Proposal 19-128

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
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SHELLFISH LABORATORY EVALUATION CHECKLIST

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**

OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: Conformity is noted by a "√"

C- Critical K - Key O - Other NA- Not Applicable

Check the applicable analytical methods:

	MPN Real-time PCR method for *Vibrio vulnificus* detection in Oysters [PART III] SmartCycler II
	MPN Real-time PCR method for *Vibrio parahaemolyticus* detection in Oysters [PART III] SmartCycler II and AB 7500 Fast
	MPN Real-time PCR method for *Vibrio parahaemolyticus* and *Vibrio vulnificus* detection in Oysters [Part III]

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PART I – Quality Assurance			
		ITEM	
CODE	REF		
		1.1 Quality Assurance (QA) Plan	
K	4, 6	1.1.1 Written Plan (Check √ those items which apply).	
			a. Organization of the Laboratory.
			b. Staff training requirements.
			c. Standard operating procedures (SOPs).
			d. Internal quality control measures for equipment, their calibration maintenance, repair, performance and rejection criteria established.
			e. Laboratory safety.
			f. Internal performance assessment.
			g. External performance assessment.
C	**4**		**1.1.2 The QA plan is implemented.**
K	6		1.1.3 The Laboratory participates in a proficiency testing program annually. Specify the program(s): _____
		1.2 Educational/Experience Requirements	
C	**State's Human Resources Department**		**1.2.1 In state/county laboratories, the supervisor must have at least a bachelor's degree in microbiology, biology or equivalent discipline with at least two years of laboratory experience.**
K	State's Human Resources Department		1.2.2 In state/county laboratories, the analysts meet the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3 In commercial laboratories, the supervisor must have at least a bachelor's degree in microbiology, biology or equivalent discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4 In commercial laboratories, the analysts must have at least a high school diploma and at least three months of experience in laboratory sciences.
		1.3 Work Area	
O	4, 6		1.3.1 Adequate for workload and storage.
K	6		1.3.2 Clean, well lighted.
K	6		1.3.3 Adequate temperature control.
O	6		1.3.4 All work surfaces are nonporous, easily cleaned and disinfected.
K	6		1.3.5 Microbiological quality of the air contains fewer than 15 colonies/plate for a 15 minute exposure determined monthly. The results are recorded and records maintained.
		1.4 Laboratory Equipment	
K	5		1.4.1 To determine the pH of prepared media and reagents, the pH meter has a standard accuracy of 0.1 pH units.
K	9		1.4.2 pH electrodes consisting of pH half-cell and reference half-cell or equivalent combination electrode free from (Ag/AgCl) or contains an ion exchange barrier preventing passage of Ag ions into the medium which may affect the accuracy of the pH reading.
K	6		1.4.3 The effect of temperature on the pH is compensated for by an internal/external ATC probe or by manual adjustment (*Circle the appropriate type of adjustment*).
K	4		1.4.4 The pH meter is calibrated daily or with each use as per product literature. Results are recorded and records maintained.
K	6		1.4.5 A minimum of two standard buffer solutions are used to calibrate the pH meter. The first is near the electrode isopotential point (pH 7). The second is near the expected sample pH (i.e. pH 4 or pH 10). Standard buffer solutions are used once and discarded.
O	4		1.4.6 Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of the slope (*Circle the method used*).
K	5		1.4.7 The balances used provide a sensitivity of at least 0.1 g at the weights of use.

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K	6		1.4.8 Balance calibrations are checked monthly according to manufacturer's specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance is verified at the weight range of use. Results are recorded and records maintained.
K	6		1.4.9 Refrigerator temperatures are monitored at least once daily on workdays. Results are recorded and records maintained.
K	1		1.4.10 Refrigerator temperatures are maintained between 0 and 4 °C, except for reagent refrigerators which are maintained between 2 and 8 °C.
C	**7**		**1.4.11 Freezer temperature is maintained at -15 °C or below.**
O	7		1.4.12 Freezer temperature is monitored at least once daily on workdays. Results are recorded and records maintained.
C	**5**		**1.4.13 The temperature of the incubator is maintained at 35 +/- 2.0 °C.**
K	6		1.4.14 Thermometers used in the air incubators are graduated at no greater than 0.5 °C increments.
K	5		1.4.15 Working thermometers are located on top and bottom shelves of use in the air incubator or appropriately placed based on the results of spatial temperature checks.
K	4, 6		1.4.16 Air incubator temperatures are taken twice daily on workdays. Results are recorded and records maintained.
C	**3**		**1.4.17 All working thermometers are appropriately immersed.**
C	**2, 20**		**1.4.18 Working thermometers are either: calibrated mercury-in-glass thermometers, calibrated non-mercury-in-glass thermometers, or appropriately calibrated electronic devices, including Resistance Temperature Devises (RTDs) and Platinum Resistance Devices (PTDs).**
C	**6, 20**		**1.4.19 A standards thermometer has been calibrated by NIST or a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at the points 0 and 35. These calibration records are maintained.**
K	3, 5		1.4.20 Standard thermometers are checked annually for accuracy by ice point determination. Results are recorded and maintained. Date of most recent determination:_____
C	**2, 20**		**1.4.21 Either mercury-in-glass thermometers, non-mercury-in-glass thermometers having the accuracy (uncertainty), tolerance and response time of mercury or low drift electronic resistance thermometers with an accuracy of ≤0.05 °C are used as the laboratory standards thermometer (*Circle the thermometer type used*).**
K	3, 8		1.4.22 All working thermometers are checked annually against the standards thermometer at temperature(s) of use. Results are recorded and records maintained.
O	6		1.4.23 Appropriate pipet aids are available and used to inoculate samples.
K	2		1.4.24 Micropipettors are calibrated annually at appropriate volumes used and checked for accuracy quarterly. Results are recorded and records maintained.
		1.5 Labware and Glassware Washing	
K	5		1.5.1 Utensils, containers, glassware and plasticware are clean borosilicate glass, stainless steel or other noncorroding material.
K	5		1.5.2 Culture tubes are new and of a suitable size to accommodate the volume for nutritive ingredients and sample.
K	5		1.5.3 Dilution bottles and tubes are made of borosilicate glass or plastic and closed with secure caps or screw caps with nontoxic liners.
K	5		1.5.4 Graduations are indelibly marked on dilution bottles and tubes or an acceptable alternative method is used to ensure appropriate volumes.
K	5		1.5.5 In washing reusable pipets, glassware and labware, a succession of at least three fresh water rinses plus a final rinse of deionized water is used to thoroughly rinse off all detergent.
C	**2**		**1.5.6 An alkaline or acidic detergent is used for washing glassware/labware.**
C	**6**		**1.5.7 With each load of labware/glassware washed, the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali as appropriate) with aqueous 0.04% bromothymol blue (BTB) solution. Results are recorded and records maintained.**
		1.6 Sterilization and Decontamination	

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K	5		1.6.1 The autoclave is of sufficient size to accommodate the workload.
K	4		1.6.2 Routine autoclave maintenance is performed and the records maintained.
C	**6, 20**		**1.6.3 The autoclave provides a sterilizing temperature of 121 ± 2 °C as determined for each load using a calibrated maximum registering thermometer. As an alternative, an appropriate temperature monitoring device is used in place of the maximum registering thermometer when these are unavailable due to the ban on mercury.**
K	6		1.6.4 An autoclave standards thermometer has been calibrated by a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at 121 °C. Calibration at 100 °C, the steam point is also recommended but not required.
K	10		1.6.5 The autoclave standards thermometer is checked every five years for accuracy at either 121 °C or at 100 °C, the steam point if the thermometer has been previously calibrated at this temperature. Date of most recent determination: _____
K	1		1.6.6 Working autoclave thermometers are checked against the autoclave standards thermometer at 121 °C yearly. Date of last check:_____
K	6		1.6.7 Spore strips/suspensions appropriate for use in an autoclave media cycle are used monthly according to manufacturer's instructions to evaluate the effectiveness of the sterilization process. Results are recorded and the records maintained.
O	6		1.6.8 Heat sensitive tape is used with each autoclave batch.
K	6		1.6.9 Autoclave sterilization records including length of sterilization, total heat exposure time and chamber temperature are maintained. Type of record: Autoclave log, computer printout or chart recorder tracings (*Circle the appropriate type or types*).
K	6		1.6.10 For dry heat sterilized material, the hot-air sterilizing oven provides heating and sterilizing temperatures in the range of 160 to 180 °C.
K	5		1.6.11 A thermometer capable of determining temperatures accurately in the range of 160 to 180 °C is used to monitor the operation of the hot air sterilizing oven.
K	8		1.6.12 Records of temperature and exposure times are maintained for the operation of the hot-air sterilizing oven.
K	6		1.6.13 Spore strips/suspensions appropriate for use in dry heat are used quarterly to evaluate the effectiveness of the sterilization process in the hot-air oven. Results are recorded and records maintained.
K	5		1.6.14 Reusable pipets are stored and sterilized in aluminum or stainless steel containers.
K	5		1.6.15 Reusable pipets (in canisters) are sterilized in a hot-air oven at 170 °C for 2 hours.
C	**2**		**1.6.16 The sterility of reusable pipets is determined with each load sterilized. Results are recorded and records maintained.**
C	**2**		**1.6.17 The sterility of autoclave sterilized disposable pipet tips and microcentrifuge tubes is determined with each load sterilized. Results are recorded and records maintained.** **If presterilized pipet tips and microcentrifuge tubes are purchased certificate should be maintained and sterility confirmed as in 1.6.18.**
C	**2**		**1.6.18 The sterility of presterilized disposable pipets, pipet tips and microcentrifuge tubes is determined with each lot received. Results are recorded and records maintained.**
K	8		1.6.19 Spent broth cultures and agar plates are properly decontaminated before disposal.
		1.7 Media Preparation	
K	13, 14		1.7.1 Alkaline peptone water (APW) is prepared from the individual components and pH adjusted appropriately.
K	6		1.7.2 Media components are properly stored in a cool dry place.
O	6		1.7.3 Media components are labeled with the analyst's initials, date of receipt and date opened.
O	6		1.7.4 Dehydrated media are labeled with date of receipt and date opened.
C	**6**		**1.7.5 Caked or expired media or media components are discarded.**

