* (green), and *vvha* (purple).

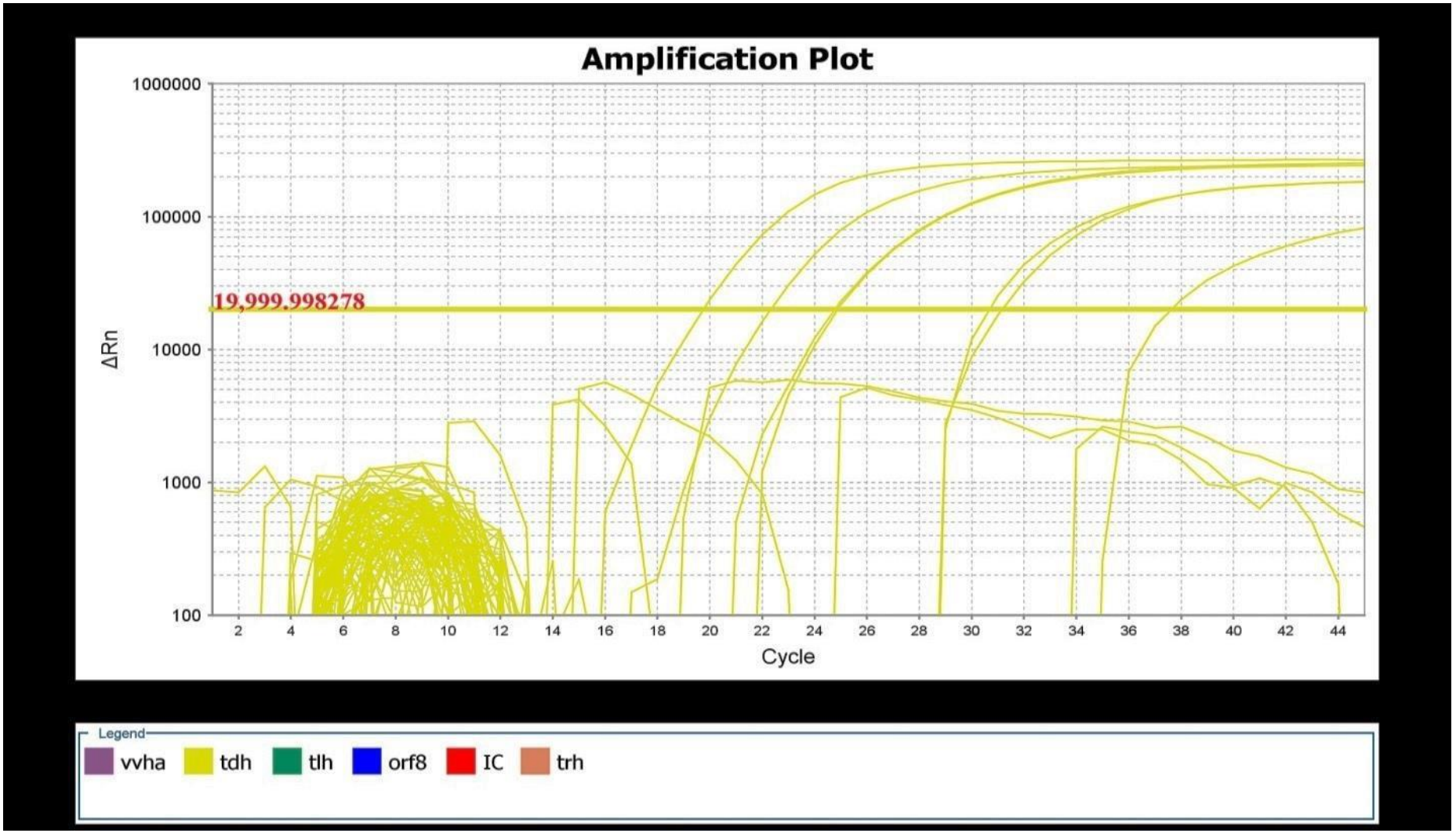


Figure 5. Thermostabile direct hemolysin (*tdh*) logarithmic amplification plot.

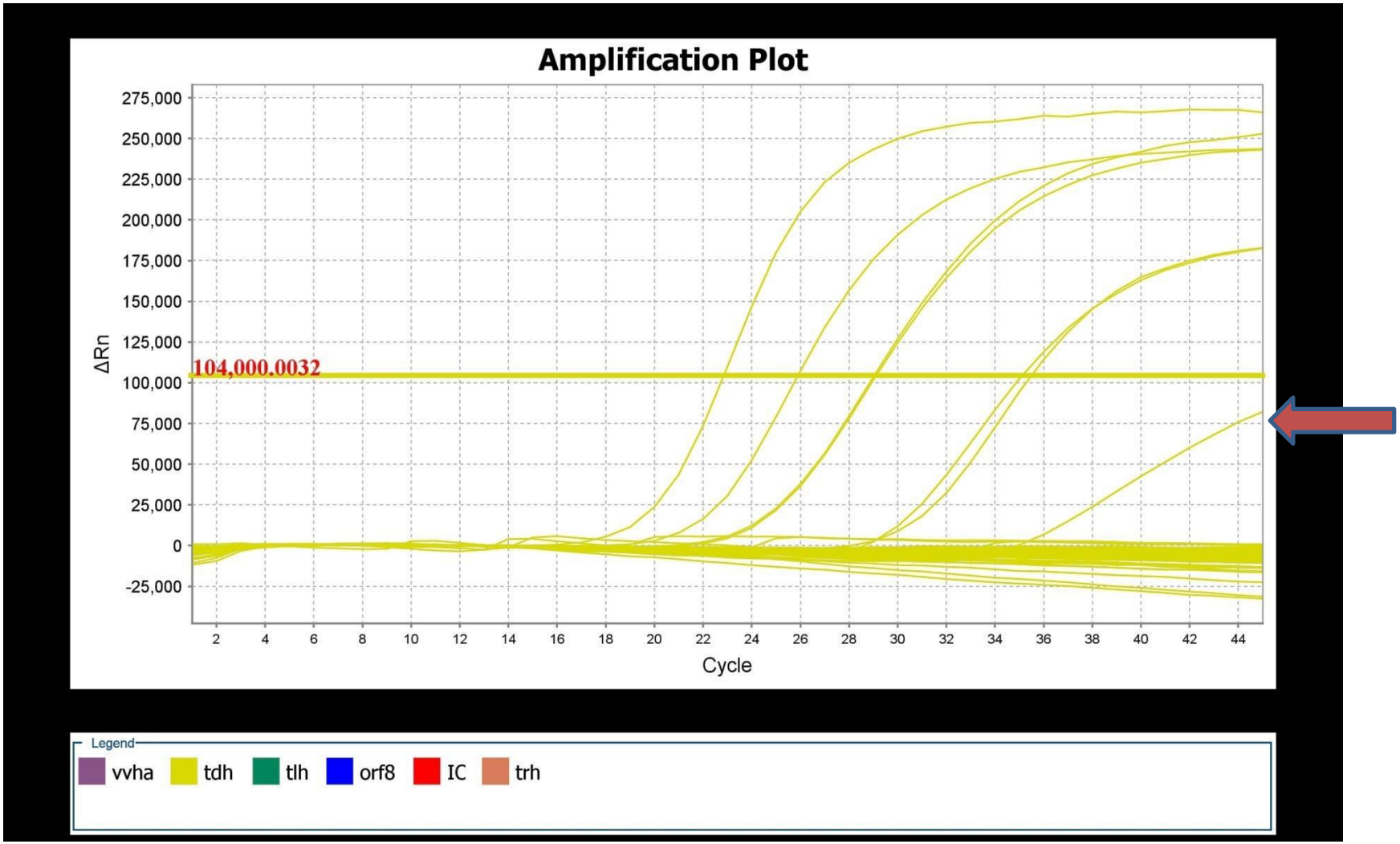


Figure 6. *tdh* linear amplification plot. Red arrow points to a reaction that was ruled negative due to 104,000 ΔRn cutoff.

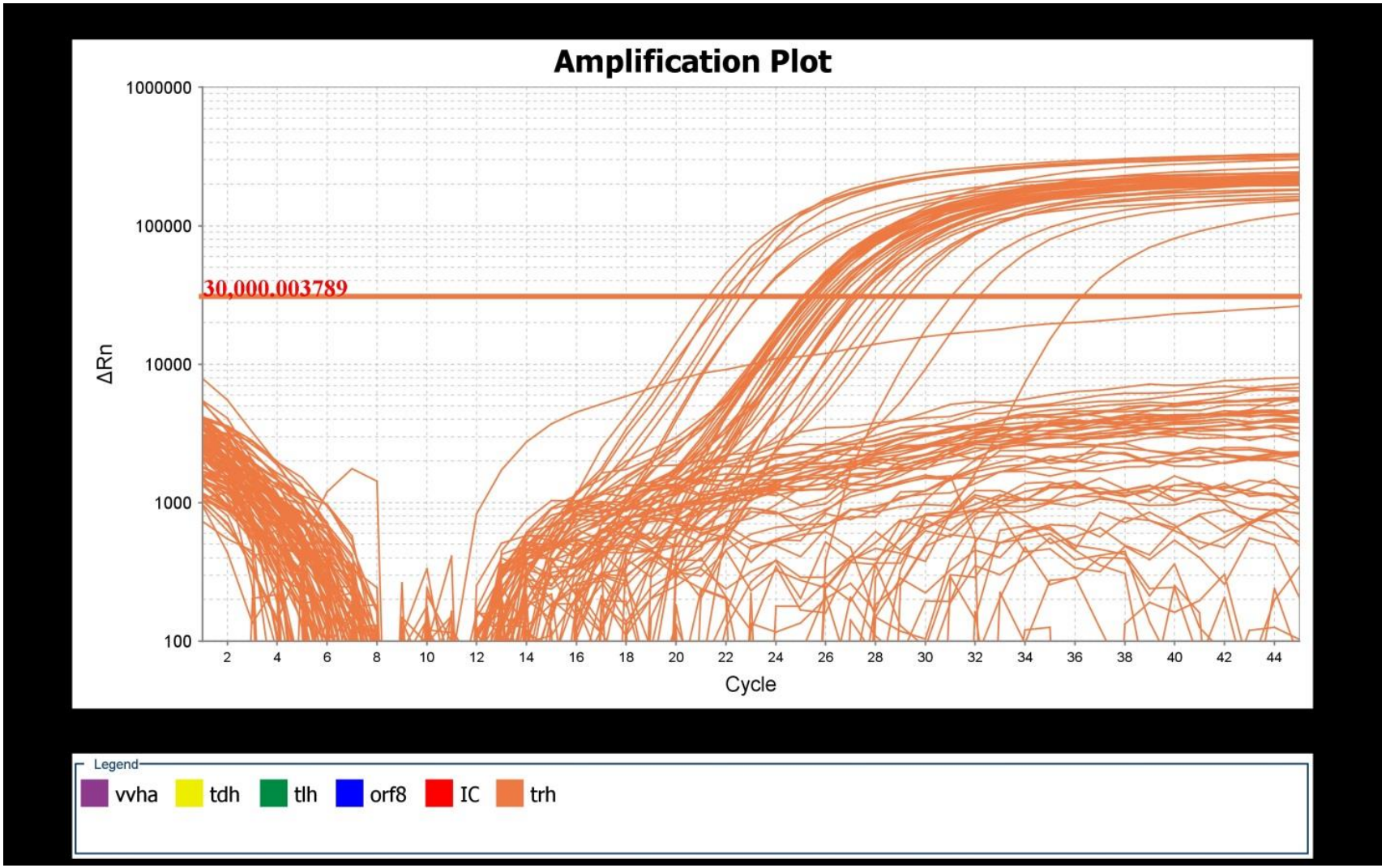


Figure 7. TDH-related hemolysin (*trh*) amplification plot. For a tube to be positive for *trh*, *tlh* must also been detected.

