Proposal Subject

MPN-Real-Time PCR for Total *V.p.*

Specific NSSP Guide Reference

Section IV. Guidance Documents
Chapter II. Growing Areas .11 Approved NSSP Laboratory Tests

Text of Proposal/ Requested Action

This method was developed by Jessica Jones (FDA Gulf Coast Seafood Laboratory) and is being submitted by the ISSC Executive Board. The Executive Board granted interim approval to this method on March 13, 2015. The Executive Board is submitting this proposal to comply with Article V. Section 1. of the ISSC Constitution, Bylaws, and Procedures.

Submitted by method developer Jessica Jones (FDA Gulf Coast Seafood Laboratory)

5. Approved Methods for Vibrio Enumeration

	Vibrio Indicator Type:	Application: PHP Sample Type: Shucked	Application: Reopening
EIA[1]	*Vibrio vulnificus (V.v.)*	X	
MPN[2]	*Vibrio vulnificus (V.v.)*	X	
SYBR Green 1 QPCR-MPN[5]	*Vibrio vulnificus (V.v.)*	X	
MPN[3]	*Vibrio parahaemolyticus (V.p.)*	X	
PCR[4]	*Vibrio parahaemolyticus (V.p.)*	X	
MPN-Real Time PCR[6]	*Vibrio parahaemolyticus (V.p.)*	X	X

Footnotes:

[1] EIA procedure of Tamplin, et al, as described in Chapter 9 of the FDA Bacteriological Analytical Manual, 7th Edition, 1992.
[2] MPN method in Chapter 9 of the FDA Bacteriological Analytical Manual, 7th Edition, May 2004 revision, followed by confirmation using biochemical analyses or by the DNA -alkaline phosphatase labeled gene probe (vvhA).
[3] MPN format with confirmation by biochemical analysis, gene probe methodology as listed in Chapter 9 of the FDA Bacteriological Analytical Manual, 7th Edition, May 2004 revision, or a method that a State can demonstrate is equivalent.
[4] PCR methods as they are listed in Chapter 9 of the FDA Bacteriological Analytical Manual, 7th Edition, May 2004 revision, or a method that a State can demonstrate is equivalent.
[5] *Vibrio vulnificus*, ISSC Summary of Actions 2009. Proposal 09-113, Page 123.

[6] MPN-real time PCR method for the *tlh* gene for total *V. parahaemolyticus* as described in Kinsey et al., 2015.

Public Health Significance

The current NSSP method for enumeration of *Vp* requires a minimum of four days from receipt of sample to results reporting. The MPN-real-time PCR method provides results in as little as 24h from receipt of sample. At the 2013 conference, proposal 13-202 was adopted which requires testing prior to reopening of growing areas closed as a result of *Vp* illnesses [Chapter II @.01.F(5)]. Availability of this more rapid method will facilitate reopening decision making.

Cost Information	This method costs ~$100 per sample for laboratory consumables, supplies, and reagents. Most equipment needed for testing is standard microbiology equipment, but purchase of a heat block (~$400) and/or centrifuge (~$2,500) may be necessary. Purchase of a real-time PCR instrument will be required ($30,000-$45,000). Additional costs for a laboratory would vary based on their operational overhead and labor.
Action by 2015 Laboratory Methods Review Committee	Recommended adoption of Proposal 15-113 as submitted and direct the Executive Office to request the submitter revise the SOP so that the BAM MPN calculator be used for determination of MPN values.
Action by 2015 Task Force I	Recommended adoption of 2015 Laboratory Methods Review Committee recommendation on Proposal 15-113.
Action by 2015 General Assembly	Adopted recommendation of Task Force I on Proposal 15-113.
Action by FDA January 11, 2016	Concurred with Conference action on Proposal 15-113.

Validation Data for MPN-Real-Time PCR for Total Vp

Name of Method Submitter: Jessica L. Jones, Ph.D.

Specific purpose or intent of the method for use in the NSSP: Seeking approval for this method as an approved limited use method that can be used as an alternate to the currently approve MPN-culture method in the NSSP. This method is appropriate for PHP validation and verification testing, as well as environmental testing such as that which may be required for the re-opening of growing areas closed due to illness.