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C	**6**		**1.7.6 Reagent water for media and diluent preparation is analyzed for residual chlorine monthly and is at a non-detectable level (≤0.1 ppm). Results are recorded and records maintained**
K	6		1.7.7 Reagent water for media and diluent preparation contains <100 CFU/mL as determined monthly using the heterotropic plate count method. Results are recorded and records maintained.
K	5		1.7.8 The volume and concentration of media in the tube is suitable for the amount of sample inoculated.
C	**6**		**1.7.9 Media broths are not in the autoclave for more than 60 minutes.**
C	**1**		**1.7.10 Media and diluent sterility is determined for each load sterilized. Results are recorded and records maintained.**
C	**1**		**1.7.11 Media productivity is determined using media-appropriate positive and negative control cultures for each lot of dehydrated media received or with each batch of media prepared when the medium is made from its individual components.**
C	**6**		**1.7.12 The pH of the prepared media is determined after sterilization to ensure that it is consistent with manufacturer requirements and/or method tolerance. Results are recorded and records are maintained.**
		1.8 Storage of Prepared Culture Media	
K	5		1.8.1 Prepared culture media are stored in a cool, clean, dry place where excessive evaporation and the danger of contamination is minimized.
K	8		1.8.2 Stored media are labeled with the storage expiration date or sterilization date.
K	5		1.8.3 Storage of prepared culture media at room temperature does not exceed 7 days.
K	2		1.8.4 Storage under refrigeration of prepared broth media with loose fitting closures does not exceed 1 month.
K	6		1.8.5 Storage under refrigeration of prepared culture media with screw- cap closures does not exceed 3 months.
K	11		1.8.6 All prepared broth media stored under refrigeration is warmed to room temperature prior to use, without exceeding incubation temperature.
PART II –Samples			
		2.1 Sample Collection, Transportation and Receipt	
C	**2, 6**		**2.1.1 A representative sample is collected and a chain of custody documenting the history of the sample(s) from collection to final disposal has been established.**
K	5		2.1.2 Shellfish samples as received are collected in clean, waterproof, puncture resistant containers loosely sealed or are rejected for regulatory analysis.
K	5		2.1.3 Shellfish samples as received are labeled with the collector's (or if PHP, company/processor and collector's) name, the source, the time and date of collection or are rejected for regulatory analysis.
C	**5**		**2.1.4 Immediately after collection, shellfish samples are placed in dry storage (ice chest or equivalent) which is maintained between 2 and 10 °C with ice or cold packs for transport to the laboratory. Once received, the samples are placed under refrigeration unless processed immediately.**
C	**1**		**2.1.5 Analysis of the samples is initiated as soon as possible after collection, but not to exceed 36 h. If processing IQF samples, samples are defrosted under refrigeration for no longer than 36 h once removed from the freezer.**
		2.2 Preparation of Samples for Analysis	
K	2, 6		2.2.1 Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes.
O	2		2.2.2 Blades of shucking knives are not corroded.
K	5		2.2.3 The hands of the analyst are thoroughly washed with soap and water or new gloves are donned, immediately prior to cleaning the shells of debris.
O	2		2.2.4 The faucet used for rinsing the shellfish does not contain an aerator.
K	5		2.2.5 Shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
K	5		2.2.6 Samples are allowed to drain in a clean container or on clean towels prior to opening
K	5, 15		2.2.7 Immediately prior to shucking, the hands or gloved hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol. The gloves if worn are latex, nitrile and/or stainless steel mesh to protect analyst's hands from injury.
C	**5**		**2.2.8 Shellfish are not shucked through the hinge.**

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C	**5**		**2.2.9 The contents of the sample (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
C	**5**		**2.2.10 A representative sample of at least 12 shellfish is used for analysis**
C	**2, 5**		**2.2.11 A quantity of meat and liquor is sufficient to cover the blender blades or additional oysters are used in order to ensure sample homogeneity.**
K	2, 13		2.2.12 The sample can be processed directly or a 1:1 dilution of shellfish:diluent made. If a dilution is made, the sample is weighed to the nearest 0.1 g and an equal amount, by weight, of diluent is added.
K	13		2.2.13 Sterile phosphate buffered saline (pH 7.4) is used as the sample diluent.
C	**5**		**2.2.14 Samples are blended for 60 to 120 seconds until homogenous.**
PART III- PCR method for *Vibrio vulnificus* and *Vibrio parahaemolyticus* detection in Oysters			
		3.1 APW Enrichment	
K	5		3.1.1 Sterile phosphate buffered saline (PBS) is used as the sample diluent.
C	**5, 15**		**3.1.2 The 1:10 dilution is prepared gravimetrically with PBS. All successive dilutions are prepared volumetrically.** **For example, if an initial 1:1 dilution of the sample was used for blending, the 1:10 dilution is prepared by adding 20 g of sample homogenate to 80 ml of PBS. If the homogenate was not diluted, the 1:10 dilution is prepared by adding 10 g of sample homogenate to 90 ml of PBS.**
C	**17**		**3.1.3 Appropriate sample dilutions are inoculated into APW.** **Specify dilution(s) used _____ Specify number of tubes per dilution _____**
C	~~**2, 15**~~**21**		**3.1.4 For *V. parahaemolyticus* analysis, a tdh+, trh+ *V. parahaemolyticus* culture diluted to <10^3 per ml is used as a positive process control. A non *V. parahaemolyticus* culture is used as a negative process control.** **For *V. vulnificus* analysis, a *V. vulnificus* culture diluted to <10^3 per ml is used as a positive process control. A non *V. vulnificus* culture is used as a negative process control.** **An uninoculated APW blank will serve as the uninoculated control.** **The process control cultures accompany the samples throughout incubation, isolation, and confirmation. Records are maintained.**
C	**13**		**3.1.5 Inoculated APW enrichment tubes are incubated at 35 +/- 2 °C.**
C	**13**		**3.1.6 Tubes are read after 18 – 24 hours of incubation. Clear tubes are negative. Turbid tubes are positive and shall be further processed.**
		3.2 PCR Reagents	
C	**14, 15**		**3.2.1 Lyophilized primers and probes are stored according to manufacturer's instructions.**
K	14, 15		3.2.2 Fluorescent probes are stored in light occluding tubes or containers.
C	~~**14, 15, 18,**~~ ~~**19**~~**21**		**3.2.3 The PCR forward and reverse primers and probes ~~are appropriate for the platform~~target.** **For Total and Pathogenic Vp Real-time PCR Method** Trh 627F: 5' ATA CCT TTT CCT TCT CCW GGT TC 3'~~tdh_269-20: 6FAM-5'-TGACATCCTACATGACTGTG-3'-MGBNFQ~~ Trh 731b R: 5' TTG TCC AGT AGT CAT CAA CGA TTG 3'~~trh_133-23: NED/TET-5'-AGAAATACAACAATCAAAACTGA-3'-MGBNFQ~~ Trh Glov R: 5' TTG TCC AAT AGT CCT CCA CAA TTG 3'~~tlh_1043: JOE/TEXAS-RED-5'-CGCTCGCGTTCACGAAACCGT-3'-BHQ2~~ WA IC F: 5' GGC GAA GCG AAT CTG GAA A 3'~~IAC_109: CY5-5'-TCTCATGCGTCTCCCTGGTGAATGTG-3'-BHQ2~~ WA IC R: 5' GGT GTA GTT GTG CGT GTA ATA TGA GA 3'~~trh_20F: 5'-TTGCTTTCAGTTTGCTATTGGCT-3'~~