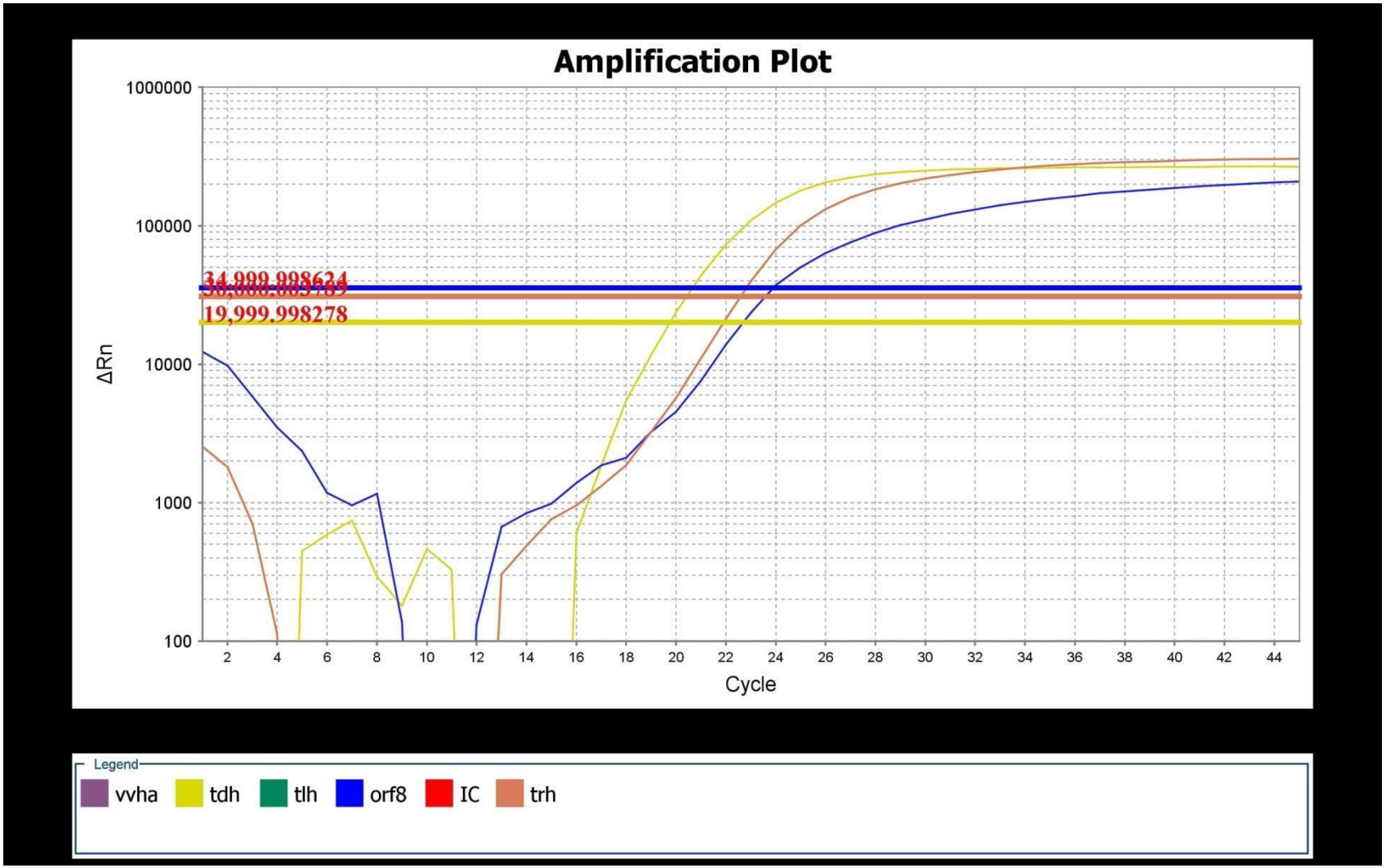


Figure 8. Positive QC for Multiplex 2. Shows amplification for *tdh* (yellow), *trh* (orange), *ORF8* (blue).

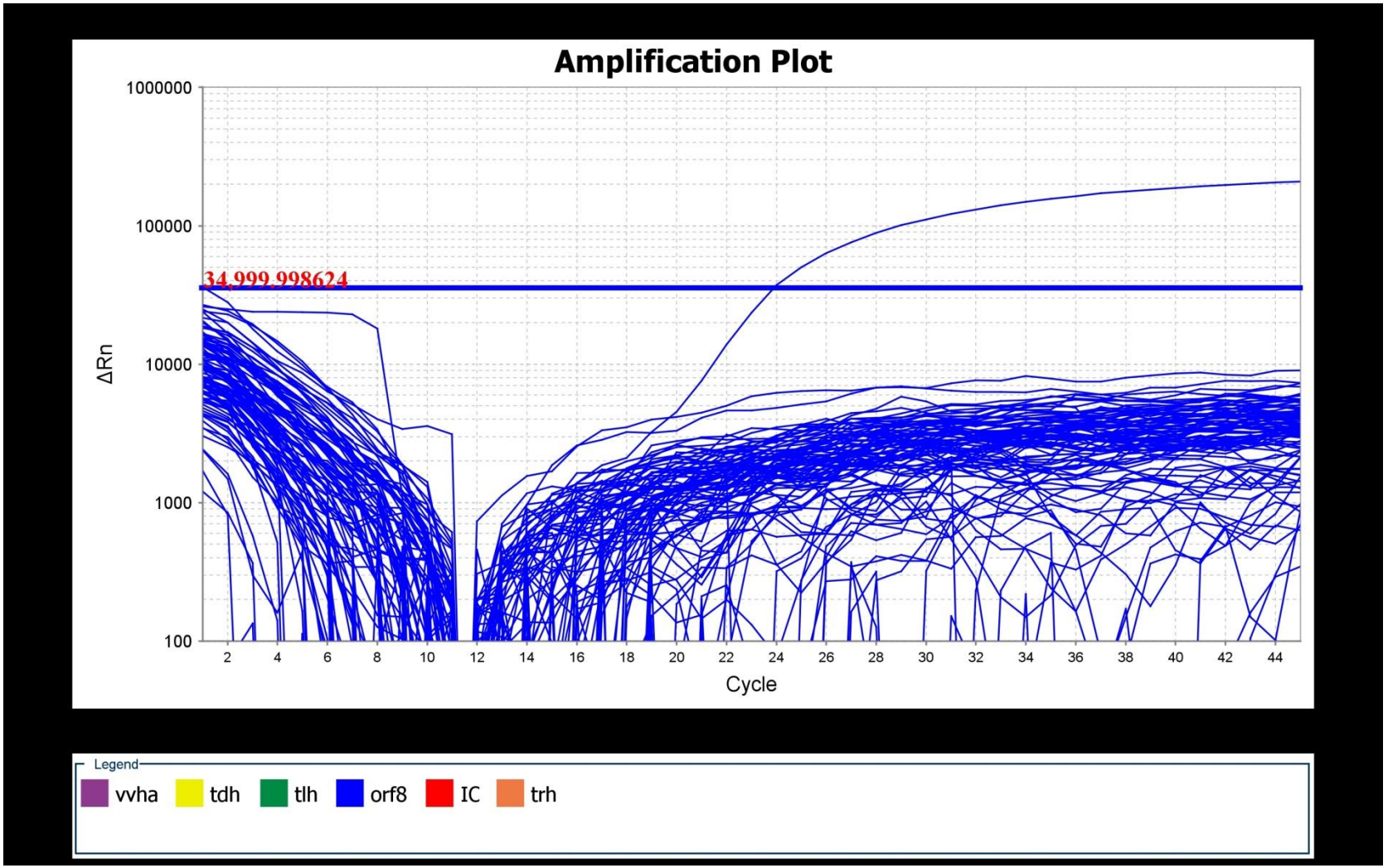


Figure 9. ORF8 gene (*ORF8*) amplification plot. This target was added to the assay to detect pandemic Vibrio, we did not have any positive wells in 2014.

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Procedure:
Vibrio parahaemolyticus enumeration and detection through MPN and real-time PCR

StarLims StarDoc ID #: _____ StarLims Version #:

Approved By:

Section Lead: _____ _____ _____
Name Signature Date

Supervisor: _____ _____ _____
Name Signature Date

Office Director: _____ _____ _____
Name Signature Date

Laboratory Director: _____ _____ _____
(if necessary)* Name Signature Date

Supersedes Procedure of: _____
(Date)

* The *Laboratory Director* will sign all procedures that are new or where there has been major changes in the procedure.

Principle

The purpose of this test is to rapidly quantify *Vibrio parahaemolyticus* (*Vp*) from oysters using a high throughput MPN based real-time PCR protocol. Culture based assays for the enumeration of *Vp* require a minimum of four days and require the Kanagawa test (media based) to detect pathogenicity. This assay was designed to provide quantitative results for total *Vibrio parahaemolyticus* (*Vp tlh+)*, known pathogenic markers of *Vibrio parahaemolyticus* (*Vp tdh*+ and *Vp trh*+), as well as some strains of potential pandemic *Vibrio* (*Vp* ORF8+). Additionally, the assay utilizes an exogenous internal control (WA IC) and an investigational marker (*vvhA*) for *Vibrio vulnificus* (*Vv*).

This test utilizes Taqman® probe real-time polymerase chain reactions to amplify 4 target genes from the *Vibrio parahaemolyticus (Vp)* genome as well as 1 target from the *Vibrio vulnificus* (*Vv*) genome.

Vp

1) The thermolabile hemolysin*, tlh* gene
2) The thermostable direct hemolysin*, tdh* gene
3) The thermostable direct related hemolysin*, trh* gene
4) The filamentous phage (f237) ORF8, gene

Vv

1) The cytolysin-hemolysin, *vvhA* gene

Pre-Analytic

1. Test Ordering Process

1.1 Test Ordering Process Flow Chart-N/A

2. Specimen Collection

2.1 Specimen Collection Process Flow Chart-N/A
2.2 Specimen Collection Procedures-N/A
2.3 Safety Considerations-N/A

3. Specimen Transport

3.1 Samples should be shipped in waterproof, puncture resistant containers. Samples should not come into direct contact with ice.

4. Specimen Receiving and Processing

4.1 Specimen Receiving and Processing Flow Chart-N/A
4.2 Specimen Receiving and Processing Procedures-N/A

5. Specimen

5.1 Criteria For Rejection of Sample
5.1.1 Samples received over 10°C are considered acceptable only under the following conditions:
5.1.1.1 The sample is shipped properly (adequate ice/cold packs) and was at an elevated temperature at collection and has had a short transit time (collected and received within 12 hours).

5.1.2 Sample processing at the lab must be initiated no more than 24 hours after collection. Samples received more than 24 hours after collection are considered unsatisfactory.

6. Other

6.1 Problems or Pitfalls-N/A

6.2 Computer Activities-N/A

Analytic

1. Reagents and Media

1.1 Materials

1.1.1 Phosphate Buffer Saline

1.1.2 Alkaline Peptone Water

1.1.3 Isopropanol, 70%

1.1.4 RNAse AWAY®

1.1.5 DNA Extraction Reagents – MagNA Pure LC

1.1.6 MagNAPure LC DNA Isolation Kit III (Bacteria, Fungi), (Roche, Cat. # 3264785)

1.1.7 PCR Reagents

1.1.7.1 Master Mix: Bioline SensiFAST™ Probe Hi-ROX Kit (BIO-82020)

1.1.7.2 Sterile Millipore water or other PCR grade water

1.1.7.3 TE buffer 1:10

1.1.7.4 Primers (See appendix A for sequences)

1.1.7.5 Probes (See appendix A for sequences)

1.2 Preparation

1.2.1 Media

1.2.1.1 Ingredients

1.2.1.1.1 Stock Buffer Solution

1.2.1.1.1.1 Potassium di-hydrogen Phosphate (KH_2PO_4) (J. T. Baker Cat. No. 3246-01 or equivalent)

1.2.1.1.1.2 Distilled Water

1.2.1.1.2 Phosphate Buffer Saline

1.2.1.1.2.1 Sodium Chloride (NaCl) (J.T. Baker Cat. No. 3264-05 or equivalent)

1.2.1.1.2.2 Stock Buffer Solution

1.2.1.1.2.3 Distilled Water

1.2.1.1.3 Alkaline Peptone Water

1.2.1.1.3.1 Peptone (BD Bacto Peptone Ref. No 211677)

1.2.1.1.3.2 Sodium Chloride (NaCl) (J.T. Baker Cat. No. 3264-05 or equivalent)

1.2.1.1.3.3 Distilled Water

1.2.1.2 Formulation and Prepartion

1.2.1.2.1 Stock Buffer Soulution

1.2.1.2.1.1 Mix 17.0 grams of KH_2PO_4 with 250mL of DI water. Adjust pH to 7.2 using 1N NaOH. Using distilled water and volumetric flask, bring volume to 500mL. Autoclave 15 minutes at 121° C. Store in refrigerator.

1.2.1.2.2 Phosphate Buffer Saline (PBS)

1.2.1.2.2.1 Mix 8.5 grams NaCl with 1.25mL of Stock Buffer Solution. Dilute to 1 liter with distilled water. Adjust pH to 7.4. Autoclave for 15 minutes @ 121° C. Final pH should be 7.2-7.5.

1.2.1.2.3 Alkaline Peptone Water (APW)

1.2.1.2.3.1 Mix 10 grams of Peptone and 10 grams of NaCl in 1 liter of distilled water. Adjust pH to 8.5 +/- .02. Autoclave 10 minutes at 121° C.

1.2.2 Reagents

1.2.2.1 Record the receipt and preparation of all reagents in the PCR reagent log book. All reagents must be labeled with a unique reagent number. The reagent number is sequentially recorded in the PCR reagent log where you can find, prep date, concentration, date received, date made, date opened, expiration date etc. The intent of the PCR reagent log book is to allow for complete reagent traceability. Therefore, the reagent numbers assigned to each item used must appear on the master mix prep sheet. Additionally the unique mastermix prep number assigned to each batch of mastermix must appear on the data sheet for all samples for which it was used. All sheets must be retained to allow traceback.