Validation Criteria Data: For evaluation of all validation criteria below, PHP oysters were obtained in the best effort to find samples free of the target organism. A different lot of PHP oysters was used for each sample. For each sample, a minimum of 10 animals were used to prepare a homogenate. The homogenate was then aliquoted and appropriate aliquots spiked, while one aliquot was left unioculated (sample blank). Spike levels were determined by spread plating dilution of the culture in triplicate onto TSA+2% NaCl. MPN-PCR analysis was conducted using both the SmartCycler and AB 7500 instruments.

1. Accuracy/Trueness: Using the data from Table 1, the differences between the spike level (plate count) and values generated by MPN-PCR on the SmartCycler and AB 7500 Fast were not significantly ($p=0.91$ and $p=0.91$, respectively). Additionally, the results from both instrument platforms we not significantly different ($p=1.00$).

The average of plate counts was 3.81 log, the average MPN from the SmartCycler (adjusted for background) was 3.99 log, and the average MPN from AB 7500 Fast (adjusted for background) was 4.00 log. Using this data, the *Accuracy/Trueness of the methods were determined to be 104% on the SmartCycler and 105% on AB 7500 Fast.*

Table 1. Data for determination of Accuracy/Trueness and Measurement Uncertainty.

Sample	Plate Count (log CFU)	Sample Blank on SC (log MPN/g)	Sample Blank on AB (log MPN/g)	Spiked Sample on SC (log MPN/g)	Spiked Sample on AB (log MPN/g)
1-2X	6.18	-0.52	<-0.52	6.04	6.04
2-2X	5.18	1.18	0.04	5.38	5.38
3-4X	3.15	0.79	-0.52	3.63	3.63
4-4X	3.15	-0.21	<-0.52	2.97	2.97
5-6X	1.23	<-0.52	-0.52	1.18	1.18
6-6X	1.23	0.88	0.88	1.97	2.18
7-2X	5.76	-0.03	0.3	6.04	6.04
8-2X	5.76	1.32	1.66	>6.04	>6.04
9-4X	3.68	<-0.52	<-0.52	3.63	3.63
10-4X	3.68	-0.52	-0.05	3.63	3.63

2. Measurement Uncertainty: Using the data from Table 1 above, measurement uncertainty was determined to be *0.16 for the SmartCycler and 0.18 for AB 7500 Fast*.

3. Precision: Using the data from Table 2, there *was no significant difference between the plate counts and the MPN values generated using the SmartCycler or the AB 7500 Fast* (ANOVA, $p>0.75$). Additionally, the difference in variance is not significant ($p>0.3$)

Table 2. Data for determination of Precision and Recovery

Sample	Aliquot	Plate Count (log CFU)	Sample on SC (log MPN/g)	Sample on AB (log MPN/g)
1	**Blank**	N/A	-0.52	<-0.52
1	**2X**	5.18	5.38	5.38
1	**2Z**	5.18	5.66	5.66
1	**4X**	3.18	2.97	2.97
1	**4Z**	3.18	2.97	2.44
1	**6X**	1.18	1.15	0.97
1	**6Z**	1.18	0.97	0.97
3	**Blank**	N/A	0.79	-0.52
3	**2X**	5.15	5.38	5.38
3	**2Z**	5.15	5.18	5.18
3	**4X**	3.15	3.63	3.63
3	**4Z**	3.15	2.88	2.88
3	**6X**	1.15	1.58	1.38
3	**6Z**	1.15	1.38	2.08
5	**Blank**	N/A	<-0.52	-0.52
5	**2X**	5.23	3.88	3.88
5	**2Z**	5.23	3.63	3.63
5	**4X**	3.23	4.38	4.38
5	**4Z**	3.23	2.97	2.97
5	**6X**	1.23	1.18	1.18
5	**6Z**	1.23	0.97	1.18
7	**Blank**	N/A	-0.03	0.30
7	**2X**	5.76	6.04	6.04
7	**2Z**	5.76	5.66	5.66
7	**4X**	3.76	3.38	3.38
7	**4Z**	3.76	3.97	3.97
7	**6X**	1.76	1.97	1.97
7	**6Z**	1.76	1.63	1.97
9	**Blank**	N/A	<-0.52	<-0.52
9	**2X**	5.68	>6.04	>6.04
9	**2Z**	5.68	5.66	5.66
9	**4X**	3.68	3.63	3.63
9	**4Z**	3.68	3.38	3.38
9	**6X**	1.68	1.63	2.38
9	**6Z**	1.68	1.38	1.63