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			Orf8 F: 5' TCA CCT GAG GAC GCA GTT ACG 3'~~trh_292R: 5' TGTTTACCGTCATATAGGCGCTT 3'~~ Orf8 R: 5' TTC AAT TGT AGA ACC GCC AGC TA 3'~~tdh_89F:5' TCCCTTTTCCTGCCCCC 3'~~ Tlh-F: 5' CCG CTG ACA ATC GCT TCT C 3'~~tdh_321R: 5' CGCTGCCATTGTATAGTCTTTATC 3'~~ Tlh-R: 5' TTT GAT CTG GCT GCA TTG CT 3'~~tlh_884F: 5' ACTCAACACAAGAAGAGATCGACAA 3'~~ TDH-F: 5' TAT CCA TGT TGG CTG CAT TC 3'~~tlh_1091R: 5' GATGAGCGGTTGATGTCCAAA 3'~~ TDH-R: 5' CGA ACA ACA AAC AAT ATC TCA TCA GA 3'~~IAC_46F: 5' GACATCGATATGGGTGCCG 3'~~ Trh Probe: 6FAM 5' TAT TTG TYG TTA GAA ATA CAA CAA T 3' MGBNFQ~~IAC_186R: 5' CGAGACGATGCAGCCATTC 3'~~ WA IC Probe :VIC 5' CGT AAG ACA ATC TGA TAG TAG T 3' MGBNFQ Orf8 Probe: NED 5' TCC TGC TGT ACT TTT AG 3' MGBNFQ Tlh Probe: 6FAM 5'ACC ACA CGA TCT GGA GCA ACG ACG MGBNFQ TDH Probe 3' VIC TGT CCC TTT TCC TGC CCC CGG 5' MGBNFQ **For Vv Real-time PCR Method** vvha-F: 5' GAT CGT TGT TTG ACC GTA AAC G 3' vvha-R 5' TGC TAA GTT CGC ACC ACA CTG T 3' vvha Probe: NED 3' CAA AAC GCT CAC AGT CG 5' MGB probe ~~vvhF 5' TGTTTATGGTGAGAACGGTGACA 3'~~ ~~vvhR 5' TTCTTTATCTAGGCCCCAAACTTG 3~~
C	14, 18		**3.2.4 Lyophilized forward and reverse primers, and probes, are hydrated with TE buffer to produce a 0.1 mM stock solution.**
C	~~14, 18~~21		**3.2.5 Storage of thawed working stocks of primers and probes are stored between 2-8°C, not to exceed 2 weeks.~~Using molecular grade, nuclease free water, primer and probe stock solutions are diluted to produce a 0.01 mM working solution.~~**
C	~~14, 18~~21		**3.2.6 Storage of aliquoted working stocks of ~~Reconstituted~~ primers and probes ~~are~~ stored in a -20 °C manual defrost freezer does not exceed 1 year.~~for up to 5 freeze thaw cycles, not to exceed two years.~~**
C	21~~, 22~~		**3.2.7 Taqman Environmental Mastermix 2.0 is stored in -20°C manual defrost freezer until first use. ~~Platinum *Taq* DNA is stored in -20 °C manual defrost freezer until first use.~~ After first use, ~~can be~~it is stored between 2-8 °C.**
C	21~~, 22~~		**3.2.8 Internal control (IC) is stored in -20°C manual defrost freezer until first use.~~PCR reagents (dNTPs, buffer, MgCl2, fluorescent dyes) are stored in -20 °C manual defrost freezer until first use.~~ After first use, they ~~can be~~are stored between 2-8 °C.**
		3.3 DNA Extraction	
C	14, 18		**3.3.1 All microcentrifuge tubes and pipet tips are sterile.**
C	14, 18		**3.3.2 Pipet tips have aerosol barriers.**
K	14, 18		3.3.3 Latex or nitrile gloves are worn throughout the extraction and PCR preparation process.
K	14, 18		3.3.4 All work surfaces, centrifuge racks and equipment used in PCR analysis are disinfected immediately prior to DNA extraction, Master Mix preparation and PCR analysis.
C	14, 18		**3.3.5 Aseptic technique is observed throughout the extraction and PCR analysis.**
C	14, 18, 21		**3.3.6 Two-hundred (200) µL~~One thousand (1000) µL~~ aliquots from each positive APW enrichment tube, including the process controls, are extracted.**
C	~~14, 18~~21		**3.3.7 For each run a specified amount of internal control (IC) is prepared such that each extracted well contains internal control DNA.~~Positive APW aliquots are placed in sterile microcentrifuge tubes and heated at 95-100 °C for 10 minutes.~~**
K	~~14, 18~~21		3.3.8 Extracts are refrigerated between 2-8°C and analyzed within 24 hrs. Frozen extracts are analyzed within 1 month of frozen storage.~~A set of positive and negative process controls are included with each batch of samples in a heating block/boiling bath.~~

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C	~~14, 18~~21		**3.3.9 A tlh+ trh+ tdh+ V. parahaemolyticus (WA4647 or equivalent), a tlh+ tdh+ Orf8+ V. parahaemolyticus (BAA-240 or equivalent), and vvha+ V. vulnificus (ATCC 27562 or equivalent) cultures are extracted and combined to serve as the positive PCR (amplification) control.~~After boiling, tubes are chilled in ice or immediately frozen in a manual defrost freezer for future analysis. Boil preps may be refrigerated not to exceed 72 hours.~~**
~~K~~	~~14, 18~~		~~3.3.10 Frozen extracts are analyzed within 6 months of frozen storage.~~
		3.4 Preparation of the Master Mix for PCR	
C	**14, 16, 18**		**3.4.1 Nuclease-free microcentrifuge tubes and pipette tips, with filters, are used in Master Mix preparation.**
C	**~~14, 16, 18~~21**		**3.4.2 For each reaction, add the specified amount of water, buffer, MgCl2, dNTPs, specific primers, nuclease probes, *Taq*, and internal control DNA is added.**
K	14, ~~21~~16, 18		3.4.3 The Master Mix is ~~gently~~ vortexed to mix constituents and then briefly spun immediately prior to dispensing aliquots to reaction tubes or plates..
C	**14, 16, 18, 21**		**3.4.4 Eighteen (18) µL ~~Twenty three (23) µL~~ of Master Mix is used for each PCR reaction.**
C	**14, 16, 18**		**3.4.5 Master Mix must be used on the day of preparation or stored at –20 °C until time of use.**
		3.5 PCR	
~~C~~	~~14, 19~~		~~**3.5.1 If previously frozen, the DNA extracts are completely thawed at temperatures no warmer than room temperature. Immediately prior to use, DNA extracts are centrifuged at >5,000 x g for 2 minutes to remove particulate matter and cell debris.**~~
C	**14, 19, 21**		**3.5.~~2~~1 Two (2) µL of DNA template is added to each reaction tube or plate well containing ~~23~~18 µL of Master Mix for a total PCR reaction volume of ~~25~~20 µL.**
C	**14, 19, 21**		**3.5.2 Two (2) µL of extracted blank APW from the uninoculated process control is added to a reaction tube or plate well containing 18µL of Master Mix.**
K	14, 19, 21		3.5.3 Two (2) µL of molecular grade, nuclease free water is added to a reaction tube or plate well containing ~~23~~18 µL of Master Mix for each batch of Master Mix prepared as a no template control.
C	**14, 19, 21**		**3.5.4 Two (2) µL of DNA template extracted from the negative process control culture is added to a reaction tube or plate well containing ~~23~~18 µL of Master Mix.**
C	**14, 19, 21**		**3.5.5 Two (2) µL of DNA template extracted from the positive process control culture is added to a reaction tube or plate well containing ~~23~~18 µL of Master Mix.**
O	14, 19, 21		3.5.6 Two (2) µL of DNA template extracted from the positive control culture (prepared separately from the positive process control) is added to a reaction tube or plate well containing ~~23~~18 µL of Master Mix as the positive PCR (amplification) control.
K	14, 19, 21		3.5.7 Immediately prior to loading the reaction tubes or plates into the instrument they are centrifuged for ~~3~~ 530 seconds to ensure that all reagents and the DNA template are in the bottom of the tube to optimize the PCR amplification process.
C	16		**3.5.8 After centrifugation, tubes or plates are inserted into the instrument.**
		3.6 PCR Amplification	
C	**14, 19**		**3.6.1 The appropriate instrument platform is used for the protocol.**
K	16		3.6.2 Manufacturer's instructions are followed in operating the instrument.
C	**14, 19**		**3.6.3 The PCR cycle parameters used are appropriate for the protocol.**
K	14, 19		3.6.4 Optical calibrations for the dyes being used are current, per the instrument manufacturer's recommendations.
C	**14, 19**		**3.6.5 The analysis settings are adjusted as specified in the protocol.**
		3.7 Computation of Results	
K	14, 19		3.7.1 All runs in which the positive control generates a Ct value for the target(s) of interest and the negative control reaction generates no Ct value for the target(s), but a Ct value for the internal control are considered valid.
C	**2**		**3.7.2 Data is quality checked by the analyst.**
C	**14, 19**		**3.7.3 All reactions in a valid run which generate a Ct value for the target(s) of interest with a sigmoidal amplification curve are considered to be positive.**

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C	**16**		**3.7.4 Any sample which does not demonstrate a sigmoidal amplification curve may have a reported positive/negative determination that is discrepant from the instrument if appropriately justified using the raw fluorescent data.**
K	16		3.7.5 All reactions in a valid run which do not generate a Ct value for the target(s) of interest, but do generate a Ct value for the internal control are considered negative.
C	**16**		**3.7.6 Any reaction in which no Ct value is generated for the target(s) of interest or the internal control is considered invalid and should be re-tested.**
C	**13**		**3.7.7 Upon determination of positive reactions, refer to the original positive dilutions of APW and record MPN values as derived from the calculator in Appendix 2 of the FDA Bacteriological Analytical Manual (BAM).**
K	13		3.7.8 For APW enrichment, results are reported as MPN/g of sample.

