Name of the New Method	MPN-Real-Time PCR Method for the Detection of Vibrio Vulnificus from Oysters
Name of the Method Developer	USFDA Gulf Coast Seafood Laboratory
Developer Contact Information	USFDA Gulf Coast Seafood Laboratory, 1 Iberville Drive, Dauphin Island, AL 36528 Jessica.jones@fda.hhs.gov

Checklist	Y/N	Submitter Comments
A. Need for the New Method		
Clearly define the need for which the method has been developed.	Y	Currently, the most common NSSP method used to detect *Vibrio vulnificus* (Vv) in oysters is MPN-culture. The method is time consuming and laborious taking a minimum of four full days to produce a result. A quicker method uses Real-time PCR for detection, currently the only NSSP approved Real-time PCR utilizes Sybr green: a non-specific DNA binding molecule, which negates the ability to multiplex thus is not permissive of the use of an internal control to assure the reaction integrity. The ability to use an internal control adds a level of reliability the use of a non-specific binder like Sybr Green cannot. Additionally, the Sybr Green method is validated for use with the Smart Cycler by Cepheid which, as of December 2018, will no longer be supported by the manufacturer. The MPN Real-time PCR method for Vv detection in oysters will utilize the AB7500 Fast, the same instrument which the NSSP-approved MPN Real-time PCR methods for Vp utilizes. Further, this method uses a specific probe targeting the vvh gene of Vv and includes an internal control in a single assay. This assay is rapid and robust producing highly reliable results in 24-36 hours.
What is the intended purpose of the method?	Y	Approved NSSP method for enumeration of Vv from oysters.

Is there an acknowledged need for this method in the NSSP?	Y	There is current methodology. This assay is quicker than the aproved culture methods and more robust than the existing real-time PCR method.
What type of method? i.e. chemical, molecular, culture, etc.	Y	MPN enrichment with molecular confirmation.

B. Method Documentation		
1. Method documentation includes the following information:		
Method Title	Y	MPN-Real-Time PCR Method for the Detection of Vibrio vulnificus from Oysters
Method Scope	Y	This method is for the detection of *Vibrio vulnificus* from oysters using the AB7500 Fast real-time PCR platform.
References	Y	Campbell, M.S., Wright, A.C., 2003. Real-time PCR analysis of *Vibrio vulnificus* from oysters. Appl Environ Microbiol 69, 7137-7144 Jones, J.L., Kinsey, T.P., Johnson, L.W., Porso, R., Friedman, B., Curtis, M., Wesighan, P., Schuster, R., Bowers, J.C., 2016. Effects of Intertidal Harvest Practices on Levels of *Vibrio parahaemolyticu*s and *Vibrio vulnificus* Bacteria in Oysters. Appl Environ Microbiol 82, 4517-4522. Kaysner, C., DePaola, A., 2004. *Vibrio*, Bacteriological Analytical Manual, 8th ed. Nordstrom, J.L., Vickery, M.C., Blackstone, G.M., Murray, S.L., DePaola, A., 2007. 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. Appl Environ Microbiol 73, 5840-5847.
Principle	Y	This method is uses an MPN format for enumeration based on molecular (PCR) detection of the vvh gene specific to Vv.

Any proprietary aspects	Y	The AB7500 Fast is a proprietary real-time PCR platform developed by Applied Biosystems and sold through ThermoFisher Scientific. The optical plates and caps or film used are proprietary to the instrument.
Equipment required	Y	Equipment is listed in Appendix A.
Reagents required	Y	Media and reagents are listed in Appendix B.
Sample collection, preservation and storage requirements	Y	Shellstock samples are bagged immediatley upon collection and labeled with collector's name, the source of harvest, sampling stations, time, and date. Samples are placed in dry storage (ice chest or equivalent) maintained between 2°C and 10°C with ice or cold packs for transport. A layer of towels, bubblewrap, or another appropriate substance will separate shellfish from contact with ice or cold packs. If collected samples are frozen (such as IQF), direct contact with ice or cold packs is not permitted. Immediately upon arrival of sample(s) to the laboratory, date, time, and initials of receiver are documented. The temperature of three shellfish, each from a separate location within each shipping container, is measured by opening the sell enough to insert a temperature probe into the meat of the shellfish. If IQF samples are received, assure samples are frozen. Store at less than -15°C until ready to process. Temperatures are taken immediately after defrosting as described above. The shellfish is discarded after temperature is measured. Once temperature of the samples upon intake is established, the samples are placed under refrigeration for not longer than 36h after collection, unless processed immeditely. Storage is documented. If processing IQF samples, samples are defrosted under refrigeration for no longer than 36h.
Safety requirements	Y	Basic Personal Protection Equipment (PPE) is needed. A chain mail glove may

		be worn during shucking. Blending is done in a biosafety hood or the blender is placed in a splash shielded containter or blender box. All biological waste is autoclaved and disposed of according to state regulations.
Clear and easy to follow step-by-step procedure	Y	Detailed procedure including sample preparation, MPN, PCR, and data analysis is included in Appendix C.
Quality control steps specific for this method	Y	Appropriately diluted process controls are used (Vv ATCC 33816 and Vp F11-3A). Appropriately diluted Internal Amplification Control (IAC) DNA is included in all PCRs. Manual review of amplification curves is conducted.