1.2.2.2 Use either filtered or sterilized MQ water from the Millipore filtration system or use commercially available PCR grade water. It is advisable to aliquot the water into smaller portions to minimize the potential for large scale contamination.

1.2.2.3 Prepare TE buffer by adding 10mL 1M Tris-HCl (pH 8.0) + 2mL 0.5M EDTA (pH 8.0). Dilute to a final volume of 1000mL MQ water. Autoclave. Store at room temperature.

1.2.2.4 Prepare supermixes containing primers and probes according to the worksheet. All sequences can be seen in appendix. Primers are diluted to 100µM stock concentrations with TE Buffer and stored at -20°C until use for supermix preparation.

1.2.2.5 Prepare exogenous control by adding 100µL TE Buffer to tube. Stock concentration will vary depending on volume synthesized. Create working concentration by diluting stock 1:10,000 in TE buffer. Freeze working concentration in 10µL aliquots (-20°C), before use add 990µL molecular grade water.

1.3 Performance Parameters-N/A

1.4 Storage Requirements

1.4.1 Container

1.4.1.1 Molecular grade water and TE Buffer can be stored in 50mL conical tubes

1.4.1.2 Primers, probes, and supermixes can be stored in 1.7mL Eppendorf tubes. Supermixes are to be stored in low light transmitting tubes.

1.4.2 Temperature

1.4.2.1 Store primers and probes at -20°C

1.4.3 Stability

1.4.3.1 Reagents free from contamination are good for 1 year unless otherwise stated by manufacturer. Stocks of primers and probes are good for 5 years in freezer (-20°C). Working concentrations of primers and probes are good for 6 months in freezer (-20°C) Thawed working concentrations (Supermix) of primers and probes are good for 2 weeks refrigerated (2-8°C).

1.4.4 Labeling

1.4.4.1 Labels should include: reagent name, date received, date prepared or reconstituted (if necessary), expiration date, and storage temperature

2. Equipment

2.1 Type of Equipment

2.1.1 Thermometer 0°C-10°C
2.1.2 Sink
2.1.3 Blender with sterile blender jars.
2.1.4 Timer
2.1.5 Vortexer
2.1.6 Bulb pipetter
2.1.7 Incubator (35°C±0.5)
2.1.8 Dry bath, 70°C
2.1.9 Refrigerator, 2-4°C
2.1.10 Freezer, -20 to -80°C (-80°C preferred)
2.1.11 Microcentrifuge
2.1.12 Autoclavable waste container
2.1.13 Biological safety cabinets (BSC) or Air Clean PCR stations
2.1.14 Rainin pipettes
2.1.14.1 P-2
2.1.14.2 P-10
2.1.14.3 P-20
2.1.14.4 P-200
2.1.14.5 P-1000
2.1.14.6 Multi-channel (8) 2µL-25µL
2.1.15 Applied Biosystems® ViiA™ 7 Real Time PCR station
2.1.16 Roche MagNaPure LC DNA purification system
2.1.17 Ice bucket/refrigerated block
2.1.18 Vortexer
2.1.19 Refrigerator, 4°C
2.1.20 Freezer, -20 to -80°C
2.1.21 Autoclavable waste container
2.1.22 Microcentrifuge
2.1.23 PCR plate centrifuge.
2.1.24 384-well microtiter-plate stand

2.2 Preparation-N/A

2.3 Performance Parameters

2.3.1 Equipment should perform within the manufacturer's specifications
2.3.2 Preventative maintenance is performed at least yearly or as needed
2.3.3 Refrigerators, incubators, and freezers are monitored daily for correct temperatures.
2.3.4 Maintenance and decontamination duties are performed on a regular basis to keep equipment in good working order and to reduce the chances of PCR contamination.

3. Supplies

3.1 Sterile scrub brushes
3.2 Sterile oyster knives

3.3 Clean specimen trays
3.4 Paper towels
3.5 Sterile wide mouth containers (1 liter capacity)
3.6 Oyster shucking block
3.7 Chain-mail glove
3.8 Dishwashing gloves
3.9 Glass or serological pipette tips
  3.9.1 25mL
  3.9.2 10mL
  3.9.3 5mL
  3.9.4 1mL (0.1mL hashes)
3.10 Rainin filtered pipette tips
  3.10.1 1000µL tips
  3.10.2 200µL tips
  3.10.3 10µL tips
3.11 Microcentrifuge tubes (1.7mL)
3.12 Reagent Tubs – Large
3.13 Reagent Tubs - Medium - 20mL
3.14 Reagent Tub Lids – Large
3.15 Reagent Tub Lids - S/M
3.16 MPLC Rxn Tips – Large
3.17 MPLC Rxn Tips – Small
3.18 MPLC Rxn Tips - L Refill
3.19 MPLC Rxn Tips - S Refill
3.20 MPLC Processing Cartridge
3.21 MPLC Sample Cartridge
3.22 Tip Stands
3.23 Waste Bags
3.24 Cartridge seals
3.25 Gloves, nitrile
3.26 384 well PCR plates
3.27 Optical covers
3.28 Cartridge seals
3.29 Sterile disposable reagent reservoirs (small volume 1-5mL)

4. Specimen

4.1 *Crassostrea gigas* (Pacific oyster)

5. Special Safety Precautions

5.1 *Vibrio* species are pathogenic and should be handled following PHL safety guidelines.
5.2 Dry baths can reach temperatures of 130° C; do not touch heated blocks. Tightly sealed tubes may burst if heated beyond safe levels
5.3 UV light can seriously burn skin and eyes. Keep safety shield lowered when UV light is on. Always keep skin covered by lab coat and gloves

6. Quality Control

6.1 Materials
  6.1.1 Pre-extracted *Vibrio parahaemolyticus* (ATCC BAA-240, WA4647) and *Vibrio vulnificus* (ATCC 27562) DNA is blended and aliquoted in 10µL aliquots and frozen at -20°C

6.1.2 Molecular grade water
6.1.3 Exogenous internal control plasmid containing a target fragment

6.2 Instructions

6.2.1 An extraction control is included in every extraction run. To prepare the extraction control material, enrich *V. parahaemolyticus* (ATCC 49398, *tlh*+, *tdh*-, *trh*-, ORF8-) overnight in APW. The material is lysed (MagNAPure protocol) and aliquoted into 1.7mL microcentrifuge tubes. Store the positive control lysate at -20°C to -80°C until needed.

6.3 Frequency

6.3.1 An NTC, exogenous internal control and positive control are included with every run

6.4 Acceptable Limits

6.4.1 A successful run should meet the following conditions:

6.4.1.1 No amplification should be present in any of the negative controls. Creeping curves without a logarithmic increase are not considered true amplification. If amplification is present in a negative control, determine the source of contamination, thoroughly clean BSC and pipettes, and discard contaminated reagents and disposables.

6.4.1.2 The positive control should show clear amplification. If NO amplification is present in the positive control for one or both of the sets, determine the problem and re-run the sample (see troubleshooting).

6.4.1.3 Wells negative for all targets should show amplification for the exogenous control (IC). The IC may be negative in positive samples.

6.5 Corrective Action

6.5.1 Runs for which the NTC is positive or the positive control and/or internal control is negative should be repeated

6.5.2 The Lead Microbiologist should be notified if any run fails QC

6.6 Recording QC Data

6.6.1 Record results for each well and record a + or – on the *Vp* worksheet

7. Calibration

7.1 Standard Prep

7.1.1 List of Standards Used
7.1.2 Directions for Preparation
7.1.3 Special Instructions
7.1.4 Degree of Accuracy
7.1.5 Storage Requirements
7.1.6 Container
7.1.7 Temperature
7.1.8 Stability
7.1.9 Labeling

7.2 Calibration Procedure

Testing Procedure

8. Quantitative Testing Procedure

8.1 Accessioning, Sample Preparation and Centrifugation

8.1.1 Samples are processed in accordance with recommended procedures described by the American Public Health Association (4). Oyster samples are removed from the shipping container and the sample submission form is located.

A laboratory worksheet is generated for each sample. Both the sample submission form and the lab worksheet are stamped in with the appropriate lab number. One oyster from each bag is opened to take tissue temperature. The temperature is recorded on the sample submission form. The bag of oysters is labeled with the sample number and placed into a 2-4°C refrigerator until they are ready to be processed.

8.2 Detailed Stepwise Procedure

8.2.1 The intent of the assay is to determine the concentration of *Vp* in the oyster tissue and liquor. Any material on the outside of the oyster that gets introduced into the interior of the animal during shucking can alter the concentration.

8.2.1.1 Clean the sink before beginning to scrub the oyster sample. The sink must be clean at this stage but sterility is not required.

8.2.1.2 Before beginning the scrubbing process, wash gloves with soap and water. Using sterile scrub brushes, each oyster is cleaned under cold running water. All barnacles, mud, vegetation and debris should be removed. Pay close attention to the hinge and shell seam. A sterile brush should only be used for one sample. Do not re-use brushes when scrubbing multiple samples.

- Any oyster that does not tightly close during handling is likely dead and should be discarded.
- In addition any oyster whose shell is broken to expose tissue should be discarded.

8.2.1.3 After cleaning each oyster place the animal upside down on a clean, labeled, paper towel lined tray. Laying the oysters upside down will prevent the liquor (fluid inside a closed oyster) from draining out of the oysters while waiting to be shucked. Clean trays must be used for each sample.

8.2.1.4 Once cleaned, return the oysters to the refrigerator to dry or towel dry them for immediate shucking.

8.2.2 In order to accurately quantify *Vp* in oyster tissue it is very important to avoid introduction of bacteria (*Vp* or other) into the oyster tissue.

8.2.2.1 The sink must be sterile before shucking can begin. Wash the sink with water or soap and water. Completely dry the sink. Wipe the sink down with 70% isopropanol and allow it to air dry.

8.2.2.2 Place a sterile pre-weighed (remove rubberband prior to weighing) tissue collection container on the sink counter.

8.2.2.3 Wipe a shucking block down with 70% isopropanol and place on the sink counter to air dry.

8.2.2.4 Place the oyster sample to be shucked on the sink counter.

8.2.2.5 Put on clean nitrile gloves. Over one of the gloves put on a chain-mail glove. The chain-mail glove should be on the hand that will not be holding the knife. Put another nitrile glove on over the chain-mail glove. Cover both hands in 70% isopropanol and allow them to air dry.

8.2.2.6 Grab and hold each oyster with the chain-mail hand and use the other hand and a sterile oyster knife to shuck each oyster.

8.2.2.7 Use the sterilized shucking block while shucking to minimize knife accidents and to protect the counter surface.

8.2.2.8 Collect all tissue and liquor (fluid) in the sterile pre-weighed container.

8.2.2.9 A fresh knife, shucking container and gloves must be used for each specimen.

8.2.2.10 The shucking block and counter must be washed and sterilized between specimens.

8.2.3 Enumeration in this assay is achieved by conducting an MPN (most probable number) analysis.

8.2.3.1 Weigh the container and shellfish tissue. Subtract the container weight from the total weight to determine the amount of oyster tissue and liquor. Record the weight of tissue on the sample worksheet.

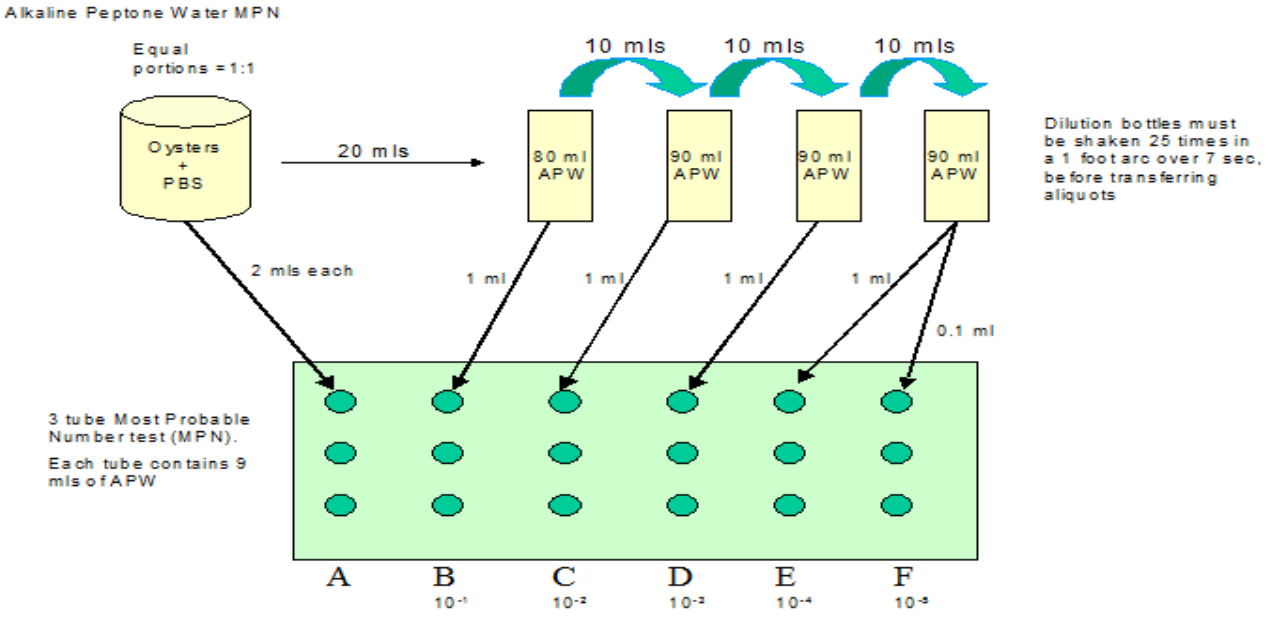
8.2.3.2 Transfer the oyster tissue and liquor to a sterile blender jar.