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LABORATORY:	**DATE of EVALUATION:**

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY of NONCONFORMITIES

Page	Item	Observation	Documentation Required

Page____of _____

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LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
MICROBIOLOGICAL COMPONENT: (Part I-III)	
A. Results Total # of Critical (C) Nonconformities in Parts I-III Total # of Key (K) Nonconformities in Parts I-III Total # of Critical, Key and Other (O) Nonconformities in Parts I-III	

B. Criteria for Determining Laboratory Status of the Microbiological Component:

1. **Does Not Conform Status**: The Microbiological component of this laboratory is not in conformity with NSSP requirements if:

   a. The total # of Critical nonconformities is **≥4 or**_____

   b. The total # of Key nonconformities is **≥13 or**_____

   c. The total # of Critical, Key and Other is **≥18** _____

2. **Provisionally Conforms Status**: The microbiological component of this laboratory is determined to be provisionally conforming to NSSP requirements if the number of critical nonconformities is **≥1**

C. Laboratory Status (*circle appropriate*)

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgment by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory
Evaluation Officer on or before_____.

Laboratory Signature: _____ Date:_____

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LABORATORY:

Page	Item	Observation

1. Purpose/Principle

The purpose of this test is to rapidly quantify *Vibrio parahaemolyticus* (*Vp*) and *Vibrio vulnificus* (*Vv*) from oysters using a high throughput MPN based real-time PCR protocol. Culture based assays for the enumeration of *Vp* and *Vv* 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 a strain of potential pandemic *Vibrio* (*Vp* ORF8+). Additionally, the assay provides quantitative results for total *Vibrio vulnificus* (*vvhA*) and utilizes an exogenous internal control (WA IC).

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

- Thermolabile hemolysin, *tlh* gene
- Thermostable direct hemolysin, *tdh* gene
- Thermostable direct related hemolysin, *trh* gene
- Filamentous phage (f237) Orf8, gene

Vv

- Cytolysin-hemolysin, *vvhA* gene

2. Scope

Rapid and early detection of these pathogens will help the shellfish industry market oysters for consumption that are within regulatory limits for these pathogens, and ensure public health safety.

3. Reagents / Media

- Master Mix: TaqMan™ Environmental Master Mix 2.0; Thermo Fisher Cat. #4396838
- Molecular PCR grade water; Thermo Fisher Cat. #SH3053802 or equivalent
- TE buffer; Thermo Fisher Cat. #BP2473500 or equivalent
- Primers (See appendix A for sequences)
- Probes (See appendix A for sequences)
- Internal Control Plasmid
- MagNAPure 96 DNA and Viral NA Small Volume kit; Roche, Cat. # 06543588001
- Alkaline Peptone Water (APW); Prepared In-house
- Phosphate Buffer Saline (PBS); Prepared In-house

Record receipt of all PCR mastermix components in the Reagent Receipt Log (P:\EHSPHL\PHL\MICRO\FOODLAB\Vibrio\Master Mix Documents\Updated Worksheets\Reagent Receipt Log). All reagents will be tracked by its lot number. The intent of the reagent receipt logbook is to allow for complete traceability. Additionally, lot numbers are recorded upon use on Master Mix Worksheet.

Prepare Primer and Probe mixes according to the Master Mix Worksheet (P:\EHSPHL\PHL\MICRO\FOODLAB\Vibrio\Master Mix Documents\Mastermix Template). Lyophilized primers are diluted to 100µM stock concentrations with TE Buffer and stored at -15°C (or below) until use. Store in low light transmitting tubes. Original stock solutions are good for 5 years unless otherwise stated by manufacturers. Working concentrations of primers and probes are good for 6 months in freezer (-15°C or below). Thawed working concentrations are good for 2 weeks refrigerated (2-8°C).

The exogenous internal control (1:100 concentration) is stored at -15°C or below. Prepare working stock by adding 990µL molecular grade H_2O to single IC tube (containing 10µL). The working stock can be stored at 2-8°C. On day of use, dilute working stock an additional 1:100. The final concentration of the final product is 1:100,000. See Appendix B for Internal Control Plasmid info.

4. Supplies / Materials

- Isopropanol, 70%
- RNAse AWAY®
- Sterile scrub brushes
- Sterile oyster knives
- Sterile wide mouth containers (1 liter capacity)
- Oyster shucking block
- Chain-mail glove
- Dishwashing gloves
- Serological pipettes (1ml, 10ml, 25ml)
- Filtered pipette tips
- Sterile dilution bottles
- Microcentrifuge tubes (1.7mL)
- 384 well PCR plates; Thermo Fisher Cat. # 4326270
- Optical covers
- MagNAPure 96 Cartridge seals; Roche Cat. # 06241638001
- MagNAPure 96 Processing Cartridge; Roche Cat. # 06241603001
- MagNaPure 96 Output plate; Roche Cat. # 06241611001
- MagNAPure 96 System Fluid; Roche Cat. # 06640729001
- MagNAPure 96 Tips 1000µL; Roche Cat. #06241620001

4.1 Bacterial Cultures

- *Vibrio parahaemolyticus* (WA4647 and BAA-240, or equivalent)
- *Vibrio vulnificus* (ATCC 29307 or equivalent)

5. Equipment

- Non-mercury glass thermometer 0°C-10°C
- High Speed blender
- Balance (±0.1 g)
- Sterile blender jars
- Timer
- Vortex mixer
- Incubator (35°C±0.5)
- Refrigerator, 2-8°C
- Freezer, -15°C to -25°C
- Biological safety cabinets (BSC) or Air Clean PCR stations
- Pipettes P-1000, P-200, P-20
- Multi-channel Pipette (8) 2µL-25µL
- Applied Biosystems® QuantStudio™ Dx™ Real-Time PCR station
- Roche MagNAPure 96 DNA purification system
- PCR plate centrifuge.

6. Safety Precautions

Vibrio species are pathogenic and should be handled following PHL safety guidelines and assay risk assessment.

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

7. Specimen Information

Samples are to be shipped properly (adequate ice/cold packs) and temperature maintained between 0 and 10°C upon arrival. Once received and logged-in, the samples are to be placed under refrigeration unless processed immediately.

Samples received over 10°C are considered acceptable only under the following conditions:

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 on the same day).

Sample processing at the lab must be initiated no more than 36 hours after collection. Samples received more than 36 hours after collection are considered unsatisfactory.

8. Quality Control

- Instructions
    - Three process controls are included in every extraction run. To prepare the process control material, enrich *V. parahaemolyticus* WA4647 (*tlh*+, *tdh*+, *trh*+) and *V. vulnificus* ATCC 29307 (vvha+) overnight in APW. The overnight enrichment is diluted and a $<10^3$ per ml culture is used as a positive control. The two organisms will also serve as negative

controls for each other. Include an uninoculated APW blank to serve as the uninoculated control. Process control cultures are to be run concurrent with the samples, and accompany the samples throughout incubation, isolation, and confirmation.

- To ensure that all MagNA Pure 96 plastics (i.e. processing cartridge, output plate, internal control tubes, 1000µl tips) and external system fluid are free of interfering contaminants, process controls will serve as quality control.
- Two amplification controls are included in every PCR run. To prepare the positive PCR control, enrich *Vibrio parahaemolyticus* (ATCC BAA-240, WA4647) and *Vibrio vulnificus* (ATCC 29307) overnight in APW. DNA is extracted individually, combined in equal parts, and divided into 10µL aliquots. Pre-extracted amplification controls should be stored frozen at -15°C or below. Expiration is one year from the date it was prepared. Sterile molecular grade water will be used for the no-template-control (NTC), and will serve as the negative amplification control.
- Certificates of analysis will be maintained in a binder within the Food lab for all pre-sterilized consumables.
- Disposable pipettes will be checked for accuracy and tested for sterility.
- Disposable pipettes (i.e. serological) used to inoculate samples and prepare reagents are checked for accuracy and tested for sterility.