4. Recovery: The average of plate counts was 3.40 log, the average MPNs (adjusted for background) were 3.06 and 3.10 log, from the SmartCycler and AB 7500 Fast, respectively. Using this data, the *Recovery of the methods were determined to be 110% on the SmartCycler and 109% on AB 7500 Fast.*

5. Specificity: Samples were prepared as above and the interfering organism was spiked at an ~4 log higher concentration than *Vibrio parahaemolyticus*. Using the data from Table 3, the average *Specificity Index for the SmartCycler was 1.01 and 1.28 for the AB 7500 Fast*; these values are not significantly different than 1 ($p>0.05$).

Table 3. Data for determination of Specificity.

	Spiked with Vp only		Spiked with Vp and Vv	
Sample	**Sample on SC (log MPN/g)**	**Sample on AB (log MPN/g)**	**Sample on SC (log MPN/g)**	**Sample on AB (log MPN/g)**
6-Blank	0.88	0.88	---	---
6-6T	1.38	2.32	0.04	0.88
6-6U	1.18	1.46	1.97	2.18
6-6W	1.38	1.58	1.18	1.58
6-6X	1.97	2.18	1.63	0.54
6-6Z	0.97	1.18	1.97	1.63

6. Working and Linear Range: Based on the data presented in Table 4, there is a significant correlation between the plate counts and MPN values generated on the SmartCycler ($p<0.001$) and the AB7500 Fast ($p<0.001$). The *correlation coefficients are 0.939 and 0.941 for the SmartCycler and AB 7500 Fast* platforms, respectively demonstrating the linearity of the method.

7. Limit of Detection: Using the data from Table 4, the *Limit of Detection of the method as implemented is determined to be 1.51 and 1.26* cells for the SmartCycler and AB 7500 Fast, respectively, which fits within the calculated 95% confidence interval of 0.62 for a theoretical limit of detection of 1.

8. Limit of Quantification/ Sensitivity: As the Limit of Detection was determined to be within the 95% confidence interval of 1 cell, the limit of quantification/sensitivity is reliant upon the number of tubes per dilution in combination of the lowest dilution examined. Using a 3-tube, multiple dilution series starting at 1g of sample, this method provides a *Sensitivity of 0.3 MPN/g of oyster tissue*.

Table 4. Data for determination of Working and Linear Range, Limit of Detection, and Limit of Quantitation/Sensitivity

Sample	**Aliquot**	**Plate Count (log CFU)**	**Sample on SC (log MPN/g)**	**Sample on AB (log MPN/g)**
1	**1X**	6.18	6.04	6.04
1	**1Z**	6.18	>6.04	>6.04
1	**2X**	5.18	5.38	5.38
1	**2Z**	5.18	5.66	5.66
1	**4X**	3.18	2.97	2.97
1	**4Z**	3.18	2.97	2.44
1	**6X**	1.18	1.15	0.97