C. Validation Criteria		
1. Accuracy / Trueness	Y	Result: 110% Data: Table 1 Spike Range: -0.35 to 6.54 Log CFU/g
2. Measurement uncertainty	Y	Result: -0.57 to 0.044 log MPN/g Data: Table 1 Spike Range: -0.35 to 6.54 Log CFU/g
3. Precision characteristics (repeatability)	Y	Results: Variance ratio is *not significant*, based on least square regression. Calculated variability of the MPN method is 0.39, with a lower 95% CI of 0.32. The theoretical variability is 0.32. Data: Table 2, Figure 1 Spike Range: 0.38 to 5.54 Log CFU/g
4. Recovery	Y	Result: 110% Is the one way ANOVA to determine the consistency of recovery significant? No. Data: Table 2 Spike Range: 0.38 to 5.54 Log CFU/g
5. Specificity	Y	*V. alginolyticus*: SI_{avg} = -1.28, p=0.42 *V. cholerae*: SI_{avg} = 1.26, p=0.09 *V. fluvialis*: SI_{avg} = -2.41, p=0.79 *V. parahaemolyticus*: SI_{avg} = 7.49, p=0.07

		Data: Table 3 Range: 0.52 to 1.53 Log CFU/g
6. Working and Linear ranges	Y	Pearson's r: 0.97 Line equation: log(MPN) = 0.44 + 0.93 x log(Plate Count) Is Pearson's r significant?:Yes Data: Table 4 and Figure 2 Range: -0.62 to 6.54 Log CFU/g
7. Limit of detection	Y	Result: 2.75 95% CI: 1.95, 3.88 Data: Table 4 and Figure 1 Range: -0.62 to 6.54 Log CFU/g
8. Limit of quantitation / Sensitivity	Y	Result: 0.3 MPN/g Data: Table 4 and Figure 1 Range: -0.62 to 6.54 Log CFU/g
9. Ruggedness	Y	Is there a significant difference between samples? Not under conditions tested. Data: Table 5 Range: 0.52 to 4.88 Log CFU/g
10. Matrix effects	Y	Effects of oyster matrix on the performance of the method was taken into consideration by using various sources of oysters for this study. Appendix D.
11. Comparability (if intended as a substitute for an established method accepted by the NSSP)	Y	No statistically significant difference between test and accepted methods. ($p<0.05$) Data: Table 6

D. Other Information		
1. Cost of the method	Y	Cost per sample for MPN: $1.05 Cost per sample for PCR: $20.55 Cost only includes reagents and consumables, infrastructure and personnel were not taken into account.
2. Special technical skills required to perform the method	Y	It is recommended that analysts have some formal training in molecular techniques or PCR, specifically.
3. Special equipment required and associated cost	Y	AB7500 FAST: $34,060.00 AB7500 FAST annual maintenance contract: $5,777.00
4. Abbreviations and acronyms defined	Y	Abbreviations and Acronyms are listed in appendix E.
5. Details of turn around times	Y	Results can be reported within 28h of

(time involved to complete the method)		sample receipt.
6. Provide brief overview of the quality systems used in the lab	Y	The laboratory adheres to the quality system standards of FDA/CFSAN, as well as those of the NSSP.

Submitters Signature	Date:
Submission of validation data and draft method to committee	Date:
Reviewing members:	
Accepted	Date:
Recommendations for further work	Date:

A. Validation Criteria

Data were generated using 20 separate lots of PHP oysters spiked with appropriate dilution(s) of a log phase culture of *Vibrio vulnificus*. Spike levels were determined by plate counts on TSA. Unless otherwise stated data was handled and analyzed as recommended in the SLV Documents for MPN Based Microbiological Methods on the ISSC website, with the exception of correcting for background using the blank sample data. The correction was not made because the levels in the blank samples were extremely low (near the LOD) and the it was more appropriate, from a statistical perspective, to not make the adjustment. For samples not detected, ½ the theoretical LOD was substituted for those values. For samples greater than the upper limit of the test, the values for the upper limit was used.

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

Sample	Plate Count (Log CFU/g)	Sample Blank (Log MPN/g)	Spiked Sample (Log MPN/g)
1	-0.35	0.31	-0.25
2	4	ND*	3.33
3	1.19	ND	1.4
4	2.92	-0.45	3.17
5	1.38	ND	1.8
6	1.06	-0.52	2.36
7	2.74	-0.52	2.64
8	4.78	ND	4.96
9	4.84	ND	5.75
10	3	ND	3.38
11	6.54	ND	6.16
12	1.11	ND	1.63
13	6.08	0.36	5.36
14	4.88	ND	5.62
15	-0.19	ND	-0.15
16	2.57	ND	2.36
17	0.97	ND	1.92
18	1.53	ND	1.17
19	1.88	-0.45	2.16
20	0.52	-0.13	0.50

*ND=Not Detected

Table 2. Data used for determination of Precision and Recovery. Samples A and B are replicate analyses of the spiked homogenate.