- Frequency
  - Process controls, exogenous internal control, and amplification controls are included with every PCR run.
  - Quality controls will be run on all media and reagents, mastermix, and primer/probe mixes prior to use or concurrent with testing.
  - Certificates of analysis (COA) for each new lot of pre-sterilized consumables will be maintained.
  - Each new lot of disposable pipettes (i.e. serological) will be tested for accuracy and checked for sterility.

- Acceptable Limits
  - See respective media, reagents, mastermix, or primer/probe QC guides for expected or acceptable results.
  - A successful PCR run should meet the following conditions:
    I. The positive controls should show clear amplification. If NO amplification is present in the positive controls for one or both multiplex's, determine the problem and re-run the sample.
    II. The negative process control should only show amplification for the exogenous internal control (IC). The no-template-control (NTC) should not show amplification for any of the targets.
    III. 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.
  - Results will not be reported without acceptable QC results.
  - All certificates for pre-sterilized consumables will be checked for conformance and initialed by the laboratorian.

- For all disposable pipette (i.e. serological) accuracy checks, a satisfactory accuracy is ±2% of volume tested.

- Corrective Action
  - PCR runs for which the NTC is positive or the positive control and/or internal control is negative should be repeated.
  - The Lead Microbiologist should be notified if any run fails QC.
  - If quality controls for mastermix, primers/probes, or disposable pipettes do not meet acceptable criteria, the QC fails and item shall not be used for testing.
  - Media/reagent quality controls not exhibiting the expected growth or reactions will be retested with fresh growth (18-24 hrs) organisms.
  - Samples tested with any failed media/reagent will be considered invalid and will be retested with new media/reagent which has passed quality control.

- Recording QC Data
  - Initial quality control data for media/reagents, mastermix, primers/probes, and disposable pipettes will be recorded on its respective QC worksheet. Records are maintained in a binder within the laboratory.
  - Record results for each PCR and Process controls by notating presence (+) or absence (–) on the sample worksheet.

9. Calibration

- **QuantStudio Dx PCR Workstation**

  Calibration kits are used to maintain the Real-Time PCR system with 384-Well Block. They include calibration plates to perform a spectral calibration with FAM™, VIC®, ROX™, SYBR® Green, TAMRA™, NED™ dyes, plates to perform region-of-interest (ROI) calibration, normalization calibration, and to run RNase P verification.

  Calibration and verification should be run at least every six months and following a Performance Maintenance. Kits are stored at -15°C to -25°C. The maximum degree of accuracy for each dye of interest in florescence emission wavelength is ±5nm. Follow manufacturer's instruction on performing calibrations.

- **Micropipettor and Thermometers**

  Micropipettors are calibrated at appropriate volumes annually and checked for accuracy quarterly. At a minimum quarterly checks are performed at 100%, 50%, and 10% of nominal volume.

  Non-mercury glass thermometers will be sent out for annual calibration and checked for accuracy quarterly by a certifying vendor on-site. Long stem digital thermometers will be replaced yearly.

  "As Found" and/or "As left" calibration data for micropipettes or thermometers must indicate that the initial calibration or recalibration passed. Acceptable tolerance limits will be obtained as pre-determined by the manufacture or ISO 17025 accredited service by the certifying vendor.

If the "As found" calibration data for micropipettes or thermometers indicate that the calibration or recalibration failed, a PHL Quality Improvement (QI) Form must be filled out for all affected samples.

Calibration/re-calibration certificates for all micropipettes and thermometers will be checked for conformance and initialed by the Supervisor or Lead prior to use. Calibration certificates are maintained in a binder within the laboratory.

10. Procedure

10.1 Sample Accessioning

a. Samples are collected, transported, and processed in accordance with Recommended Procedures for the Examination of Sea Water and Shellfish described by the American Public Health Association[iii].
b. Oyster samples are removed from the shipping container and the sample submission form is located. At a minimum the sample submission forms must contain the following information: collector's name, harvest area, sampling station, time and date of collection.
c. A laboratory testing worksheet is generated for each sample.
d. Both the sample submission form and the testing worksheet are stamped with the appropriate laboratory number.
e. One oyster from each bag is opened to take tissue temperature. The temperature is recorded on the sample submission form.
f. The bag of oysters is labeled with its associated sample ID and placed into a 2-8°C refrigerator unless processed immediately.

10.2 Sample Preparation- Scrubbing

a. The intent of the assay is to determine the concentration of *Vp* and *Vv* 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.
b. The sink must be clean before scrubbing can begin. Wash the sink with water or soap and water.
c. The gloved hands of the analyst are to be washed with soap immediately prior to cleaning the shells of debris. The gloves worn are latex, nitrile and/or stainless steel mesh to protect analyst's hands from injury.
d. Using sterile scrub brushes, each oyster is cleaned under cold running water. All barnacles, mud, vegetation and debris should be removed.
   Note: The faucet used for rinsing the shellfish should not contain an aerator. 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.
e. A representative sample of at least 12 shellfish is used for analysis.
f. After cleaning each oyster place the animal upside down on a clean paper towel lined tray. Ensure that you have labeled the tray with corresponding sample number.

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.

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

10.3 Sample Preparation- Shucking

a. In order to accurately quantify *Vp* and *Vv* in oyster tissue it is very important to avoid introduction of bacteria (*Vp* or other) into the oyster tissue.
b. The sink must be disinfected 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.
c. Place a sterile pre-weighed tissue collection container on the sink counter.
d. Disinfect a shucking block by washing with soap and water, and wiping down with 70% isopropanol. Place block on the sink counter to air dry.
e. Place the oyster sample to be shucked on the sink counter.
f. Put on clean nitrile gloves.
g. 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.
h. Put another nitrile glove on over the chain-mail glove. Cover both hands in 70% isopropanol and allow them to air dry.
i. Grab and hold each oyster with the chain-mail hand and use the other hand and a sterile oyster knife to shuck each oyster.
j. A fresh knife, shucking container and gloves must be used for each sample.
k. Use the disinfected shucking block while shucking to minimize knife accidents and to protect the counter surface.
l. Collect all tissue and liquor (fluid) in the sterile pre-weighed container. 10^{-1}
m. The shucking block and counter must be washed and sterilized between samples.

10.4 Sample Processing- Setting up MPN

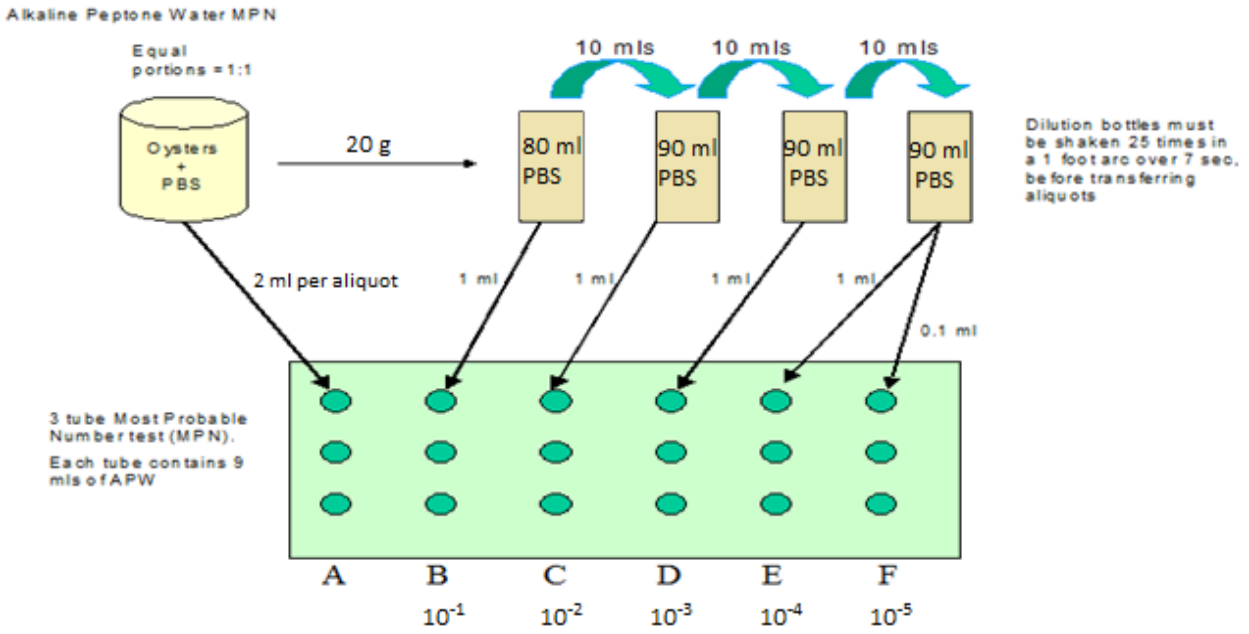
Enumeration in this assay is achieved by conducting an MPN (most probable number) analysis.

a. Weigh the shellfish tissue collection container. Subtract the original container weight from the total weight to determine the amount of oyster tissue and liquor.
b. Record the weight of tissue on the sample worksheet.
c. Transfer the oyster tissue and liquor to a sterile blender jar.
d. Add an equal weight of diluent (PBS) to the sample container. If needed, PBS can be used to rinse any residual tissue from the container just as long as a 1:1 dilution can be maintained (±0.1g).
e. Transfer the PBS to the blender jar. Record the weight of PBS used on the sample worksheet.
f. Blend the shellfish sample with PBS at high speed for 90 seconds (60 to 120 seconds is acceptable).