1	**6Z**	1.18	0.97	0.97
1	**7X**	0.18	<-0.52	-0.13
1	**7X**	0.18	-0.14	-0.04
3	**1X**	6.15	>6.04	>6.04
3	**1Z**	6.15	6.04	6.04
3	**2X**	5.15	5.38	5.38
3	**2Z**	5.15	5.18	5.18
3	**4X**	3.15	3.63	3.63
3	**4Z**	3.15	2.88	2.88
3	**6X**	1.15	1.58	1.38
3	**6Z**	1.15	1.38	2.08
3	**7X**	0.15	1.18	0.97
3	**7Z**	0.15	0.97	1.32
5	**1X**	6.23	6.04	6.04
5	**1Z**	6.23	>6.04	>6.04
5	**2X**	5.23	3.88	3.88
5	**2Z**	5.23	3.63	3.63
5	**4X**	3.23	4.38	4.38
5	**4Z**	3.23	2.97	2.97
5	**6X**	1.23	1.18	1.18
5	**6Z**	1.23	0.97	1.18
5	**7X**	0.23	0.18	-0.04
5	**7Z**	0.23	0.78	0.63
7	**1X**	6.76	>6.04	>6.04
7	**1Z**	6.76	>6.04	>6.04
7	**2X**	5.76	6.04	6.04
7	**2Z**	5.76	5.66	5.66
7	**4X**	3.76	3.38	3.38
7	**4Z**	3.76	3.97	3.97
7	**6X**	1.76	1.97	1.97
7	**6Z**	1.76	1.63	1.97
7	**7X**	0.76	1.97	1.63
7	**7Z**	0.76	1.08	0.63
9	**1X**	6.68	6.04	6.04
9	**1Z**	6.68	>6.04	>6.04
9	**2X**	5.68	>6.04	>6.04
9	**2Z**	5.68	5.66	5.66
9	**4X**	3.68	3.63	3.63
9	**4Z**	3.68	3.38	3.38
9	**6X**	1.68	1.63	2.38
9	**6Z**	1.68	1.38	1.63
9	**7X**	0.68	1.63	1.38
9	**7Z**	0.68	0.88	0.63

9. Ruggednes: Replicate spiked aliquots from each sample were processed with different batches of media/ lots of reagents at the same time. Different samples were processed on different days. Using the data in Table 5, there was *no significant difference (p>0.80) between batches/lots* of media and reagents on either instrument platform.

Table 5. Data for determination of Ruggedness.

	Replicate 1 (X)		Replicate 2 (Z)	
Sample	**Sample on SC (log MPN/g)**	**Sample on AB (log MPN/g)**	**Sample on SC (log MPN/g)**	**Sample on AB (log MPN/g)**
2	5.38	5.38	4.97	4.97
4	2.97	2.97	2.97	2.97
6	1.97	2.18	0.97	1.18
8	>6.04	>6.04	6.04	6.04
10	3.63	3.63	3.63	3.63

10. ***Matrix Effects:*** Effects of oyster matrix on the performance of the method was taken into consideration in testing all of the above criteria by using the sample blank.

11. Additional Data: *Inclusivity/Exclusivity*. The primers and probes utilized in this method have been tested against DNA extracts from the isolates listed in the table below. All 76 of the *Vibrio parahaemolyticus* isolates were positive and all 30 of the non-*V. parahaemolyticus* isolates were negative, demonstrating 100% inclusivity and exclusivity for these sequences.

Species	**Strain ID**	**Isolation Location**	**Isolation Date**	**Isolation Source**	***tlh***	***tdh***	***trh***
Vibrio parahaemolyticus	V05/011	Norway	Unk*	Clinical	+	-	+
Vibrio parahaemolyticus	V05/067	Spain	Unk	Clinical	+	+	-
Vibrio parahaemolyticus	K5278	USA, WA	Unk	Clinical	+	+	+
Vibrio parahaemolyticus	F1103A	USA, WA	Unk	Environmental	+	+	+
Vibrio parahaemolyticus	V05/071	Portugal	Unk	Environmental	+	-	+
Vibrio parahaemolyticus	V05/081	Italy	Unk	Clinical	+	+	-
Vibrio parahaemolyticus	V05/014	Norway	Unk	Clinical	+	+	+
Vibrio parahaemolyticus	FIHES98V103204	Japan	Unk	Clinical	+	-	-
Vibrio parahaemolyticus	0337-2111 (K1311)	USA, AK	2004	Environmental	+	-	+
Vibrio parahaemolyticus	0872-2247-2 (K1321)	USA, AK	2004	Environmental	+	-	+
Vibrio parahaemolyticus	TX2103	USA, TX	1998	Clinical	+	+	-
Vibrio parahaemolyticus	DI0B9 3/16	USA, AL	1999	Environmental	+	+	+
Vibrio parahaemolyticus	AQ4913	Unk	Unk	Clinical	+	+	+
Vibrio parahaemolyticus	KXV 755	Unk	Unk	Clinical	+	+	+
Vibrio parahaemolyticus	V05/010	Norway	Unk	Clinical	+	+	-
Vibrio parahaemolyticus	K5208	USA, AK	2007	Clinical	+	+	+
Vibrio parahaemolyticus	K5330	USA, TX	2007	Clinical	+	-	+
Vibrio parahaemolyticus	SPRC 10295	USA, WA	Unk	Clinical	+	+	+
Vibrio parahaemolyticus	48057	USA, WA	Unk	Clinical	+	+	+
Vibrio parahaemolyticus	AN2189	Bangladesh	Unk	Clinical	+	+	-
Vibrio parahaemolyticus	0330020030B (K1295)	USA, AK	Unk	Environmental	+	+	-
Vibrio parahaemolyticus	KXV0627	Unk	Unk	Clinical	+	+	-
Vibrio parahaemolyticus	1300-A2-1 (K1316)	USA, AK	2004	Environmental	+	+	+
Vibrio parahaemolyticus	K4859	USA, HI	2007	Clinical	+	-	-