Sample	Plate Count (Log CFU/g)	Sample Blank (Log MPN/g)	Spiked Sample A (Log MPN/g)	Spiked Sample B (Log MPN/g)
1	0.66	0.31	0.87	1.36
1	2.66	0.31	2.96	2.63
1	4.66	0.31	4.63	5.31
3	1.19	ND	1.63	1.17
3	3.19	ND	1.72	2.96
3	5.19	ND	3.96	5.17
5	0.38	ND	0.63	0.96
5	2.38	ND	2.63	2.17
5	4.38	ND	3.96	4.36
7	0.74	-0.52	1.32	1.31
7	2.74	-0.52	2.96	2.32
7	4.74	-0.52	4.97	5.35
9	0.84	ND	2.04	1.33
9	2.84	ND	3.66	3.38
9	4.84	ND	5.87	5.62
11	1.54	ND	1.63	1.63
11	3.54	ND	3.63	3.36
11	5.54	ND	5.34	5.62
13	1.08	0.36	1.36	1.96
13	3.08	0.36	2.96	3.16
13	5.08	0.36	5.18	4.97
15	0.81	ND	0.63	0.87
15	2.81	ND	2.63	3.96
15	4.81	ND	4.97	4.63
17	0.97	ND	1.97	1.87
17	2.97	ND	4.38	3.97
17	4.97	ND	5.87	5.887
19	0.88	-0.45	1.63	0.87
19	2.88	-0.45	3.36	3.36
19	4.88	-0.45	5.62	5.62

*ND=Not Detected

Figure 1. Plot of data from Table 2 by different concentrations (Low, Medium, and High). An alternative approach from ISSC recommendations to evaluating precision was used as a generalized least square regression with heterogenous variance structure was deemed a more appropriate test to estimate variance components for method error at different concentrations and then test whether or not method error varies significantly by concentration level. The output estimates of the variance components of the fit of two different models and then a comparison of those fits. One model has different parameters for method variation for each level (L, M, H) and the other constrains that variation to be the same across levels. The 1st model (null) estimates a common method error SD as 0.387 (same as the nested ANOVA). The 2nd model (full) estimates different method error SDs as 0.3217, 0.4688 and 0.3558 at levels L, M, and H respectively. Both models fit the same main effects (Levels nested within Samples) to remove that variation from what remains to determine method error estimates. A likelihood ratio test is used to compare the difference in the fit between the two models. The test statistic is the likelihood ratio between the two models and this is distributed as a Chi-square with 2 degrees of freedom (the difference in the number of parameters between the two models, 3 vs 1 variance parameters). The test statistic has a value of 1.58 and the p-value is 0.54 indicating no significant difference between the fits and hence no strong statistical evidence that method error varies across levels (L, M, H). The MSE for the residuals is 0.15. This corresponds to a SD of 0.39, which is only slightly higher than the theoretical method error SD (0.32), with a lower 95% confidence limit of 0.32.