8.2.3.3 Add an equal weight of PBS to the sample container (the PBS can be used to rinse any residual tissue from the container). Transfer the PBS to the blender jar. Record the weight of PBS used on the sample worksheet.

8.2.3.4 Blend the shellfish sample with PBS (now a 1:2 dilution) at high speed for 90 seconds.

8.2.3.5 The resulting homogenate should be relatively smooth. If the blender isn't generating a smooth homogenate, it is advisable to service the blender (sharpen/replace blades).

8.2.3.6 From this homogenized sample, set up a 3-tube most probable number (MPN) serial dilution series. Use PBS for making dilutions and alkaline peptone water (APW) as the enrichment broth in each of the MPN tubes.



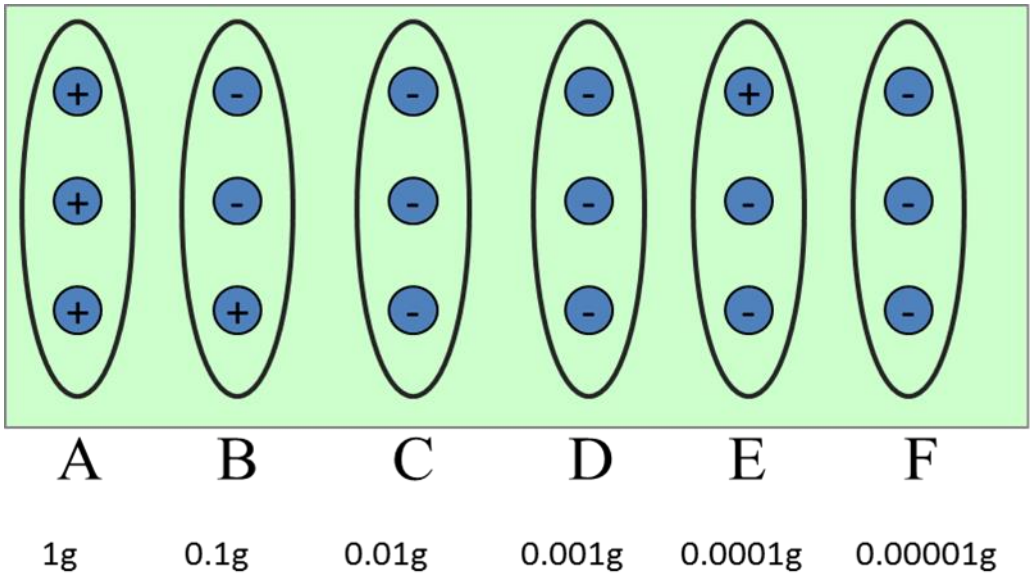
8.2.3.7 Incubate at 35°C± for 18 to 24 hours. Write sample number, date, and time placed in incubator on tube A1.

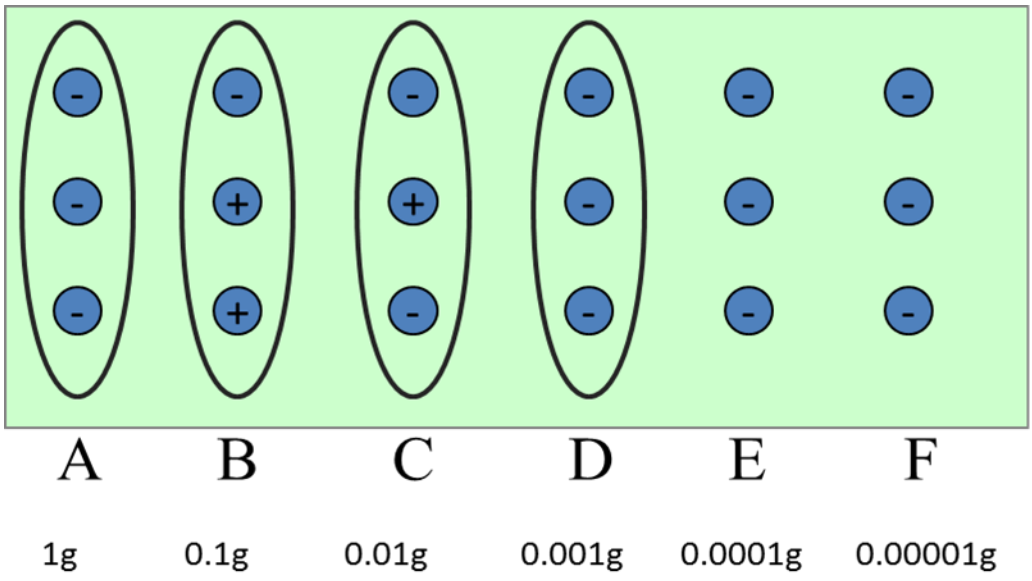
8.2.3.8 Following the 18-24 hour incubation step, each APW tube must be checked for growth. Use the following criteria to select tubes for further testing.

8.2.3.9 Examine all tubes for turbidity. Examine each tube with a light source shining through the tube.

8.2.3.10 Record all positive and negative results on the lab worksheet.

8.2.3.11 The following examples illustrate the selection process. Each tube is labeled as +/- for turbidity. The dilutions circled should be selected for further testing.





8.2.3.12 If one tube in a given three tube dilution series is positive for turbidity all tubes in that series must be tested (you will still record the actual positive or negative values).

8.2.3.13 In addition, test one complete dilution series beyond the last series that contained any growth and all tubes of higher concentration.

8.2.4 DNA Extraction

8.2.4.1 DNA extraction must be initiated within the 18-24 hour incubation window.

8.2.4.2 Reagent prep should be carried out in the Pre-PCR Lab in order to minimize the potential for contamination. The lysis procedure itself may be carried out in the DNA extraction room or in the main lab on the bench. Once complete, the material is considered stable and may be stored at 2-4°C for 1-

2 days or frozen at -20 to -80°C for extended periods. NOTE: Multiple freeze thaw cycles should be avoided due to potential DNA degredation.

8.2.4.3 Each tube that is selected for additional testing will require one 1.7mL microcentrifuge tube that was pre-loaded with lysis buffer and proteinase K (proK). (130µL lysis buffer + 20µL proK provided in MagNA Pure kit)

8.2.4.4 Label each tube with the sample number and tube ID.

8.2.4.5 Vortex each APW tube that is to be extracted briefly.

8.2.4.6 Pipette 1mL from each APW tube to its labeled 1.7mL tube.

8.2.4.7 Briefly vortex each 1.7mL tube.

8.2.4.8 Place the 1.7mL tubes in the 65°C dry bath for 10 minutes.

8.2.4.9 The lysed tubes are now ready for DNA extraction. **NOTE:** Due to the high number of DNA extractions that are required as part of this assay, the Roche MagNA Pure was chosen for initial validation.

8.2.4.10 Lysed material from each 1.7mL tube should be manipulated in either the DNA extraction room or the template addition lab.

8.2.4.11 At this point due to the high number of lysates it is necessary to create a document to track the location of each lysate. This document is referred to as the “MagNA Pure Plate Map”.

8.2.4.12 After the MagNA Pure Plate Map is created, load the MagNA Pure cartridge accordingly. Loading should take place in a BSC or an AirClean hood. 200µL of each lysate should be added to the plate. Include 200 ul of pre-lysed *Vp* culture and 200µL of sterile PCR grade water. These will act as controls for the assay.

8.2.4.13 Once loaded, seal the MagNA Pure cartridge with an adhesive cartridge seal.

8.2.4.14 Label the plate with the sample number, date, initials and the label “Pre-Ext”. The specimen can now be loaded onto the MagNA Pure.

8.2.4.15 Turn on both the computer and the MagNA Pure.

8.2.4.16 Ensure that the correct protocol is selected “DNA III Bacteria”.

8.2.4.17 Enter in your sample order. Be sure that the correct cartridge wells are highlighted on the screen. Incorrect set up here will lead to a failed extraction.

8.2.4.18 Sample volume should be entered as 200µL.

8.2.4.19 Elution volume should be entered as 100µL.

8.2.4.20 Click the “Stage Set-up” button.

8.2.4.21 Begin adding in the appropriate plastics. Click the items on the screen as you add them to the MagNA Pure. Make sure that the discard bag is not too full before you begin your extraction.

8.2.4.22 Once all plastics are added and the discard bag is replaced remove the reagent tray from the machine.

8.2.4.23 Add the amount requested of each color coded reagent to the appropriate size of tub and apply a lid (with holes for tip access). Using the wrong tub will likely lead to a failed extraction.

8.2.4.24 Before adding the Magnetic Glass Particles (MGPs), vortex them for 15-20 seconds to completely suspend them. Once the particles are completely re-suspended in solution, rapidly deliver the appropriate volume to the correct tub and cover. It is important to get the extraction started soon after the beads are pipetted as they will rapidly settle out of suspension.

8.2.4.25 Once the reagent tray is completely loaded, place the tray in the machine.

8.2.4.26 Remove the cartridge seal from the lysate cartridge and add it to the machine. Discard the seal into an autoclavable container.

8.2.4.27 Ensure that all plastics, reagents and sample cartridges are in place and accounted for on the computer screen.

8.2.4.28 Close the door and press the start extraction button.

8.2.4.29 Note the time that the run will be completed. The final extracted template DNA will be refrigerated on-board the MagNA Pure until it is removed. It is however not advisable to leave the extract uncovered for any length of time.

8.2.4.30 Once completed, open the door, remove the extracted DNA, immediately seal the cartridge with a new cartridge seal, and refrigerate at 2-4°C until ready for PCR (if PCR is to be completed in 1-2 days). If PCR will not be complete in the next two days freeze the DNA at -20 to -80°C.

8.2.4.31 Remove all soiled plastics, replace used tips and run the decontamination protocol from the MagNA Pure main screen.

8.2.5 PCR Mastermix Preparation

8.2.5.1 Mastermix preparation is performed in the Pre-PCR Lab biological safety cabinet or Airclean hood. This includes primer and probe manipulations and mastermix loading into the PCR plate. Thorough decontamination before and after use of the room is advisable. **NOTE**: A person who has previously in the same day worked in the Template addition lab should not re-enter the Pre-PCR lab.

8.2.5.2 Turn on the ABI® ViiA™7 machine and the computer.

8.2.5.3 Prepare a PCR platemap. Always include a positive and a negative control for each MagNA Pure plate that was extracted.

- Negative control: No DNA, milli-Q water (from MagNA Pure cartridge)
- Positive control: *tlh*+ *V. parahaemolyticus* ATCC 49398 (from MagNA Pure cartridge)

8.2.5.4 Prepare the mastermix according to the mastermix prep sheet (Appendix C). Determine how many reactions you will need and calculate how much of each component you must use. It is advisable to prepare several reactions more than are needed to account for pipetting variability.

8.2.5.5 Once prepared, briefly vortex to completely mix the components.

8.2.5.6 Add the mastermix to a sterile disposable reagent reservoir.

8.2.5.7 Using a pipetter (multichannel advisable), add 18µL of mastermix to each appropriate well (384 well plate) according to the PCR platemap.

8.2.5.8 Once the plate is loaded with mastermix, place the plate in a biological transport container (sealed box) and take the plate into the Template addition lab. It is important to quickly add the template, seal the plate and initiate PCR. Therefore make sure that the extracted DNA is ready before setting up your PCR reactions.