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

g. 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. See Figure below.

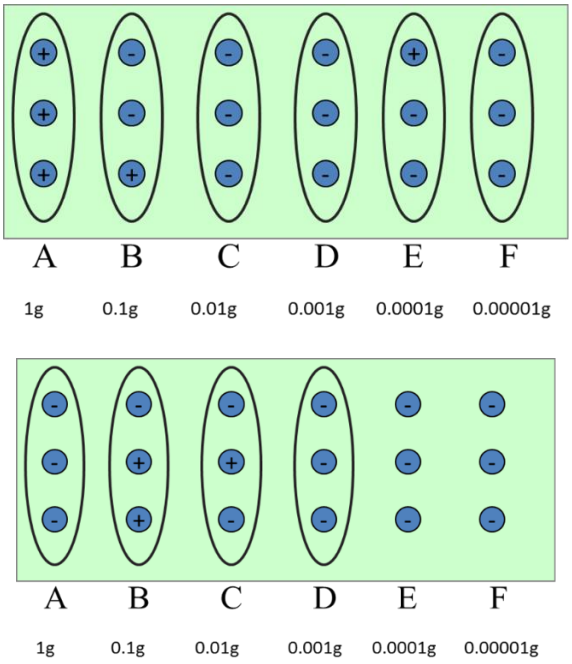
The initial 1:10 dilution is prepared gravimetrically with PBS (20 g of sample homogenate to 80 mL of PBS). Record the weight and volume used on the sample worksheet for the sample worksheet. All successive dilutions are prepared volumetrically.



h. Incubate at 35°C± 1 for 18 to 24 hours. Write sample number, date, time, and analyst initial on the A1 tube, prior to placing in incubator.

10.5 Reading MPN

a. Each APW tube must be checked for growth following 18-24 hrs. of incubation. Use the following criteria to select tubes for further testing.
   i. Examine all tubes for turbidity. Examine each tube with a light source shining through the tube.
   ii. Record all positive and negative results on the lab worksheet.
   iii. The following examples illustrate the selection process. Each tube is labeled as +/- for turbidity. The dilutions circled should be selected for further testing.



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

10.6 DNA Extraction

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

Reagent prep should be carried out in the Pre-PCR room in order to minimize the potential for contamination. Once extraction is complete, the material is considered stable and may be stored at 2-8°C for 1-2 days or frozen at -20°C to -80°C for extended periods. NOTE: Multiple freeze thaw cycles should be avoided due to potential DNA degradation.

a. Due to the high number of sample tubes it is necessary to create a document to track the location of each one. This document is referred to as the "MagNA Pure Plate Map".
b. After the MagNA Pure Plate Map is created, load the MagNA Pure 96 cartridge accordingly. 200µL of each sample should be added to the 96 well cartridge. Include 200µL of *Vp* culture, 200µL of *Vv* culture, and 200µL of uninoculated APW. These will act as process controls for the assay.
c. Once loaded, seal the MagNA Pure cartridge with an adhesive cartridge seal.
d. Label the cartridge with the sample number, date, initials and label as "Pre-Extract". The specimen can now be loaded onto the MagNA Pure 96 instrument.
e. Confirm that the MagNA Pure 96 instrument and its linked computer are turned on.
f. Ensure that the correct MagNA Pure kit is selected "DNA/Viral SV 2.0".
g. Select the protocol "Pathogen Universal 200.3.1"
h. Sample volume should be entered as 200µL.
i. Elution volume should be entered as 100µL.

j. Next to the Internal Control section, click the More Options icon. Scan the barcode located on the IC tube. Enter the number of tests. This will determine the amount of IC needed. Since IC is prepped for single use, the auto-filled expiration date is not relevant.
k. 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.
l. Click the "Stage Set-up" button.
m. Begin adding in the appropriate reagents/plastics in accordance with the outlined requirements on the MagNA Pure load screen (software).
n. Once the reagent trays are completely loaded and the tips are adequately filled, place the remaining trays back into the instrument.
o. Remove the cartridge seal from the processing cartridge and place into the instrument. Discard the seal into an autoclave waste container.

p. Ensure that all plastics, reagents and sample cartridges are in place and accounted for on the computer screen.
q. Close the door and press the "start extraction" button.
r. Note the time that the run will be completed. The final extracted template DNA will be refrigerated on-board the MagNA Pure 96 instrument until it is removed. It is however not advisable to leave the extract uncovered for any length of time.
s. Once completed, open the door, remove the extracted DNA, immediately seal the cartridge with a new cartridge seal, and refrigerate at 2-8°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°C to -80°C.

10.7 MagNAPure 96 Waste Removal and Decontamination.

a. Remove all soiled plastics, replace used tips, and wipe the trays with 10% bleach, isopropyl alcohol and RNAse Away using Manufacturer's suggested cleaning procedure. Run the UV decontamination protocol.
b. If waste bottle is full, follow MagNAPure 96 Waste Disposal Procedure listed below.
    I. Instrument will indicate waste container is full.
    II. Attach empty waste container to instrument and secure caps on full container.
    III. Carry full waste container with caps closed to the sink, set inside sink, and remove small cap.
    IV. Tip container onto side with small cap and allow to drain into sink. As it drains, you may need to tilt the container to ensure complete drainage of liquids.
    V. Spray sink and container with 70% Isopropanol.
    VI. Pour entire bottle of 70% Isopropanol (~ 500ml) into container, secure cap, and carefully invert to mix.
    VII. Allow Isopropanol to sit for 10 min.
    VIII. Spray sink and container again with 70% Isopropanol and wipe down container. Pour Isopropanol from inside container into the sink and secure caps.
    IX. Rinse sink and exterior of container with water.
    X. Spray exterior of container with 10% bleach, allow 3 minute contact time, and then rinse with water.
    XI. Container can be stored in lab with secured caps until next use.
c. For routine MagNA Pure 96 maintenance, follow the MagNA Pure 96 Daily Maintenance Log (for start-of-day and end-of-day instructions) and MagnNA Pure 96 Post Run Cleaning Log

instructions. Forms can be found in the Master Document Control or link to the following address:

P:\EHSPHL\PHL\MICRO\COMMON\ENTERICS - FOOD\QC\Media QC\501.4206.docx
P:\EHSPHL\PHL\MICRO\COMMON\ENTERICS - FOOD\QC\Media QC\501.4207.docx

10.8 PCR Mastermix Preparation

Mastermix preparation is performed in the Pre-PCR room, within an Airclean hood. This includes primer and probe manipulations and mastermix loading onto the PCR plate. Thorough decontamination before and after use of the Airclean hood is advisable.
Note: A person who has previously in the same day worked with amplicon should not re-enter the Pre-PCR lab.

a. Prepare a PCR platemap using the MasterMix Prep worksheet. (P:\EHSPHL\PHL\MICRO\FOODLAB\Vibrio\Master Mix Documents\Mastermix Template). Be sure to include positive and negative process controls, in addition to a positive and negative amplification control.
b. Using the worksheet determine the number of reactions needed. This will help you determine how much of each mastermix component will be required. It is advisable to prepare several reactions more than are needed to account for pipetting variability.
c. Once in the Pre-PCR room, follow mastermix recipe and MagNAPure plate map to prepare Multiplex 1 and 2. The mastermix can be prepared in a microcentrifuge tube or sterile disposable reagent reservoir.
d. Briefly vortex (swirl, pipette up and down, or equivalent) to completely mix the components.
e. Using a pipette (multichannel advisable), add 18µL of mastermix to each appropriate well (384 well plate) according to the PCR platemap.
f. Once the 384-well plate is loaded with mastermix, cover the plate with aluminum foil, place the plate in a biological transport container (sealed box), and transport to an available AirClean hood within the Food laboratory (Alternatively the Template Addition Room can be used).