Vibrio parahaemolyticus	K5435	USA, WA	Unk	Clinical	+	-	+
Vibrio parahaemolyticus	K5439	USA, WA	2007	Clinical	+	+	-
Vibrio parahaemolyticus	0330-2006 (K1296)	USA, AK	2004	Environmental	+	-	+
Vibrio parahaemolyticus	V05/062	Spain	Unk	Clinical	+	+	-
Vibrio parahaemolyticus	DI0E12 5/26	USA, AL	Unk	Environmental	+	+	+
Vibrio parahaemolyticus	08880200901 (K1198)	USA, AK	2004	Environmental	+	+	+
Vibrio parahaemolyticus	Isolate 1	Australia	2010	Environmental	+	-	-
Vibrio parahaemolyticus	V05/020	Spain	Unk	Environmental	+	-	-
Vibrio parahaemolyticus	V05/072	Portugal	Unk	Environmental	+	-	-
Vibrio parahaemolyticus	V05/070	Portugal	Unk	Environmental	+	-	+
Vibrio parahaemolyticus	V05/017	Norway	2002	Clinical	+	+	-
Vibrio parahaemolyticus	V05/065	Spain	1998	Clinical	+	+	-
Vibrio parahaemolyticus	K4842	USA, MD	2006	Clinical	+	-	+
Vibrio parahaemolyticus	K4557	USA, LA	2006	Clinical	+	-	-
Vibrio parahaemolyticus	K4637	USA, NY	2006	Clinical	+	+	-
Vibrio parahaemolyticus	VPHY 145	Thailand	Unk	Clinical	+	+	-
Vibrio parahaemolyticus	VPHY 123	Thailand	Unk	Clinical	+	+	-
Vibrio parahaemolyticus	AO024491	Bangladesh	Unk	Clinical	+	+	-
Vibrio parahaemolyticus	AP9251	Bangladesh	Unk	Clinical	+	+	-
Vibrio parahaemolyticus	K4639	USA, NY	2006	Clinical	+	+	+
Vibrio parahaemolyticus	AP 11243	Bangladesh	Unk	Clinical	+	+	-
Vibrio parahaemolyticus	V05/080	Adriatic Sea	Unk	Environmental	+	-	-
Vibrio parahaemolyticus	V05/018	Norway	2006	Clinical	+	-	+
Vibrio parahaemolyticus	11/001	Peru	2006	Clinical	+	+	-
Vibrio parahaemolyticus	K4760	USA, VA	2006	Clinical	+	-	-
Vibrio parahaemolyticus	V05/026	United Kingdom	Unk	Environmental	+	-	-
Grimontia hollisae	98A1960	Unk	Unk	Unk	-	+	-
Photobacteria damselae	Hw-33-5	Unk	Unk	Unk	-	-	-
Vibrio metschnikovii	2908-8	Unk	Unk	Unk	-	-	-
Vibrio fluvialis	DAL197	Unk	Unk	Unk	-	-	-
Vibrio alginolyticus	ATCC 33787	Unk	Unk	Unk	-	-	-
Vibrio alginolyticus	1296-A2-1	USA, AK	2004	Environmental	-	-	+
Vibrio alginolyticus	2208-1B	USA, AK	2004	Environmental	-	-	+
Vibrio fluvialis	DAL506	Unk	Unk	Unk	-	-	-
Vibrio furnissii	1955-83	Unk	Unk	Clinical	-	-	-
Vibrio fluvialis	1959-82	Unk	Unk	Clinical	-	-	-
Grimontia hollisae	2039	Unk	Unk	Unk	-	-	-
Grimontia hollisae	89A4206	Unk	Unk	Unk	-	-	-
Photobacteria damselae	FT-452	Unk	Unk	Unk	-	-	-
Photobacteria damselae	BR-907	Unk	Unk	Unk	-	-	-
Photobacteria damselae	BR-D1-100	Unk	Unk	Unk	-	-	-
Vibrio vulnificus	99-780 DP-E1	USA, LA	1999	Food, oyster	-	-	-
Vibrio vulnificus	98-624 DP-C9	USA, TX	1998	Food, oyster	-	-	-
Vibrio vulnificus	99-581 DP-C7	USA, LA	1999	Food, oyster	-	-	-
Vibrio vulnificus	99-796 DP-E7	USA, FL	1999	Food, oyster	-	-	-
Vibrio vulnificus	99-584 DP-B12	USA, TX	1999	Food, oyster	-	-	-
Vibrio vulnificus	98-640 DP-E9	USA, LA	1998	Food, oyster	-	-	-
Vibrio vulnificus	99-743 DP-B6	USA, TX	1999	Food, oyster	-	-	-
Vibrio vulnificus	98-783 DP-A1	USA, LA	1998	Food, oyster	-	-	-
Vibrio vulnificus	CDC 9149-95	USA	1995	Clinical	-	-	-
Vibrio cholerae	CDC 3569-03	Unk	2003	Clinical	-	-	-
Vibrio cholerae	C-6706	Unk	Unk	Unk	-	-	-
Vibrio cholerae	CDC F851	Unk	Unk	Clinical	-	-	-