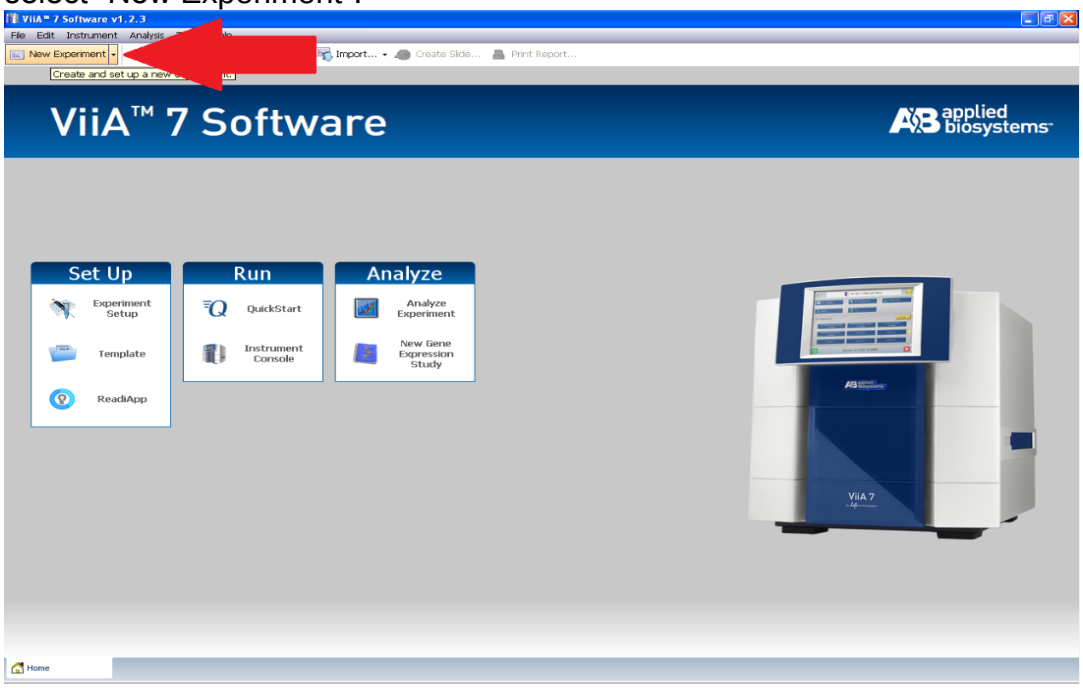
8.2.5.9 Add the extracted DNA template to the appropriate wells according to your PCR platemap. Use 2µL of DNA for a total reaction volume of 20µL.

8.2.5.10 Once all wells are loaded including the positive and negative controls, seal the plate with an optical seal. Avoid touching either side of the seal as the adhesive side will come into contact with your reactions (poses a

contamination risk) and the outside must be clear of smudges to allow accurate readings. Apply the seal using the plastic applicator supplied with the instrument. Take care to completely seal each well. Any unsealed well will rapidly evaporate during PCR and lead to inaccurate results.

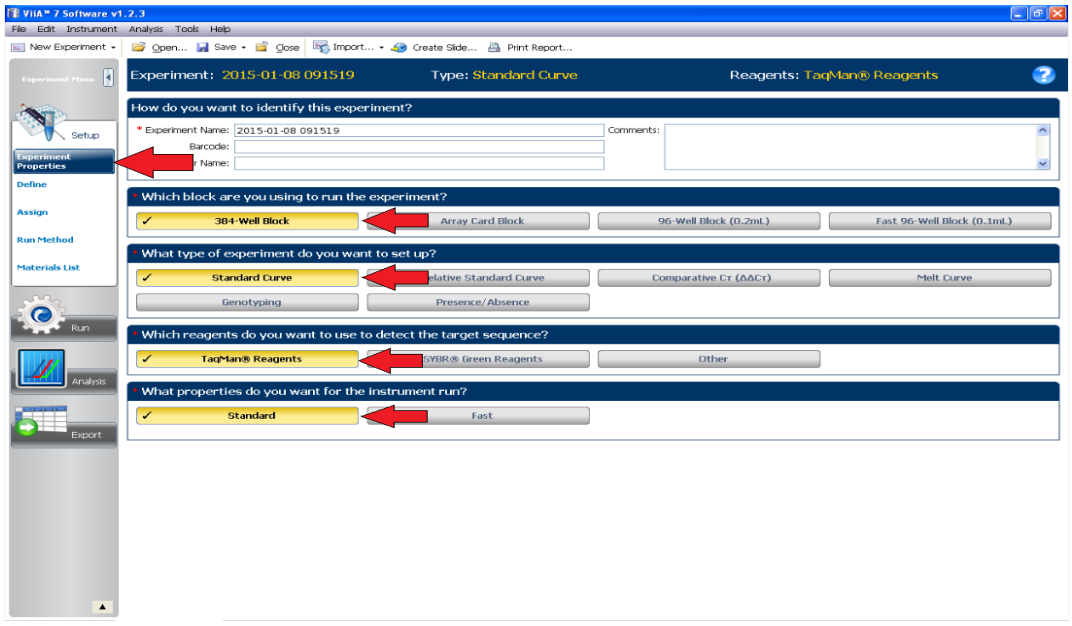
8.2.5.11 Once the plate is sealed, centrifuge the plate briefly to remove bubbles from the wells and ensure that the template is in contact with the reaction mix.

8.2.5.12 Return to the ABI ViiA™7. Open the SDS software, under the File menu select “New Experiment”.

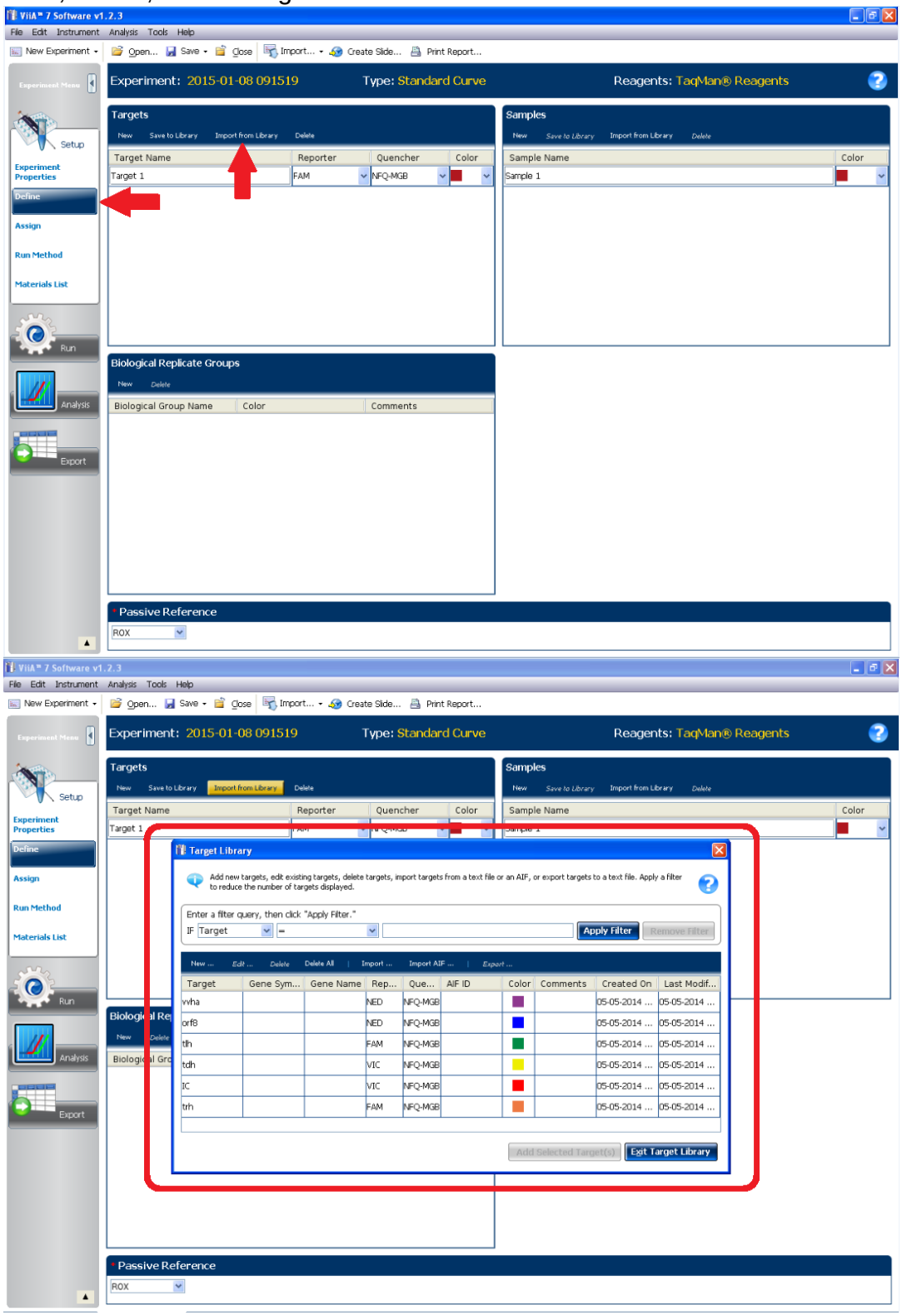


8.2.5.13 Rename experiment with the appropriate sample numbers and date of run.

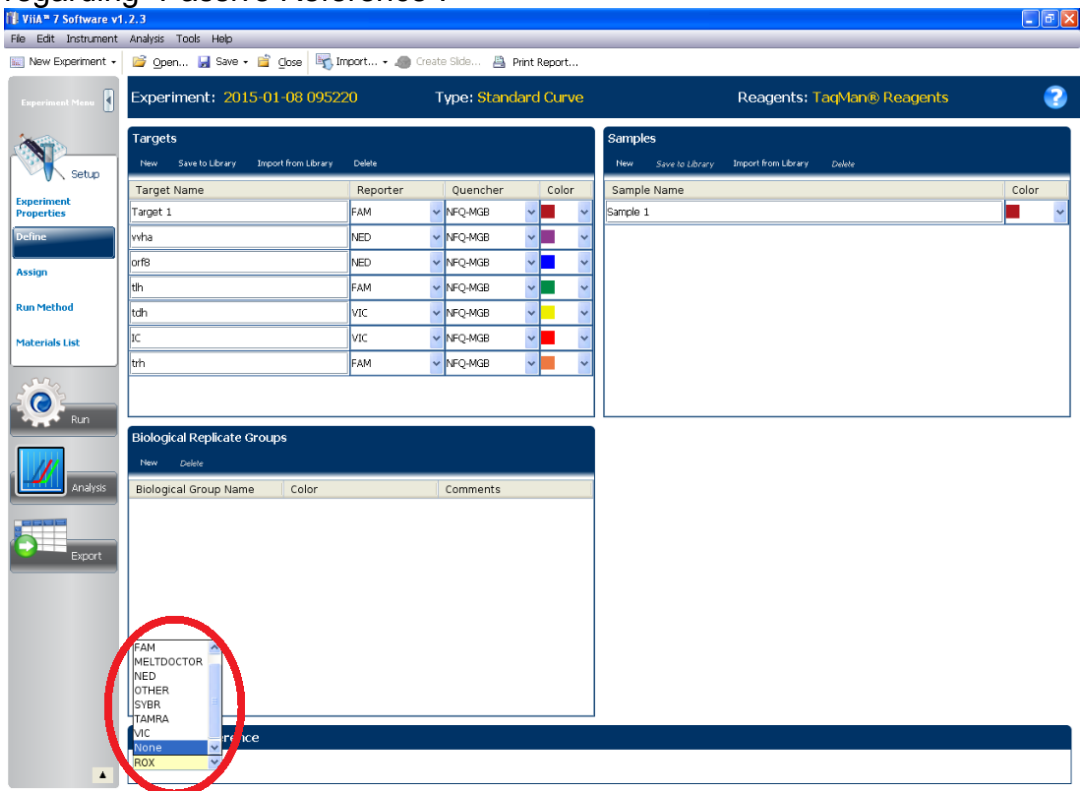
8.2.5.14 Under the tab “Experiment Properties” ensure “384-Well Block”, “Standard Curve”, TaqMan® Reagents”, and “Standard” (for run mode) are selected.