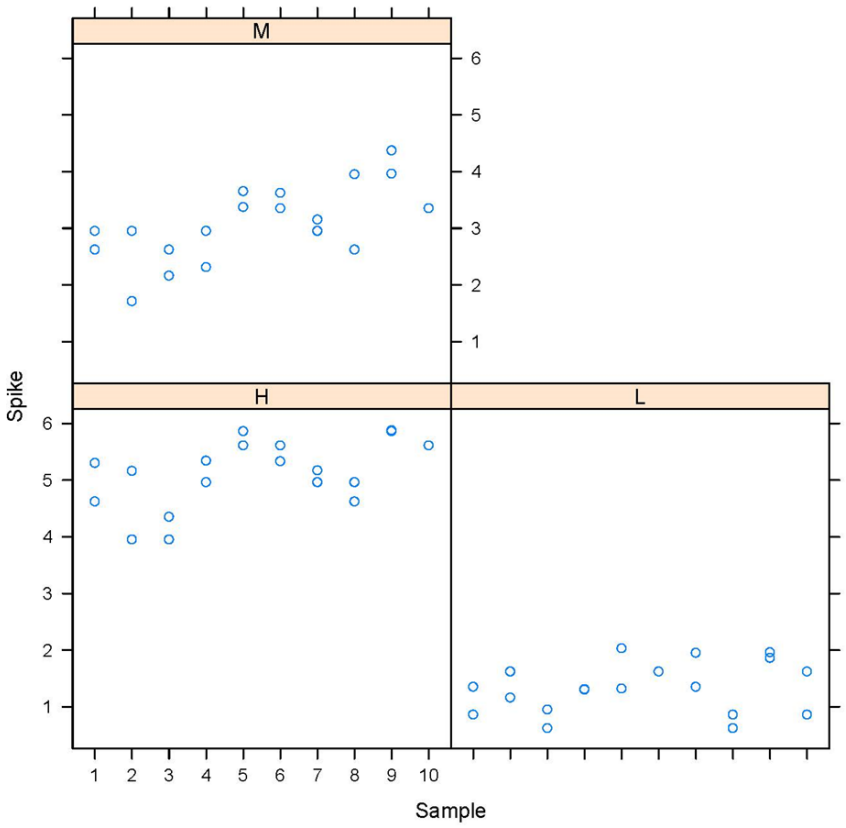

Table 3. Data used for determination of Specificity. Spike samples A-E are replicate analyses of the homogenate spiked only with Vv. Dual spike samples A-E are replicate analyses of the same homogenate spiked with Vv and the interfering organism.

Sample	Interfering Organism	Interfering Organism Plate Count (Log CFU/g)	*Vibrio vulnificus* Plate Count (Log CFU/g)	Sample Blank (Log MPN/g)	Spike Sample A (Log MPN/g)	Spike Sample B (Log MPN/g)	Spike Sample C (Log MPN/g)	Spike Sample D (Log MPN/g)	Spike Sample E (Log MPN/g)	Dual Spike Sample A (Log MPN/g)	Dual Spike Sample B (Log MPN/g)	Dual Spike Sample C (Log MPN/g)	Dual Spike Sample D (Log MPN/g)	Dual Spike Sample E (Log MPN/g)
6	*Vibrio parahaemolyticus*	5.49	1.06	-0.52	2.36	2.38	2.38	2.38	2.66	1.63	0.31	0.19	1.32	0.19
12	*Vibrio cholerae*	6.75	1.11	ND	1.63	1.63	1.63	1.63	1.96	1.36	0.96	1.36	1.63	1.63
18	*Vibrio fluvialis*	6.83	1.53	ND	1.17	1.36	1.63	1.36	1.86	1.96	1.96	1.63	-0.03	0.06
20	*Vibrio alginolyticus*	6.17	0.52	-0.13	0.45	0.96	0.31	0.45	0.45	0.96	0.44	0.43	-0.04	0.3

Table 4. Data used for determination of Working and Linear Ranges, Limit of Detection, and Limit of Quantitation/Sensitivity. Samples A and B are replicate analyses of the spiked homogenate. The LOQ is determined by the amount of inoculum used in the lowest dilution of the MPN, so long as the LOD is not statistically different than 1. As tested with a starting inoculum of 1g, the LOD of this method is 0.3 MPN/g

Sample	Plate Count (Log CFU/g)	Sample Blank (Log MPN/g)	Spike Sample A (Log MPN/g)	Spike Sample B (Log MPN/g)
1	-0.35	0.31	-0.45	-0.04
1	0.66	0.31	0.87	1.36
1	1.66	0.31	1.63	3.06
1	2.66	0.31	2.96	2.63
1	4.66	0.31	4.63	5.31
1	5.66	0.31	5.92	6.16
3	0.19	ND	0.86	0.63
3	1.19	ND	1.63	1.17
3	2.19	ND	2.36	2.87
3	3.19	ND	1.72	2.96
3	5.19	ND	3.96	5.17
3	6.19	ND	6.16	6.16
5	-0.62	ND	0	-0.45
5	0.38	ND	0.63	0.96
5	1.38	ND	1.63	1.96
5	2.38	ND	2.63	2.17
5	4.38	ND	3.96	4.36
5	5.38	ND	4.97	4.63
7	-0.25	-0.52	0.87	0.19
7	0.74	-0.52	1.32	1.31
7	1.74	-0.52	1.96	2.96
7	2.74	-0.52	2.96	2.32
7	4.74	-0.52	4.97	5.35
7	5.74	-0.52	5.92	6.16
9	-0.15	ND	0.31	0.17
9	0.84	ND	2.04	1.33
9	1.84	ND	1.87	2.04
9	2.84	ND	3.66	3.38
9	4.84	ND	5.87	5.62
9	5.84	ND	5.87	6.16
11	0.54	ND	0.36	0.63
11	1.54	ND	1.63	1.63
11	2.54	ND	2.36	2.87
11	3.54	ND	3.63	3.36

11	5.54	ND	5.34	5.62
11	6.54	ND	6.16	6.16
13	0.08	0.36	0.36	0.63
13	1.08	0.36	1.36	1.96
13	2.08	0.36	2.36	2.17
13	3.08	0.36	2.96	3.16
13	5.08	0.36	5.16	4.97
13	6.08	0.36	6.16	4.56
15	-0.19	ND	-0.45	0.16
15	0.81	ND	0.63	0.87
15	1.81	ND	1.45	0.54
15	2.81	ND	2.63	3.96
15	4.81	ND	4.97	4.63
15	5.81	ND	5.92	6.16
17	-0.03	ND	0.96	1.17
17	0.97	ND	1.96	1.87
17	1.97	ND	2.97	2.97
17	2.97	ND	4.38	3.97
17	4.97	ND	5.87	5.87
17	5.97	ND	6.16	6.16
19	-0.12	-0.45	-0.04	0.17
19	0.88	-0.45	1.63	0.87
19	1.88	-0.45	1.96	2.36
19	2.88	-0.45	3.36	3.36
19	4.88	-0.45	5.62	5.62
19	5.88	-0.45	6.16	6.16

Figure 2. Plot of data from Table 4 for determination of LOD/LOQ.