Vibrio cholerae	SJ 21	USA, CA	Unk	Environmental	-	-	-
Vibrio cholerae	CDC 3541-98	Unk	1998	Clinical	-	-	-
Vibrio cholerae	CDC 3525-97	Unk	1997	Clinical	-	-	-
Vibrio parahaemolyticus	0331-2017B	USA, AK	2004	Environmental	+	+	+
Vibrio parahaemolyticus	Isolate 11	Australia	2010	Environmental	+	+	-
Vibrio parahaemolyticus	CA012017	USA, CA	2012	Food, oyster	+	+	+
Vibrio parahaemolyticus	78024600C2	USA, CA	2013	Food, oyster	+	+	+
Vibrio parahaemolyticus	78024600C3	USA, CA	2013	Food, oyster	+	+	+
Vibrio parahaemolyticus	77545901A1	USA, CA	2013	Food, oyster	+	+	+
Vibrio parahaemolyticus	77545901A2	USA, CA	2013	Food, oyster	+	+	+
Vibrio parahaemolyticus	FDA_R10	USA, FL	2007	Food, oyster	+	+	+
Vibrio parahaemolyticus	FDA_R16	USA, FL	2007	Food, oyster	+	+	+
Vibrio parahaemolyticus	FDA_R31	USA, LA	2007	Food, oyster	+	+	+
Vibrio parahaemolyticus	FDA_R32	USA, LA	2007	Food, oyster	+	+	+
Vibrio parahaemolyticus	FDA_R26	USA, NJ	2007	Food, oyster	+	+	+
Vibrio parahaemolyticus	FDA_R51	USA, AL	2007	Food, oyster	+	+	+
Vibrio parahaemolyticus	FDA_R47	USA, AL	2007	Food, oyster	+	+	+
Vibrio parahaemolyticus	FDA_R149	USA, FL	2007	Food, oyster	+	+	+
Vibrio parahaemolyticus	CDC_K4763	USA, VA	2006	Clinical	+	+	+
Vibrio parahaemolyticus	CDC_K5009G	USA, MA	2006	Clinical	+	+	+
Vibrio parahaemolyticus	CDC_K5009W	USA, MA	2006	Clinical	+	+	+
Vibrio parahaemolyticus	CDC_K4636	USA, NY	2006	Clinical	+	+	+
Vibrio parahaemolyticus	CDC_K4639G	USA, NY	2006	Clinical	+	+	+
Vibrio parahaemolyticus	CDC_K4639W	USA, NY	2006	Clinical	+	+	+
Vibrio parahaemolyticus	CDC_K5073	USA, MD	2007	Clinical	+	+	+
Vibrio parahaemolyticus	CDC_K5276	USA, NY	2007	Clinical	+	+	+
Vibrio parahaemolyticus	CDC_K5067	USA, SD	2007	Clinical	+	+	+
Vibrio parahaemolyticus	CDC_K5280	USA, WA	2007	Clinical	+	+	+
Vibrio parahaemolyticus	CDC_K5306	USA, GA	2007	Clinical	+	+	+