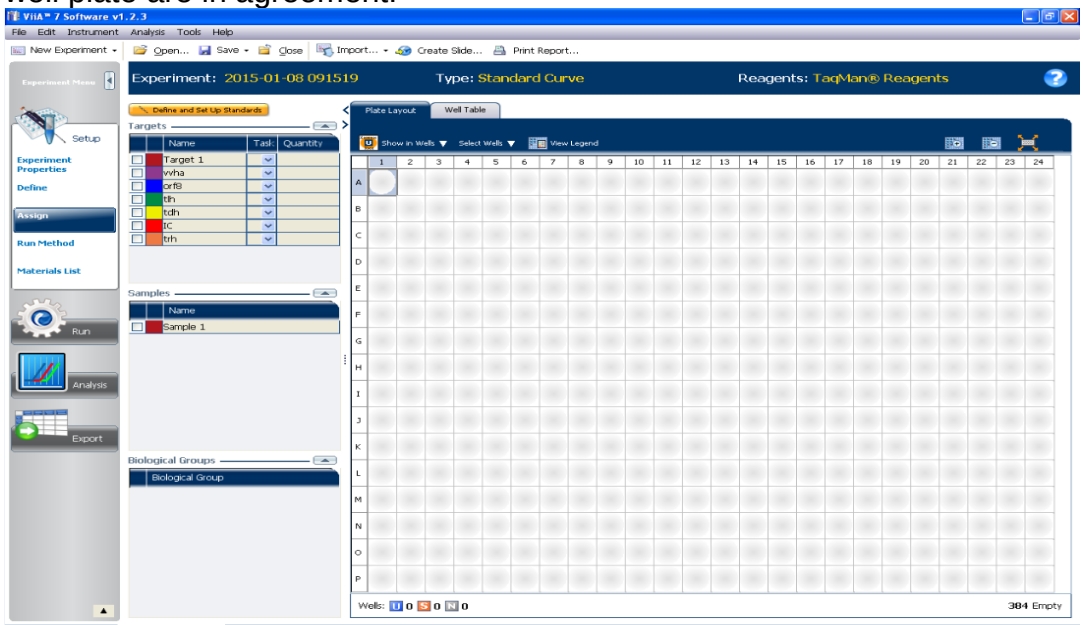
8.2.5.15 The “Define” tab is used to select the targets of detection. The targets are saved to the library. Import targets from library. Select the *tlh, tdh, trh, vvha,* ORF8, and IC targets.



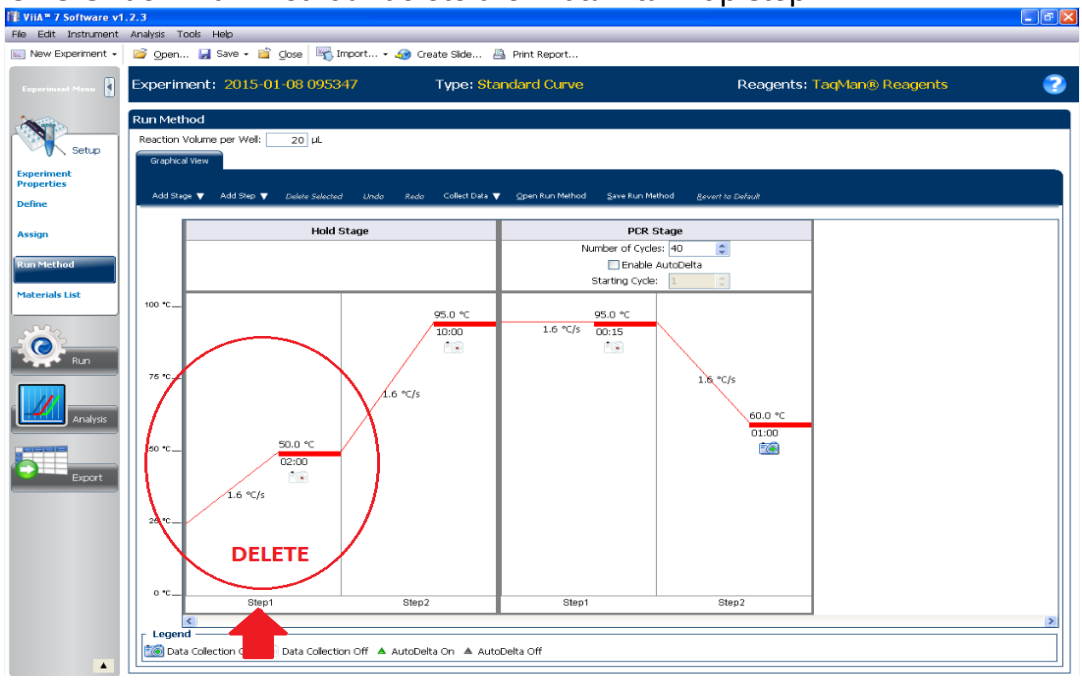
8.2.5.16 At the bottom of the screen, select “NONE” from the drop down menu regarding “Passive Reference”.



8.2.5.17 Select the “Assign” tab and assign the appropriate wells with the corresponding targets of interest. Be sure to double check the map and 384-well plate are in agreement.

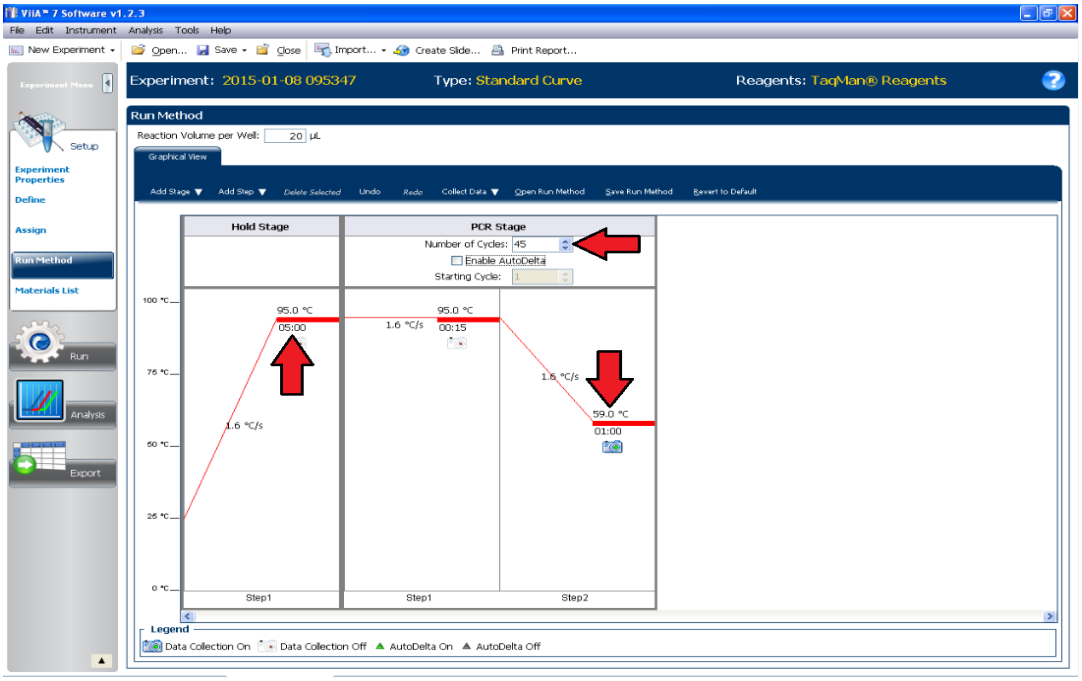


8.2.5.18 Under “Run Method” delete the initial warm-up step.



After doing so the parameters are the following:

- 95° C for 5 minutes
- 45 cycles of
  - 95° C for 15 seconds
  - 59° C for 60 seconds

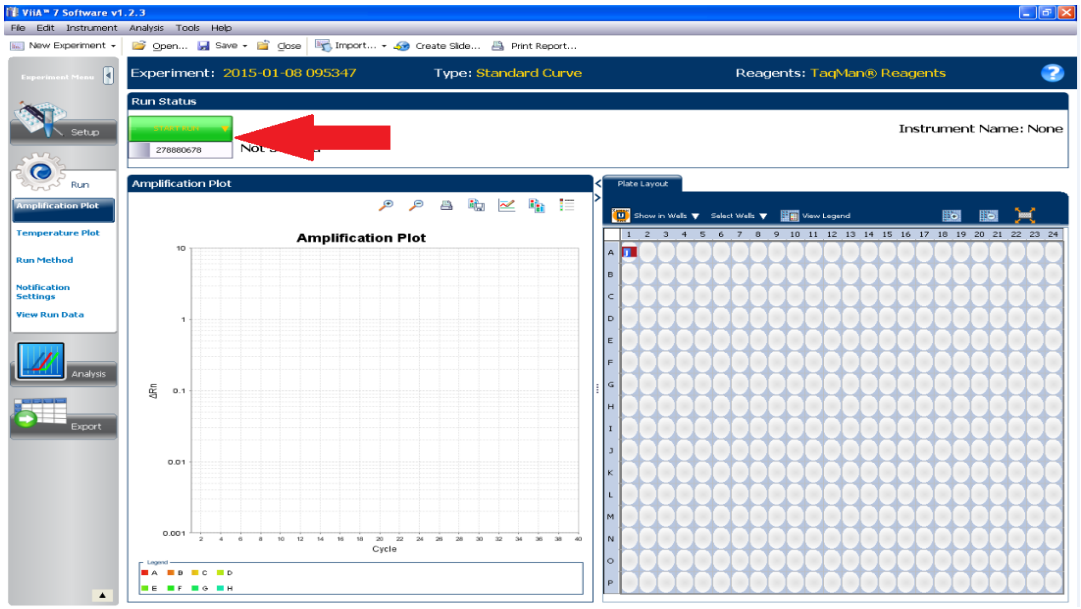


8.2.5.19 Turn on the machine user interface but touching the touchscreen. To open loading tray, touch the eject button.

8.2.5.20 Load the plate and close tray.

8.2.5.21 From the PC, click the “Run” tab and find the green “START RUN” button. Select the appropriate machine from the drop down menu and click

the “START RUN” button.



8.2.5.22 Save run file using sample numbers and date.

8.3 Measurements from Instruments

8.3.1 Once run is complete, remove plate from instrument and discard in appropriate waste receptacle.

8.3.2 Select “Analysis Settings” on the Amplification Plot screen.

8.3.3 Change all thresholds and baseline settings to “manual” and set all thresholds to 20,000 and leave baseline settings at 3 to 15.

8.3.4 Apply analysis settings and exit to Amplification Plot screen.

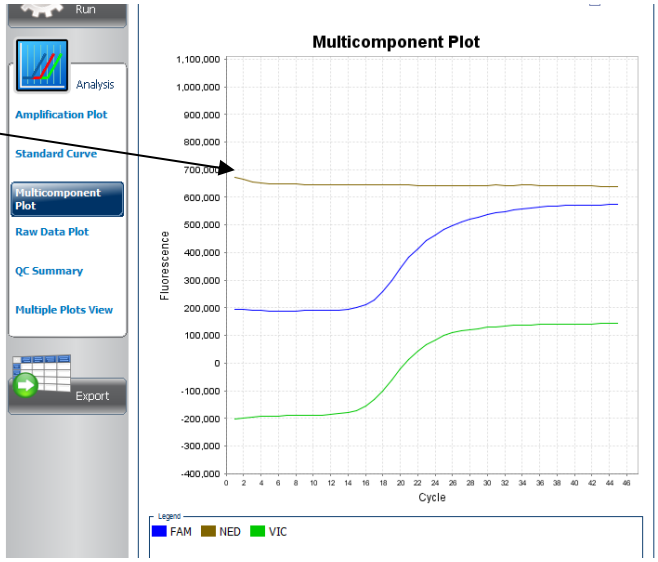
8.3.5 Select all wells in the plate by clicking in the upper left box of the plate layout.

8.3.6 View each target individually and make necessary changes to the threshold and baseline. The threshold should be set above background levels. It may be necessary to change baseline settings to lower background levels.

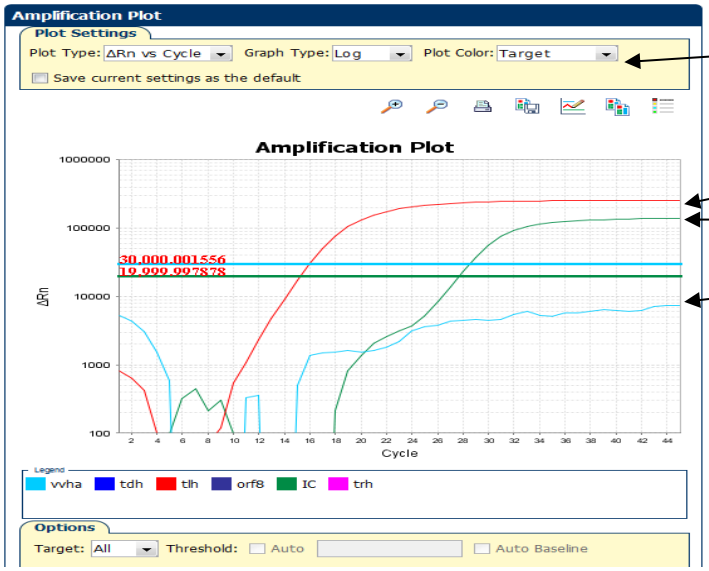
8.3.7 Acceptable threshold settings range from 10,000 to 50,000, if setting the threshold outside these values please consult with the lead or supervisor.

8.3.8 If baseline changes are necessary - view individual wells in the Multicomponent Plot screen. Change baseline settings as needed to exclude early background noise.

Exclude early background noise when setting baseline



8.3.9 Once threshold and baseline are set at appropriate levels, record results from each well for every target. Targets within the amplification plot may have high background in the early stages of the run (i.e. <10 cycles). Disregard background that crosses the threshold before cycle 10. Change the Plot color to "Target" to help read results.