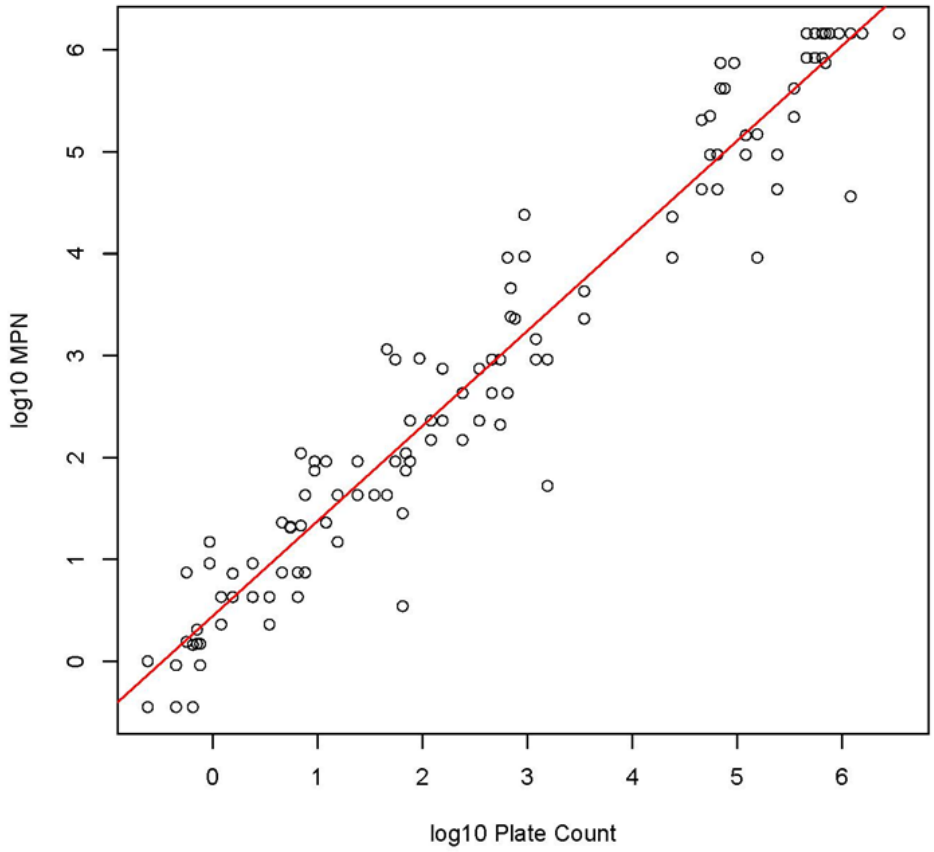

Table 5. Data used for determination of Ruggedness. Results reported as log MPN/g of *V. vulnificus* for each variation of the method SOP.

Sample	Plate Count (Log CFU/g)	Sample Blank (Log MPN/g)	Media/ Reagents		Oysters shucked and held prior to blending				RT 30 m Post-blending	MPN incubation			Boil prep time		Master Mix stored frozen				Master Mix thawed and re-frozen			Master Mix at RT 0.5-1.5h
			Lot 1	Lot 2	4C 1h	4C 3h	RT 30m	RT 1h		35C >24h	RT 18-24h	39C 18-24h	5m	30m	5d	3d	2d	1d	4X	3X	2X	
2	4.00	0.52	3.33	5.04	3.38	4.04	3.66	4.04	4.04	5.04	4.04	3.66	4.04	4.04	3.33	3.06	3.33	3.33	3.33	3.33	3.33	2.54
4	2.92	-0.45	3.17	3.17	2.66	3.04	3.38	2.36	3.17	2.97	3.38	3.38	3.17	3.38	3.17	3.17	3.17	3.17	3.17	3.17	3.17	3.17
6	1.06	-0.52	2.36	2.36	0.06	-0.45	0.31	1.87	0.36	0.54	1.53	0.63	2.66	2.38	2.66	2.38	2.38	2.38	2.66	2.66	2.38	2.38
8	4.78	ND	4.96	4.63	4.38	417	4.38	4.63	4.63	4.59	4.38	4.63	4.63	4.96	4.63	4.63	4.63	4.63	4.63	4.63	4.63	4.63
10	3.00	ND	3.38	3.38	2.97	2.38	3.66	2.63	3.16	3.17	2.96	2.63	3.36	3.36	3.36	3.36	3.36	3.36	2.96	3.36	3.36	3.36
12	1.11	ND	1.63	1.63	1.63	0.87	1.86	1.36	1.63	1.3	1.17	1.16	1.63	1.63	1.96	1.63	1.63	1.63	1.63	1.63	1.63	1.63
14	4.88	ND	5.62	5.34	4.97	4.97	4.97	4.97	4.97	5.34	4.63	4.63	5.62	5.62	5.62	5.62	5.62	5.62	5.62	5.62	-0.52	5.62
16	2.57	ND	2.36	2.36	1.36	1.96	2.17	2.36	2.63	2.63	2.63	3.36	2.36	2.36	2.36	2.36	2.36	2.36	2.36	2.36	2.36	2.36
18	1.53	ND	1.17	1.36	1.63	1.36	1.86	1.36	1.63	1.63	1.63	2.17	1.32	1.63	1.96	1.63	1.63	1.96	1.96	1.96	1.96	1.96
20	0.52	-0.13	0.45	0.96	0.31	-0.04	0.96	0.19	-0.04	1.17	0.58	0.36	0.32	0.17	0.45	0.45	1.63	0.45	0.45	1.63	0.45	0.45