*Unk = unknown; data not available.

Step-by-step procedure including equipment, reagents and safety requirements necessary to run the method:

1. Special Equipment, Media, and Reagents
   - 1.1. Heat block (100°C) or boiling water bath
   - 1.2. Eppendorf 5415D centrifuge or equivalent (capable of 13,000x*g*)
   - 1.3. Mini-centrifuge
   - 1.4. SmartCycler II (Cepheid, Sunnyvale, CA) OR AB 7500 (Life Technologies, Foster City, CA)
   - 1.5. SmartCycler tubes OR AB 7500 Fast reaction plates or 8-tube strips
   - 1.6. Micropipetters (volume ranges from 0.1µl to 1000µl) with filter tips
   - 1.7. Oligonucleotide primers (desalted) and nuclease-style probes (HPLC purified) in 10µM working solutions - sequences provided below in Table 1
   - 1.8. Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA)
   - 1.9. 50 mM MgCl2 (Invitrogen, or equivalent)
   - 1.10. dNTP's, mixed equal concentration (Roche, or equivalent)
   - 1.11. ROX reference dye (if using the AB 7500)
   - 1.12. Internal Amplification Control (IAC) DNA (BioGX, Birmingham, AL)
   - 1.13. PCR-grade water

1.14. Alkaline peptone water (APW) - 10 g peptone, 10 g NaCl, 1L d. water, dissolve ingredients, then adjust pH to 8.5±0.2 and autoclave 15 min at 121°C

1.15. Phosphate buffered saline (PBS) - 7.650 g NaCl, 0.724 g Na2HPO4 anhydrous, 0.210 g KH2PO4, 1L d. water, dissolve ingredients then adjust pH to 7.4 and autoclave 15 min at 121°C

2. Outlined Procedure

2.1. Preparation of shellfish

2.1.1. Hands of examiner must be scrubbed thoroughly with soap and potable water; latex or nitrile gloves should be worn while cleaning oysters.

2.1.2. Scrape off growth and loose material from shell, and scrub shell stock with sterile stiff brush under running water.

2.1.3. Place clean shellstock on clean towels or absorbent paper.

2.1.4. Change gloves and brushes between samples.

2.1.5. Protective chain mail glove can be used under a latex glove; outer gloves should be changed between samples.

2.1.6. Tare a sterile blender.

2.1.7. Using a sterile oyster knife, insert the point between the shells on the ventral side, about ¼ the distance from the hinge to the bill; alternately, knife can be inserted after making small opening with sterile bone cutting forceps.

2.1.8. Cut adductor muscle from upper flat shell and pry the shell wide enough to drain shell liquor into the blender.

2.1.9. The upper shell can then be pried loose at hinge and discarded.

2.1.10. The whole animal (including adductor muscle) should be transferred to the sterile blender after severing the adductor muscle connection to the lower shell.

2.1.11. A minimum of 12 animals or 200g is required.

2.1.12. Blend without adding diluent for 60-120 sec at 14,000 rpm.

2.2. Preparation of MPN Enrichment Series

2.2.1. Prepare a 1:10 dilution of the homogenate by transferring 1 g (weighing is required for accurate volumetric transfer) of the homogenate to 9 ml of PBS. Additional 10-fold dilutions can be prepared volumetrically (i.e., 1 ml of 1:10 to 9 ml of PBS for a 1:100 dilution).