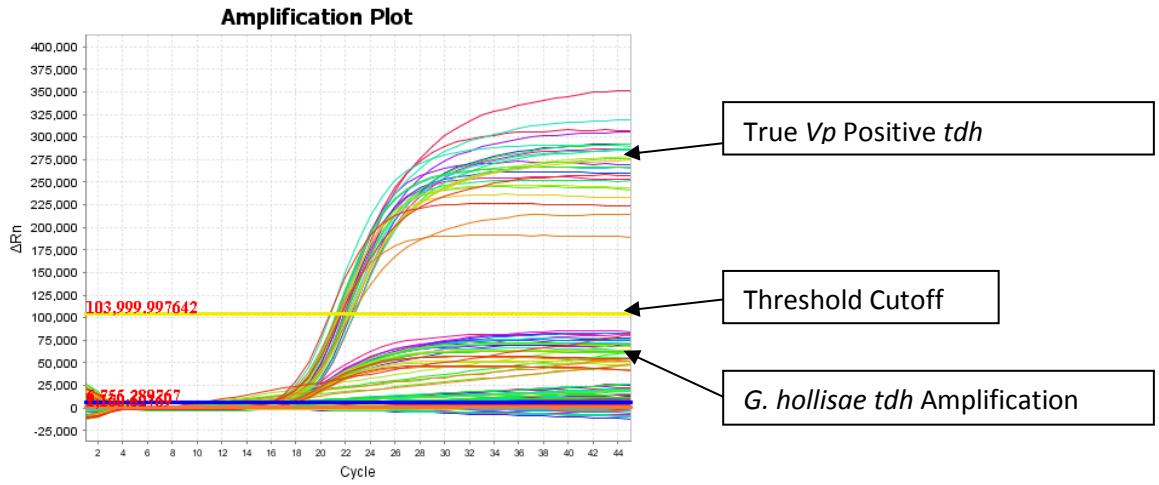


Plot Color = Target

Positive = amplification above threshold

Negative = No amplification

8.3.10 When reading *tdh* amplification results a ΔRn of greater than 104,000 (read in Linear setting) is necessary for a positive result. This cutoff will exclude any *G. hollisae tdh* amplification.



Post Analytic

1. Interpretation & Reporting of Results

1.1 Procedure for Abnormal Results

1.1.1 Abnormal MPN Index

1.1.1.1 If abnormal results appear to be caused by cross contamination (i.e. late CT value) rerun real-time PCR in duplicate of suspected contaminated wells

1.1.1.2 If duplicate results are in agreement, report these results. If the duplicates differ, report the results in agreement with ORIGINAL rtPCR run.

1.1.2 Positive pathogenic markers (*tdh, trh,* ORF8) in absence of *Vibrio parahaemolyticus* marker (*tlh)*

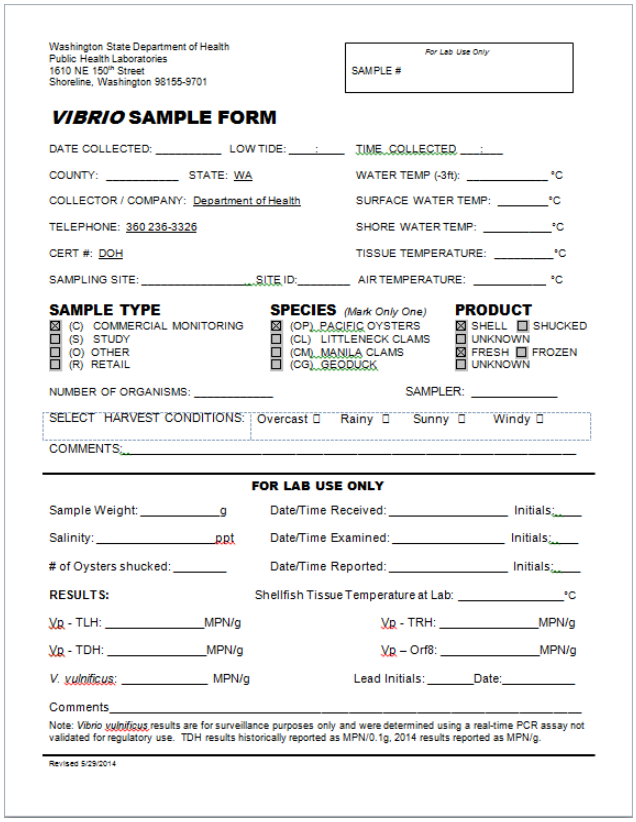
1.1.2.1 Current findings do not support pathogenic markers being present without *Vp* being present. Real-time PCR reactions resulting in this situation should be re-run upon Lead Microbiologist discretion.

1.1.2.2 The presence of the *trh* gene in the absence of the *tlh* gene has been documented. This is due to the *Vibrio parahaemolyticus trh* gene having 98% homology with the *trh* gene of *Vibrio alginolyticus*[i]. Any *trh* positive wells must be *tlh* positive as well.

1.2 Reporting Format

1.2.1 Real-time PCR results should be reported in MPN per gram according to FDA BAM 3 tube MPN chart

1.2.1.1 Sample Submission Form



Washington State Department of Health
Public Health Laboratories
1610 NE 150th Street
Shoreline, Washington 98155-9701

For Lab Use Only
SAMPLE #

VIBRIO SAMPLE FORM

DATE COLLECTED: _____ LOW TIDE: ___:___ TIME COLLECTED ___:___

COUNTY: _____ STATE: WA WATER TEMP (-3ft): _____ °C

COLLECTOR / COMPANY: Department of Health SURFACE WATER TEMP: _____ °C

TELEPHONE: 360.236-3326 SHORE WATER TEMP: _____ °C

CERT #: DOH TISSUE TEMPERATURE: _____ °C

SAMPLING SITE: _____ SITE ID:_____ AIR TEMPERATURE: _____ °C

SAMPLE TYPE
☒ (C) COMMERCIAL MONITORING
☐ (S) STUDY
☐ (O) OTHER
☐ (R) RETAIL

SPECIES *(Mark Only One)*
☒ (OP) PACIFIC OYSTERS
☐ (CL) LITTLENECK CLAMS
☐ (CM) MANILA CLAMS
☐ (CG) GEODUCK

PRODUCT
☒ SHELL ☐ SHUCKED
☐ UNKNOWN
☒ FRESH ☐ FROZEN
☐ UNKNOWN

NUMBER OF ORGANISMS: _____ SAMPLER: _____

SELECT HARVEST CONDITIONS: Overcast ☐ Rainy ☐ Sunny ☐ Windy ☐

COMMENTS:_____

FOR LAB USE ONLY

Sample Weight: _____g Date/Time Received: _____ Initials:____

Salinity: _____ppt Date/Time Examined: _____ Initials:____

# of Oysters shucked: _____ Date/Time Reported: _____ Initials:____

RESULTS: Shellfish Tissue Temperature at Lab: _____°C

Vp - TLH: _____MPN/g Vp - TRH: _____MPN/g

Vp - TDH: _____MPN/g Vp – Orf8: _____MPN/g

V. vulnificus: _____ MPN/g Lead Initials: _____Date:_____

Comments_____

Note: *Vibrio vulnificus* results are for surveillance purposes only and were determined using a real-time PCR assay not validated for regulatory use. TDH results historically reported as MPN/0.1g, 2014 results reported as MPN/g.

Revised 5/29/2014

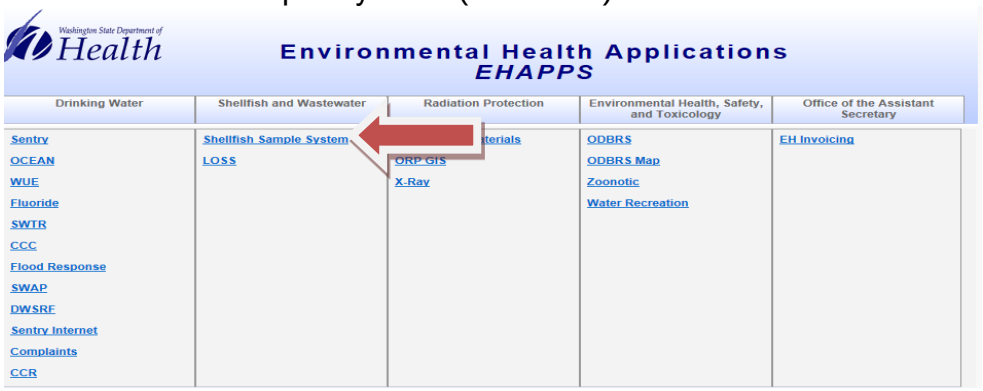
1.3 Prioritizing Results-N/A

1.4 Entering Laboratory Results in Reporting System

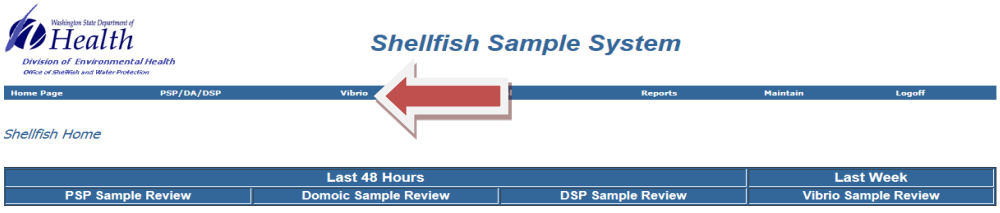
1.4.1 Office of Shellfish and Water Protection database

1.4.1.1 Access to the database must be authorized. Lead Microbiologist will facilitate the authorization process.

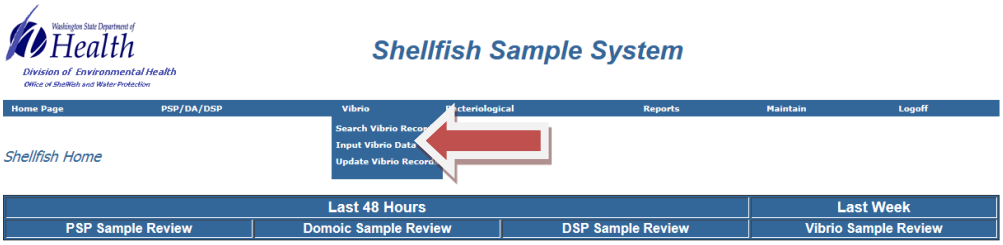
1.4.1.2 After entering web address, find column “Shellfish and Wastewater” and click “Shellfish Sample System” (red arrow)



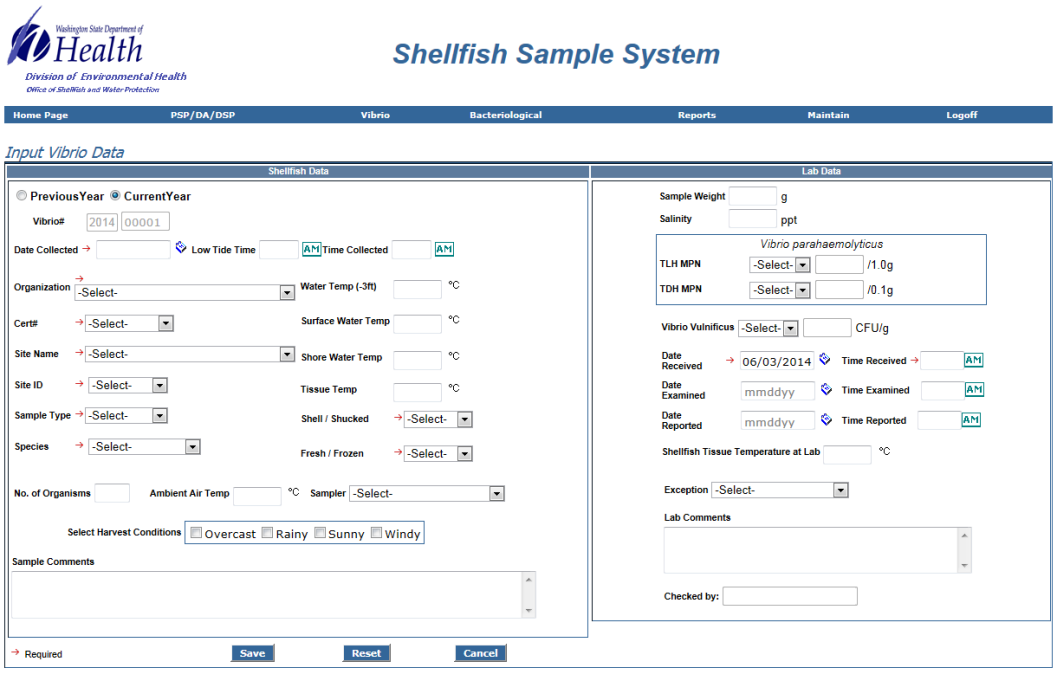
1.4.1.3 Along the top, hover mouse over “Vibrio” (red arrow)



1.4.1.4 Click “Input Vibrio Data” (red arrow)



1.4.1.5 The sample number will auto-populate. Enter all information and data provided from Vibrio Sample Submission form. Enter final results if available.