Table 6. Data used for determination of Comparability. Samples of with naturally incurred *V. vulnificus* were analyzed by both the test (MPN-real-time PCR) and NSSP (MPN-culture, with DNA probe confirmation) methods.

Sample	Real-Time PCR (Log MPN/g)	DNA Probe (Log MPN/g)
AL18-121	3.87	4.17
AL18-122	4.17	3.31
AL18-123	4.17	3.31
AL18-130	3.96	3.17
AL18-131	3.96	2.96
AL18-132	4.06	**>4.06**
AL18-139	3.96	3.96
AL18-148	1.98	3.96
AL-1	3.63	3.96
AL-2	2.63	3.32
SC-1	1.63	2.36
SC-2	2.36	2.36
SC-3	0.58	3.16
NC-1	2.45	3.36
NC-2	3.63	3.63
NC-3	3.17	3.17
NC-4	2.96	3.63
NC-5	2.63	3.63
NC-6	2.63	3.63
NC-7	2.36	4.31
VA-1	3.63	3.63
VA-2	-0.45	2.63
VA-3	2.44	2.17
WA-1	ND	1.36
WA-2	ND	-0.03
WA-3	ND	1.36
WA-4	ND	-0.52
WA-5	ND	1.32
CA-1	ND	-0.04
CA-2	ND	ND
OR-1	ND	ND
OR-2	ND	ND

Appendix A. Equipment Required.

Blender
Oyster knife
Shucking knife
Nitrile or Latex gloves
Soap
Stiff bristled brush
Chain mail glove (optional)
Bone cutting forceps (optional)
Test tubes (FisherScientific, 14-961-32, or equivalent)
Tube closures (FisherScientific, 14-957-92K, or equivalent)
Test tube racks (FisherScientific, 14-809-64, or equivalent)
Sterile stripettes (FisherScientific, 07-200-574, or equivalent) or pipet tips
Pipette-Aid or micropipettor (capable of 1000 µl)
Balance with a sensitivity of at least 0.01g
Incubator capable of maintaining 35±2°C
Heat block (95-100°C) or boiling water bath
Eppendorf 5415D centrifuge or equivalent (capable of >10,000x*g*)
Microcentrifuge tubes (USA Scientific, 1620-2799, or equivalent)
Mini-centrifuge (USA Scientific, 2631-0006, or equivalent)
AB 7500 Fast System (Life Technologies, Foster City, CA)
MicroAmp Fast Optical 96 Well Reaction Plate 0.1mL (Cat# 4346907) or MicroAmp Fast 8-tube strips (0.1 mL) (Cat# 4358293)
MicroAmp Optical 8-Cap Strip (Cat# 4323032) or Optical Adhesive Film (Cat# 4311971)
Micropipettors (volume ranges from 0.1 – 1000 µl)
Filtered, DNase/RNase-free pipette tips
Refrigerator capable of maintaining 2-8°C
Freezer capable of maintaining <-15°C
Stripfuge or 96 well plate centrifuge
Ice bucket (optional)
Tube and plate racks
PCR hoods with UV light

Appendix B. Media and Reagents Required.