10.9 Template Addition

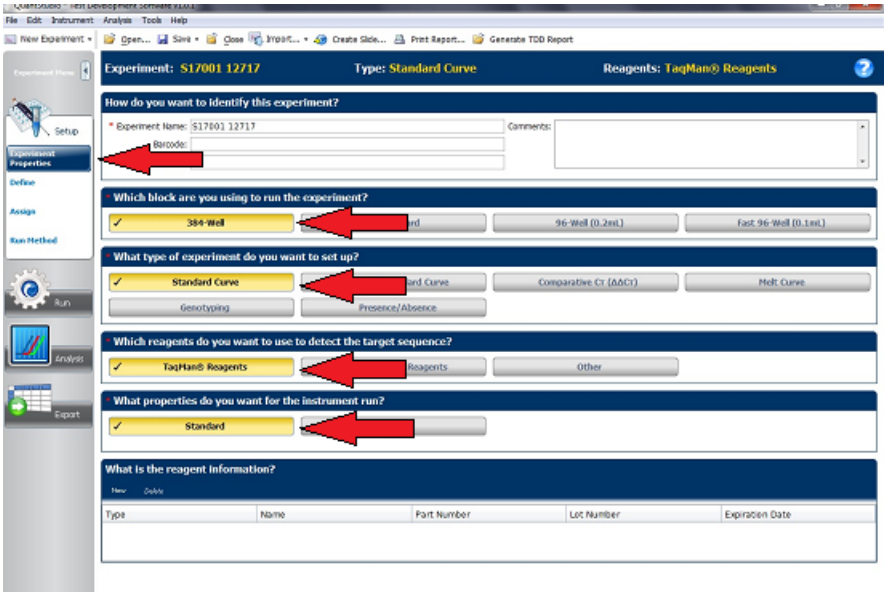
a. Add the extracted DNA template to the appropriate wells according to your PCR platemap. Use 2µL of DNA for a total reaction volume of 20µL.
b. Once all wells are loaded including the positive and negative amplification controls, seal the plate with an optical seal.
Avoid touching either side of the 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.
c. Centrifuge the plate briefly to remove bubbles from the wells and ensure that the template is in contact with the reaction mix.

10.10 Setting up Real-Time PCR Station

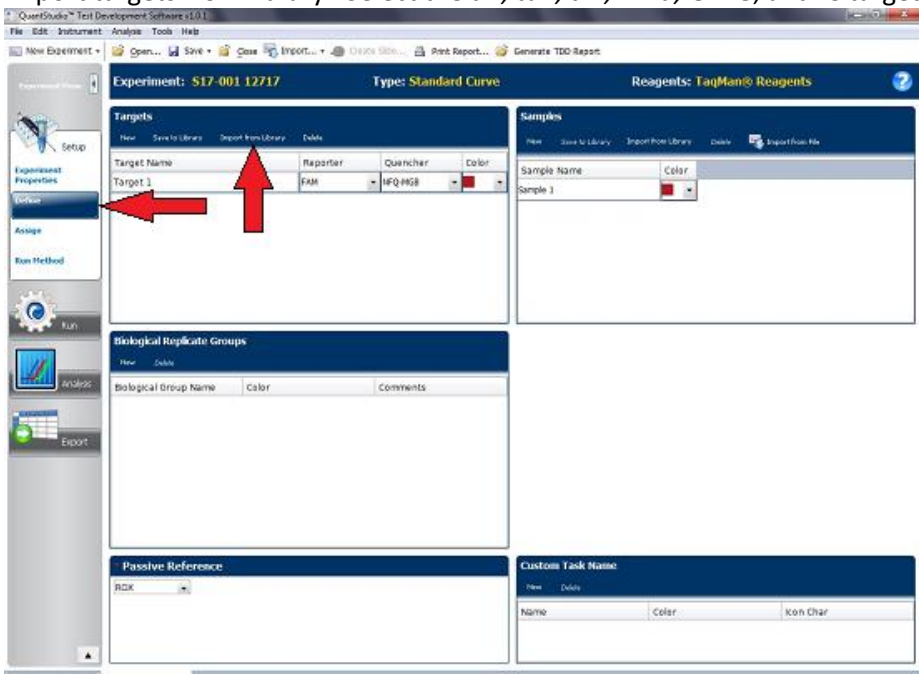
a. Turn on the Applied Biosystems® QuantStudio™ Dx™ instrument and the computer.
b. Open the Test Development software, under the File menu select “New Experiment”.

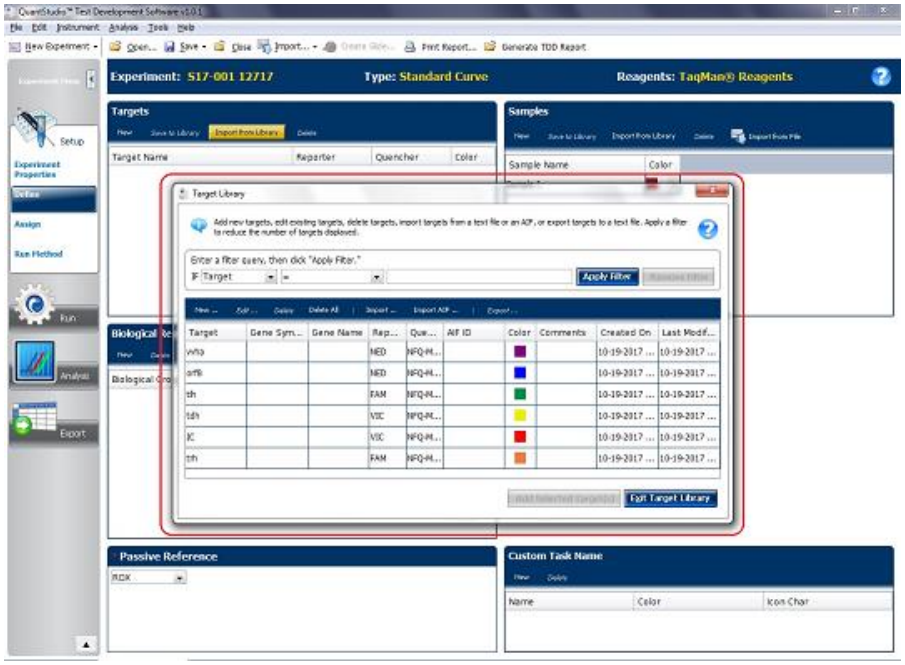


c. Rename experiment with the appropriate sample numbers and date of run.
d. Under the tab “Experiment Properties” ensure “384-Well Block”, “Standard Curve”, TaqMan® Reagents”, and “Standard” (for run mode) are selected.

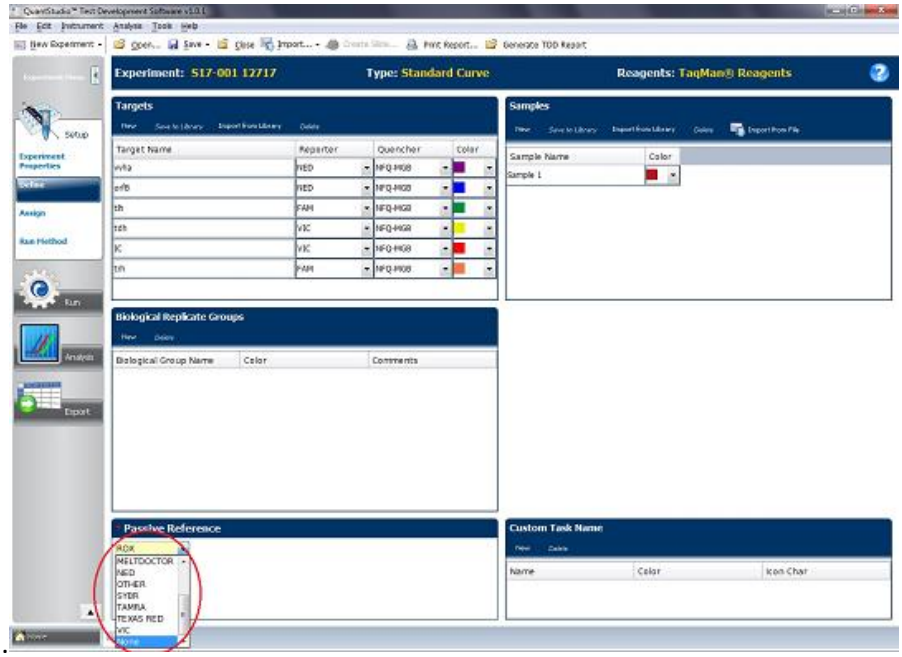


e. The “Define” tab is used to select the targets of detection. The targets are saved to the library. Import targets from library. Select the *tlh, tdh, trh, vvha,* ORF8, and IC targets.