1.5 Notification of Test Results

1.5.1 Fax

1.5.1.1 Test results are faxed to the Office of Shellfish and Water Protection after Lead Microbiologist approval and signature.

1.5.1.2 Fax cover sheet and *Vibrio* sample submission form(s) to X2257. Completely fill out cover sheet and note which sample numbers are contained in fax (i.e. *Vibrio* S14-001 through S14-003).

1.5.2 Phone

1.5.2.1 For STAT results (per request of OSWP), the Lead Microbiologist will contact the appropriate personnel at OSWP

1.6 Archiving Results & Retention

1.6.1 Filing Results

1.6.1.1 Results are to be filed in filing cabinet located in Food and Shellfish Bacteriology Laboratory.

1.6.1.2 File approved samples that have NOT been entered into the Office of Shellfish and Water Protection (OSWP) database in the folder titled “Results NOT Entered in Database”

1.6.1.3 File approved samples that have been entered into the OSWP database into the folder titled “Entered in Database-Need Lead Approval”. The lead microbiologist will then file completed samples into appropriate folder.

1.6.2 Retention

1.6.2.1 Sample results and all paperwork pertaining to samples will be kept in Food and Shellfish Bacteriology Laboratory file cabinet for a minimum of seven (7) years.

1.6.2.2 OSWP database is maintained by OSWP

2. Calculations

2.1 Instructions

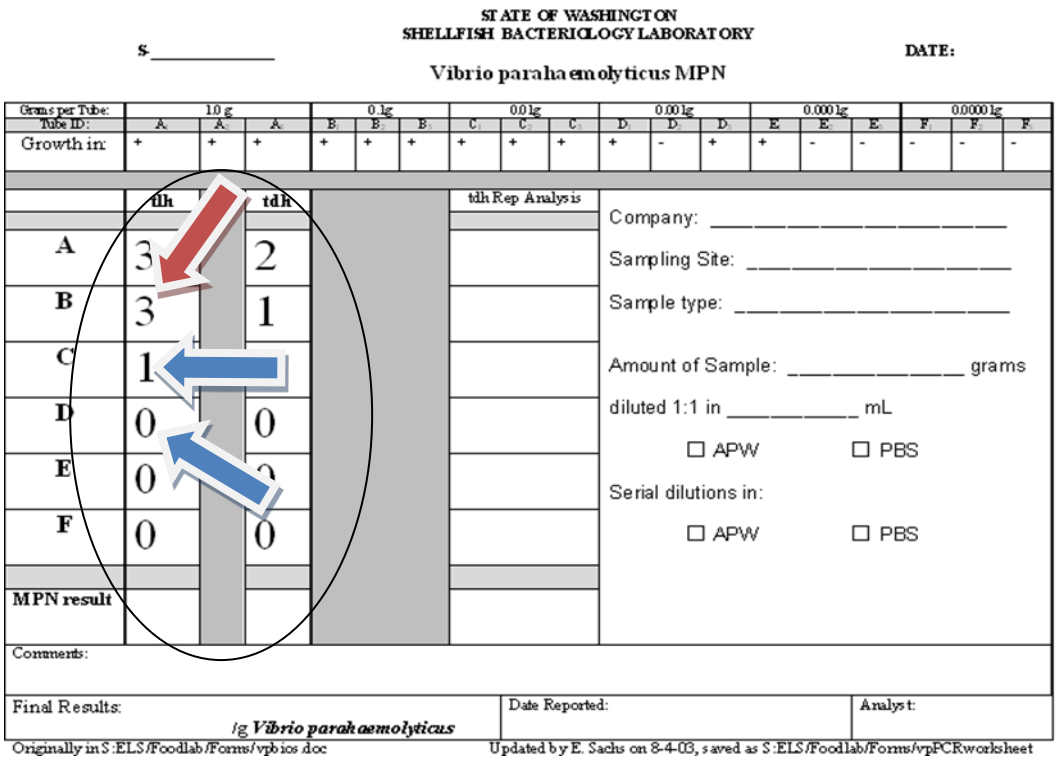
2.1.1 To calculate the concentration we must first determine the MPN index for all targets (*tlh*, *tdh, trh*, *ORF8*, and *vvhA*).

2.1.2 The MPN index is a 3 digit number where each digit represents the number of positive tubes in a given dilution

2.1.3 The first dilution set used in the MPN index is always the most dilute set of three tubes that are all positive for a given target. The next two digits represent the number of positive tubes in the next two dilution series.

2.1.3.1 Examples

2.1.3.1.1 The circled columns represent the number of positive (by PCR) tubes in each dilution set. The MPN index for TLH in the example below is 3-1-0. The first number is selected by finding the most dilute set of tubes that are all positive (red arrow). The next two numbers in the index are the numbers of positive tubes in the next two dilution sets (blue arrows).


STATE OF WASHINGTON
SHELLFISH BACTERIOLOGY LABORATORY
S-_____ DATE:
Vibrio parahaemolyticus MPN

Grams per Tube:	1.0 g			0.1g			0.01g			0.001g			0.0001g			0.00001g		
Tube ID:	A	A	A	B	B	B	C	C	C	D	D	D	E	E	E	F	F	F
Growth in:	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-

	tlh	tdh
A	3	2
B	3	1
C	1	
D	0	0
E	0	0
F	0	0
MPN result		

tdh Rep Analysis

Company: _____
Sampling Site: _____
Sample type: _____
Amount of Sample: _____ grams
diluted 1:1 in _____ mL
☐ APW ☐ PBS
Serial dilutions in:
☐ APW ☐ PBS

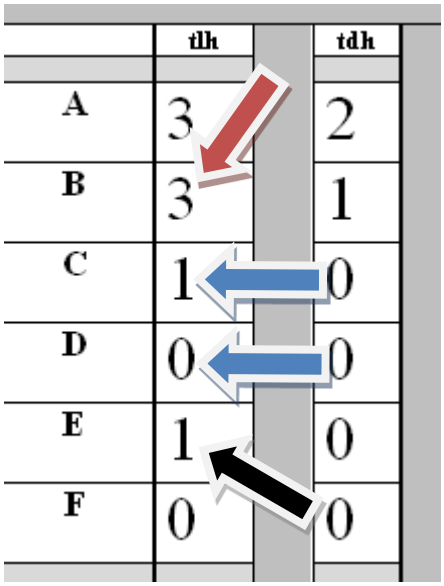
Comments:

Final Results: /g *Vibrio parahaemolyticus* | Date Reported: | Analyst:

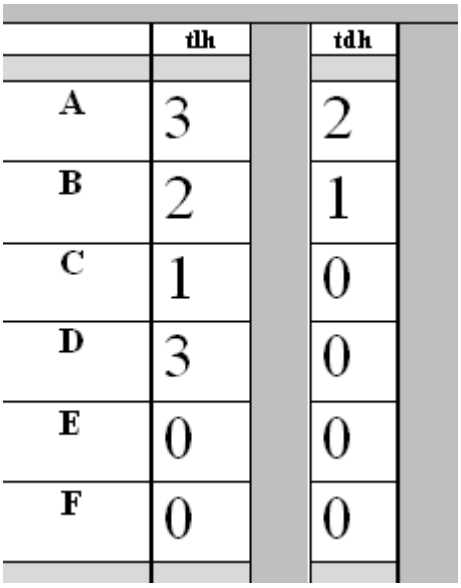
Originally in S:ELS/Foodlab/Forms/vpbios.doc Updated by E. Sachs on 8-4-03, saved as S:ELS/Foodlab/Forms/vpPCRworksheet

2.1.3.1.2 The following sample has an MPN index of 3-1-1. The first number is selected by finding the most dilute set of tubes that are all positive (red arrow). Typically, the next two numbers in the index are the numbers of positive tubes in the next two dilution sets (blue arrows). However, when there is a positive tube that exists beyond the selected dilution sets (black

arrow), that tube is added to the positive total of the last selected dilution set.



2.1.3.1.3 The following results are unable to generate an MPN index. Unusual MPN indexes are typically due to some sort of contamination. It may be necessary to re-extract and/or re-run PCR. If this does not resolve the issue, further investigation is required to determine the source of contamination.



2.1.4 Once the MPN index has been determined, calculate the concentration by using the MPN chart. According to the 3 tube MPN chart from the FDA Bacteriological Analytical Manual, an index of 3-1-1 corresponds to a concentration of 75 MPN/g of shellfish tissue. Always ensure that you have accounted for any dilutions when determining final concentrations. Since the amount of tissue in our MPN index matches the column headings on the MPN chart, in this case all dilutions are accounted for

2.1.4.1 MPN Chart from FDA BAM

Table 1. For 3 tubes each at 0.1, 0.01, and 0.001 g inocula, the MPNs per gram and 95 percent confidence intervals.

Pos. tubes			MPN/g	Conf. lim.		Pos. tubes			MPN/g	Conf. lim.	
0.10	0.01	0.001		Low	High	0.10	0.01	0.001		Low	High
0	0	0	<3.0	–	9.5	2	2	0	21	4.5	42
0	0	1	3.0	0.15	9.6	2	2	1	28	8.7	94
0	1	0	3.0	0.15	11	2	2	2	35	8.7	94
0	1	1	6.1	1.2	18	2	3	0	29	8.7	94
0	2	0	6.2	1.2	18	2	3	1	36	8.7	94
0	3	0	9.4	3.6	38	3	0	0	23	4.6	94
1	0	0	3.6	0.17	18	3	0	1	38	8.7	110
1	0	1	7.2	1.3	18	3	0	2	64	17	180
1	0	2	11	3.6	38	3	1	0	43	9	180
1	1	0	7.4	1.3	20	3	1	1	75	17	200
1	1	1	11	3.6	38	3	1	2	120	37	420
1	2	0	11	3.6	42	3	1	3	160	40	420
1	2	1	15	4.5	42	3	2	0	93	18	420
1	3	0	16	4.5	42	3	2	1	150	37	420
2	0	0	9.2	1.4	38	3	2	2	210	40	430
2	0	1	14	3.6	42	3	2	3	290	90	1,000
2	0	2	20	4.5	42	3	3	0	240	42	1,000
2	1	0	15	3.7	42	3	3	1	460	90	2,000
2	1	1	20	4.5	42	3	3	2	1100	180	4,100
2	1	2	27	8.7	94	3	3	3	>1100	420	–

2.2 Equations

2.3 Computer Validation Protocol

2.4 Examples

3. Expected Values-N/A

4. Method Limitations

4.1 Reportable Range

4.1.1 *tlh*: <0.3 MPN/g to >110,000 MPN/g

4.1.2 *tdh*: <0.3 MPN/g to >110,000 MPN/g

4.1.3 *trh*: <0.3 MPN/g to >110,000 MPN/g

4.1.4 *ORF8*: <0.3 MPN/g to >110,000 MPN/g

4.2 Interfering Substances

4.2.1 Upon validation, *Grimontia hollisae* (formerly *Vibrio hollisae*) has been found to have cross reactivity with the real-time PCR assay. *G. hollisae* shows slight amplification for the *tdh* marker. See discussion in Analytic 8.3.10 on how to determine true *Vp tdh* amplification vs. *G. hollisae* amplification.

4.2.2 As noted in the Post Analytic 1.1.2.2, a strain of *Vibrio alginolyticus* possesses a *trh* gene with 98% homology to the *trh* gene in *Vibrio parahaemolyticus*. Most probable number values for *trh* should be reported only if *tlh* is present in the

corresponding tube. Tubes only positive for *trh* should not be accounted for when generating the MPN value.

5. Procedure Notes

- **5.1** Reason for Special Precaution
- **5.2** Possible Sources of Errors
- **5.3** Helpful Hints
- **5.4** Situations That May Influence Test
- **5.5** Applications
- **5.6** Turn Around Time
  - **5.6.1** Samples results from samples that meet all criteria of acceptable submission should be reported to the Office of Shellfish and Water Protection within 3 days
- **5.7** Alternative Back up Procedures
- **5.8**
- **5.9** Definitions

6. References

- **6.1** Manufacturer Product Literature
- **6.2** Textbooks
- **6.3** Standards Publications
- **6.4** Written Personal Communications
- **6.5** Research

7. Supplemental Materials

- **7.1** Manufacturer Product Instructions
- **7.2** Flow Diagrams
- **7.3** Method Evaluation Assessment Sheet
- **7.4** Proficiency Provider

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[i] González-Escalona, Narjol, George M. Blackstone, and Angelo DePaola. "Characterization of a Vibrio alginolyticus strain, isolated from Alaskan oysters, carrying a hemolysin gene similar to the thermostable direct hemolysin-related hemolysin gene (trh) of Vibrio parahaemolyticus." Applied and environmental microbiology 72.12 (2006): 7925-7929.