APW, prepared according to BAM manual, Chapter 9, Vibrio (M10).
PBS, prepared according to BAM manual, Chapter 9, Vibrio (R59).
Platinum *Taq* DNA polymerase kit (ThermoFisher,10966026) Invitrogen, Carlsbad, CA):
Includes Taq, PCR Buffer, 50mM $MgCl_2$
PCR Nucleotide Mix (DNTP's) (Sigma Aldrich, 11814362001)
ROX reference dye (ThermoFisher,12223012)
Internal Amplification Control (IAC) DNA (BioGX, Birmingham, AL)
PCR-grade water (Ambion AM9937, or equivalent)
Crushed ice (optional)
Tris pH 8.0 (ThermoFisher, AM9855G, or equivalent)
Oligonucleotide primers (desalted) – see Table
Nuclease-style probes (HPLC purified) – see Table

	Sequence (5' to 3')	Modifications
*vvh*F	TGTTTATGGTGAGAACGGTGACA	--
*vvh*R	TTCTTTATCTAGGCCCCAAACTTG	--
vvh Probe	CCGTTAACCGAACCACCCGCAA	5Cy5-3IAbRQSp[a]
IAC 46G	GACATCGATATGGGTGCCG	--
IAC 186R	CGAGACGATGCAGCCATTC	--
IAC Probe	TCTCATGCGTCTCCCTGGTGAATGTG	56-JOEN-3IABkFQ[b]

[a] Iowa Black RQ-Sp
[b] Iowa Black FQ

Appendix C. Detailed Procedure.

1. **Shellfish Preparation:**
    a. Scrape off growth and loose material from shell and scrub shell stock with sterile stiff brush under running water.
    b. Place clean shellstock on clean towels or absorbent paper.
    c. Change gloves and brushes between samples.
    d. Protective chain mail glove can be used under a latex or nitrile glove; outer gloves should be changed between samples and disinfected with alcohol immediately prior to analysis.
    e. Tare a sterile blender.
    f. 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.
    g. Cut adductor muscle from upper flat shell and pry the shell wide enough to drain shell liquor into the blender.
    h. The upper shell can then be pried loose at hinge and discarded.
    i. The whole animal (including adductor muscle) should be transferred to the sterile blender after severing the adductor muscle connection to the lower shell.
    j. A minimum of 12 animals is used.
    k. blend for 60-120 sec. If sample requires dilution, an equal weight of sterile PBS is used. After blending, homogenized sample is further processed within 20 minutes.
2. **MPN for Vibrio Analysis**
    a. Prepare a 1:10 dilution of the homogenate by transferring 1g (weighing is required for accurate transfer) of the homogenate to 9 mL of PBS.
        i. If diluent was used, transfer 2 g of 1:1 homogenate to 8 mL of PBS. Additional 10-fold dilutions can be prepared volumetrically (i.e., 1 mL of 1:10 to 9mL of PBS for a 1:100 dilution).
        ii. Volume of PBS is critical, so tubes must be aseptically filled after sterilization of diluent.
    b. Transfer 1g of homogenate to APW, in triplicate (this should be done by weight to ensure accurate transfer).
    c. Inoculate 1 ml portions of the 1:10, 1:100, 1:1000, 1:10,000, 1:100,000, and 1:1,000,000 dilutions (from step 2.a.) into APW, in triplicate, for as many dilutions as deemed necessary for the sample.
    d. Inoculate appropriate process controls into properly labeled tubes of APW.
    e. Leave one APW tube un-inoculated as a blank.
    f. Incubate APW overnight (18-24h) at 35 ±2°C.
    g. Confirm presence of Vv in each turbid tube by Real-Time PCR as described below.
    h. Determine MPN estimate for each sample using the draft "Dilution Selection Tool" to select appropriate dilutions. Use the standard table or calculator tool available in the BAM, Appendix 2 and report as MPN/g of shellfish.

3. **Preparation of DNA Extracts**
    a. Transfer 1mL from each MPN tube with visible growth (turbidity) to a microcentrifuge tube.
    b. Boil (heat to 95-100°C) the sample aliquots from APW tubes, including the process controls and blank, for 10 min. Ensure that one set of process controls is included with each set of samples in a heat block.
    c. Immediately plunge into ice until cold, or freeze at <-15°C.
    d. If extracts were previously frozen, ensure they are completely thawed (not exceeding room temperature) before proceeding.
    e. Centrifuge samples for 2 min at >10,000 x *g*. Use 2µL of supernatant as template in the real-time PCR reaction as detailed below.
    f. DNA extracts can be stored at 4°C for up to 3d or at <-15°C for up to 6 months.
4. **Preparation of PCR**
    a. Prepare mastermix in the clean hood and using aerosol resistant pipette tips. Use DNAse and RNAse free consumables.
        i. Refer to the Table below for component concentrations.
        ii. Briefly mix tubes of individual components.
        iii. Briefly centrifuge the tubes (2-3 sec) in a mini centrifuge.
        iv. Combine components (except for IAC DNA) into an appropriately sized tube.