Table 1. Oligonucleotide sequences

	Sequences (5'---->3')	Modifications
tlh 884F	ACTCAACACAAGAAGAGATCGACCA	----------
tlh 1091R	GATGAGCGGTTGATGTCCAA	----------
tlh Probe	CGCTCGCGTTCACGAAACCGT	5'TexasRed-3'BHQ2[a,b]
tlh Probe	CGCTCGCGTTCACGAAACCGT	5'JOE-3'BHQ2[c]
IAC 46F	GACATCGATATGGGTGCCG	----------
IAC 186R	CGAGACGATGCAGCCATTC	----------
IAC probe	TCTCATGCGTCTCCCTGGTGATGTG	5'Cy5-3'BHQ2

[a] BHQ2=black hole quencher 2

[b] When run on the SmartCyclers

[c] When used with *V. parahaemolyticus* primers and probes

2.2.2. Transfer 3 aliquots of 1 g of homogenate to 9 ml of APW (this should be done by weight to ensure accurate transfer). Inoculate 3 x 1 ml portions of the 1:10, 1:100, 1:1000, 1:10,000, 1:100,000, and 1:1,000,000 dilutions into 10 ml of APW for the -1 thru -6 samples.

2.2.3. Incubate APW overnight (18-24h) at 35 ±2°C.

2.3. Preparation of DNA Extracts

2.3.1. Transfer 1ml from each MPN tube with visible growth to a microcentrifuge tube.

2.3.2. Boil (or heat to 100°C) 1-ml aliquot of sample (from MPN enrichment) for 10 min.

2.3.3. Immediately plunge into ice until cold.

2.3.4. Centrifuge samples for 2 min at 14-16,000 x g. Use 2 µl of supernatant as template in the real-time PCR reaction as detailed below.

2.3.5. DNA extracts can be stored at 4°C for up to 24 h or at -20°C.

2.4. Preparation of PCR

2.4.1. Prepare master mix in a clean hood or area and always use aerosol resistant pipette tips for PCR.

2.4.2. To a clean microfuge tube, add the following volumes of each reagent (µl) per reaction: 2.5 PCR buffer, 2.5 $MgCl_2$, 0.75 dNTPs, 0.5 tlhF primer, 0.5 tlhR primer, 0.19 IACF primer, 0.19 IACR primer, 0.38 tlh probe, 0.38 IAC probe, 2 IAC DNA, 0.22 Platinum *Taq*.

2.4.3. To the master mix for the SmartCycler, add 12.9 µl PCR-grade water per reaction to complete the master mix.

2.4.4. To the master mix for the AB 7500, add 12.2 µl PCR-grade water and 0.6 µl of ROX reference dye to complete the master mix.

2.4.5. Flick tube to mix and briefly spin (2-3 sec) in a pop spinner.

2.4.6. Add 23 µl of master mix to each reaction tube or well.

2.4.7. Add 2 µl of supernatant from each boiled DNA extract sample to a reaction tube or well.

2.4.8. Add 2 µl of a Vp control template to a reaction tube or well as a positive control.

2.4.9. Add 2 µl of PCR-grade water to a tube or well as a negative control.

2.4.10. Load sample tubes or 96-well plate to instrument and start cycling with the following conditions: hold at 95°C for 60sec, followed by 45 cycles of 95°C for 5 sec, 59°C for 45 sec.

2.4.11. The read stage for the instrument should be programmed to be the 59°C for 45 sec.

2.5. Data Analysis

2.5.1. For results analysis, default instrument settings will be used, except the threshold is set at 15 on the SmartCycler; the threshold is set at 0.02 and background end cycle set at 10 on the AB7500.

2.5.2. Any sample that crosses the threshold in the appropriate channels/filters will be considered positive.

2.5.3. If the IAC is negative, and the target is negative, the test should be considered invalid.

2.5.4. Calculate the MPN-PCR estimate as described in Appendix 2 of the BAM.