PCR Mastermix

Component	Units	Final Concentration	Vol/Rxn (µL)
PCR H2O	--	--	12.22
PCR Buffer	X	1.000	2.500
$MgCl_2$	mM	5.000	2.500
dNTPs (mixed equal conc of each)	mM	0.300	0.750
Forward Primer *vvh*F	µM	0.300	0.750
Reverse Primer *vvh*R	µM	0.300	0.750
Forward Primer IAC 46F	µM	0.075	0.188
Reverse Primer IAC 186R	µM	0.075	0.188
Probe *vvh* Cy5	µM	0.200	0.500
Probe IAC JOE	µM	0.150	0.375
Platinum Taq	Units/µL	1.120	0.220
ROX (passive reference dye) 1:1 dilution	--	0.03	0.060

    b. After the mastermix is compiled, move to a template hood and add the appropriate amount of IAC DNA to the mastermix. Use an IAC concentration that will amplify between 24-29 cycles.
    c. The completed mastermix should be used the day of preparation or frozen until use. Mastermix can be frozen at this point or after it has been aliquoted into the reaction tubes or wells.

d. Flick mastermix tube to mix. If previously frozen as a single tube, ensure the mastermix is completely thawed (not exceeding room temperature).
e. Briefly centrifuge mastermix (2-3 sec) in a mini centrifuge.
f. Add 23µL of mastermix to each reaction tube or well. If previously frozen after aliquoting to individual tubes or wells, ensure the mastermix is completely thawed (not exceeding room temperature) and then briefly centrifuge (2-3 sec) before proceeding.
g. Add 2µL of supernatant from each boiled DNA extract sample (including process controls and APW blank) to a reaction tube or well.
h. Add 2µL of positive control template (boiled cells of strain VV ATCC 33816) to a reaction tube or well as a PCR positive control.
i. Add 2µL of PCR-grade water to a tube or well as a PCR negative control.
j. Centrifuge sample tubes or 96-well plate briefly (2-3 sec) to ensure reagents and sample are settled to the bottom.
k. Load sample tubes or 96-well plate to instrument and start cycling with the cycling parameters listed in Table below.
l. The read stage for the instrument should be programmed to the extension phase.

Cycling Parameters

	Temp (°C)	Time (s)	
Initial Denature	95	60	--
Denature	95	15	
Anneal	57	15	x45
Extend	72	25	

5. **Data Analysis:**
   a. For results analysis, default instrument settings will be used, except the threshold is set at 0.02 and background end cycle set from 3 to 10 on the AB7500.
   b. Positive/negative results will be recorded based on the instrument determinations. Analyst will review amplification data for all samples and can record a positive/negative determination discrepant with the instrument output if supported by the raw fluorescence data.
   c. If both the IAC and target are negative, the reaction should be considered invalid, and the sample re-tested.
   d. If the negative PCR control reaction is positive, all positive samples in the same run must be considered invalid, and can be re-tested.
   e. If the positive PCR control reaction is negative, all negative samples in the same run must be considered invalid, and can be re-tested.

Appendix D. Source of matrix for spike samples.

Sample	PHP Type	Date of Harvest	Location of Harvest	Process Date	Vv Strain
1	HPP	2017-10-15	Area 3. LA	2017-10-18	07-2405
2	HPP	2017-10-21	Area 5. LA	2017-10-25	K4776
3	IQF	2017-05-17	Area 8. LA	2017-05-28	R844-G9
4	Irradiated	2017-11-02	Area 3. LA	2017-11-07	R19-C1
5	HPP	2017-11-13	Area 19. LA	2017-11-15	K4633
6	IQF	2017-06-12	Area 9. LA	2017-06-15	R84-F1
7	HPP	2017-12-03	Area 3. LA	2017-12-06	07-2405
8	Irradiated	2018-01-03	Area 3. LA	2018-01-07	K4776
9	HPP	2018-04-15	Area 19. LA	2018-04-18	R844-G9
10	IQF	2018-01-14	Area 3. LA	2018-01-18	R19-C1
11	HPP	2018-05-19	Area 19. LA	2018-05-23	K4633
12	IQF	2018-03-07	Area 9. LA	2018-03-08	R84-F1
13	HPP	2018-06-17	Area 12. LA	2018-06-20	07-2405
14	IQF	2017-12-01	Area 3. LA	2017-12-04	K4776
15	HPP	2018-07-01	Area 3. LA	2018-07-05	R844-G9
16	IQF	2017-12-16	Area 3. LA	2017-12-18	R19-C1
17	HPP	2018-07-29	Area 3. LA	2018-08-01	K4633
18	IQF	2017-12-16	Area 3. LA	2017-12-18	R84-F1
19	HPP	2018-08-12	Area 3. LA	2018-08-16	07-2405
20	IQF	2017-12-20	Area 9. LA	2017-12-21	K4776

Appendix D. Abbreviations and Acronyms.

APW – Alkaline Pepton Water
ATCC – American Tissue Culture Collection
BAM – Bacteriological Analytical Manual
CFU – Colony Forming Unit
DNA – Deoxyribonucleic Acid
EDTA- Ethylene diamine tetraacetic acid
IAC – Internal Amplification Control
MPN- Most Probable Number
NPC – Negative Process Control
NSSP – National Shellfish Sanitation Program
PBS- Phosphate Buffered Saline
PCR – Polymerase Chain Reaction
PPC – Positive Process Control
RNA- Ribonucleic Acid
Tris – tris(hydroxymethyl)aminomethane
Vv- Vibrio vulnificus