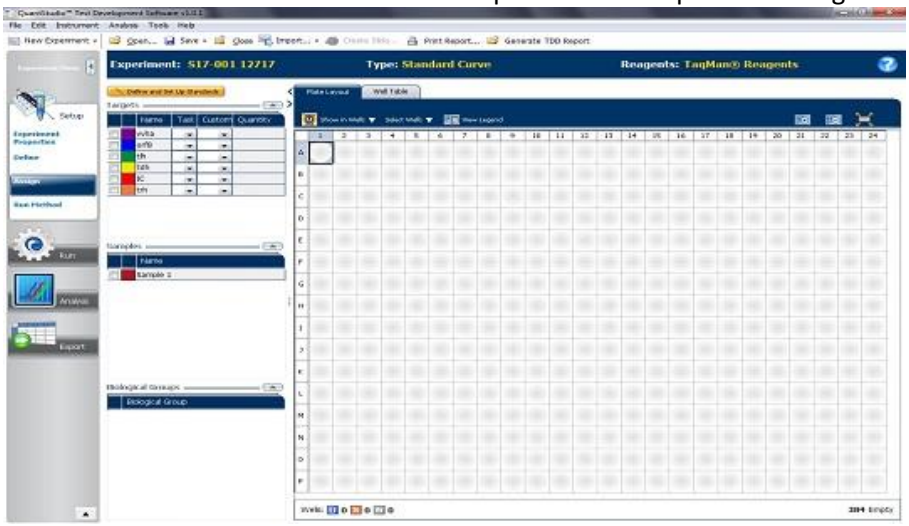




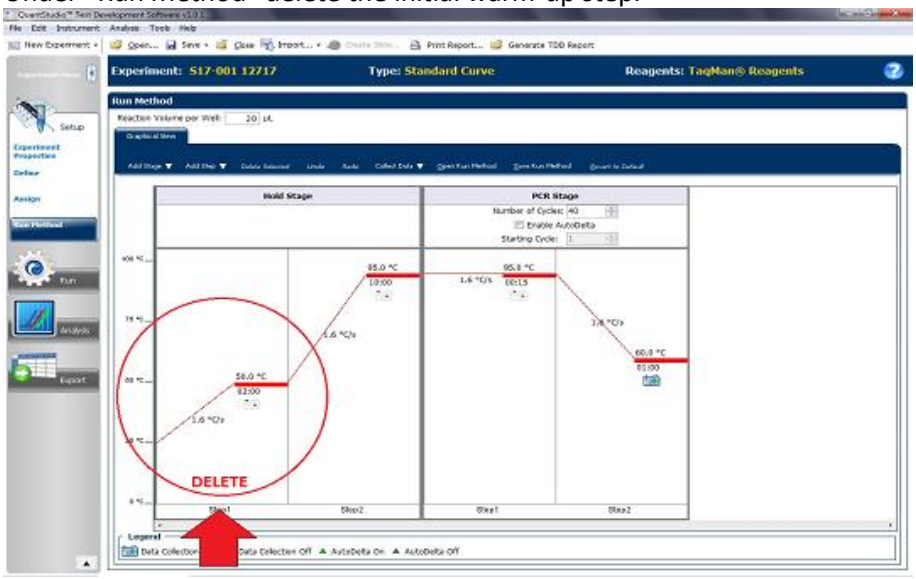
f. At the bottom of the screen, select “ROX” from the drop down menu regarding “Passive Reference”



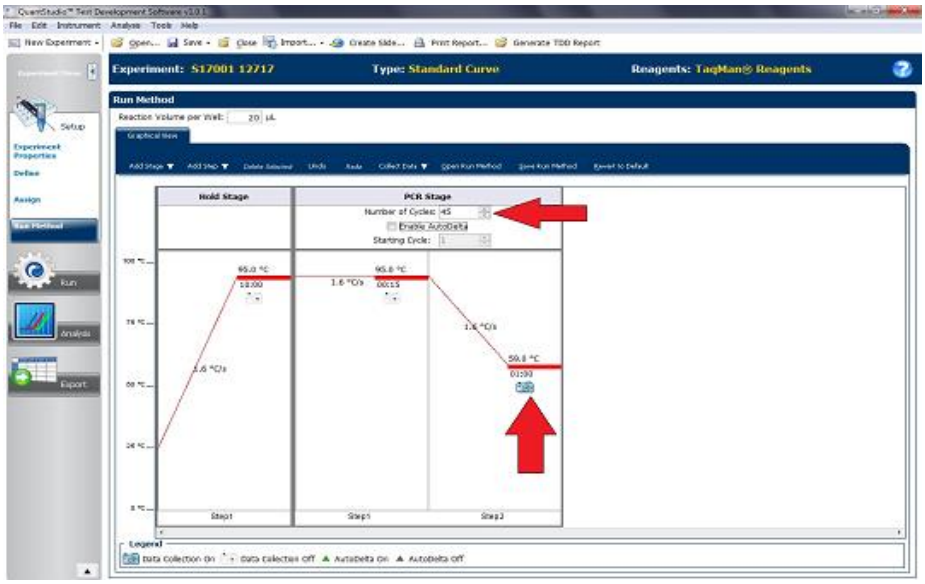
g. Select the “Assign” tab and assign the appropriate wells with the corresponding targets of interest. Be sure to double check the map and 384-well plate are in agreement.



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



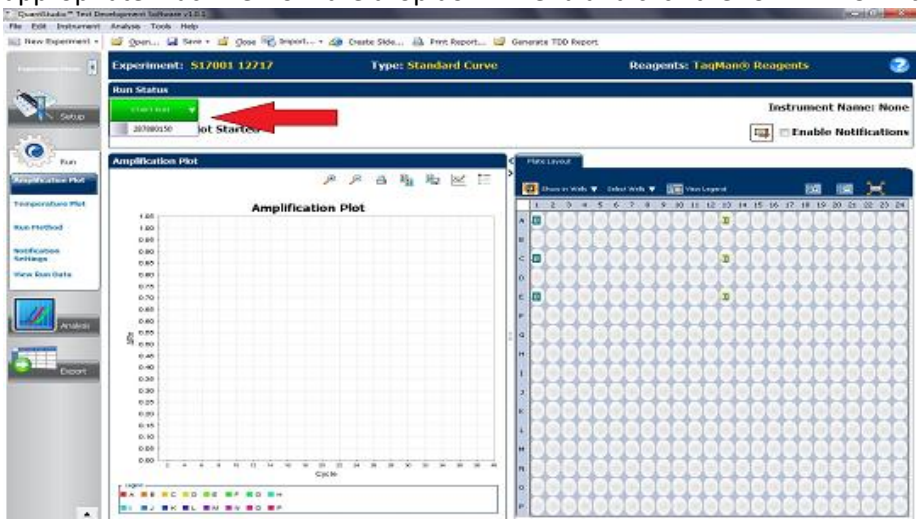
i. After doing so the parameters are the following:
   95° C for 10 minutes
   40 cycles
   95° C for 15 seconds
   59° C for 60 seconds



j. Turn on the instrument user interface by touching the touchscreen. To open loading tray, touch the eject button.

k. Load the plate and close tray.

l. 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.



m. Save run file using sample numbers and date.

11. Waste Management

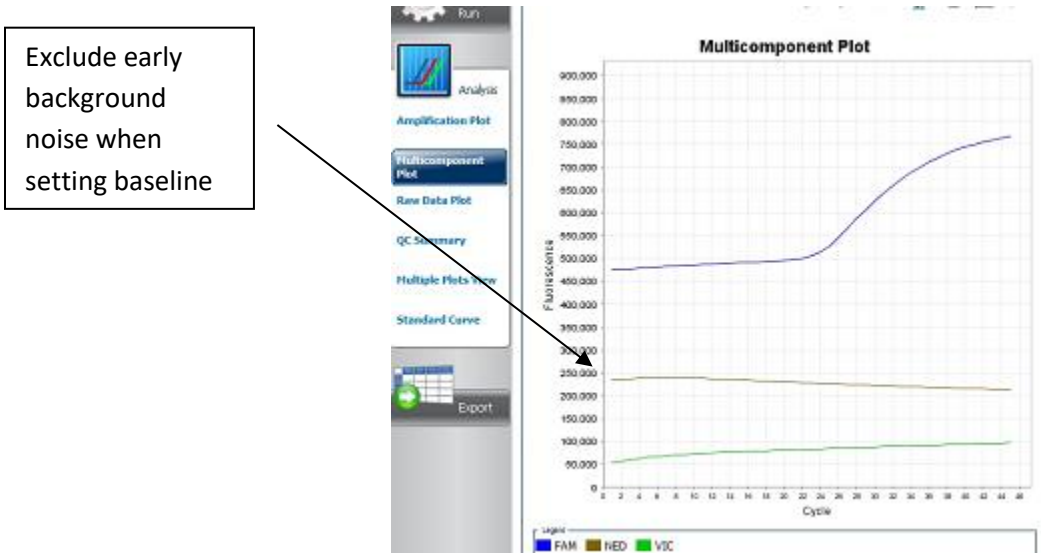
The biological material is rendered non-hazardous through use of the MagNA Pure 96 System and associated reagents[ii]. The reference refers to the MagNA Pure Compact System, however; all of these instruments share the same buffer system, process, and concentrations. The remaining waste is considered flammable by Department of Ecology Standards and will be collected as hazardous waste for disposal.

All other plastics and glassware containing the sample will be treated by autoclaving using appropriate conditions.

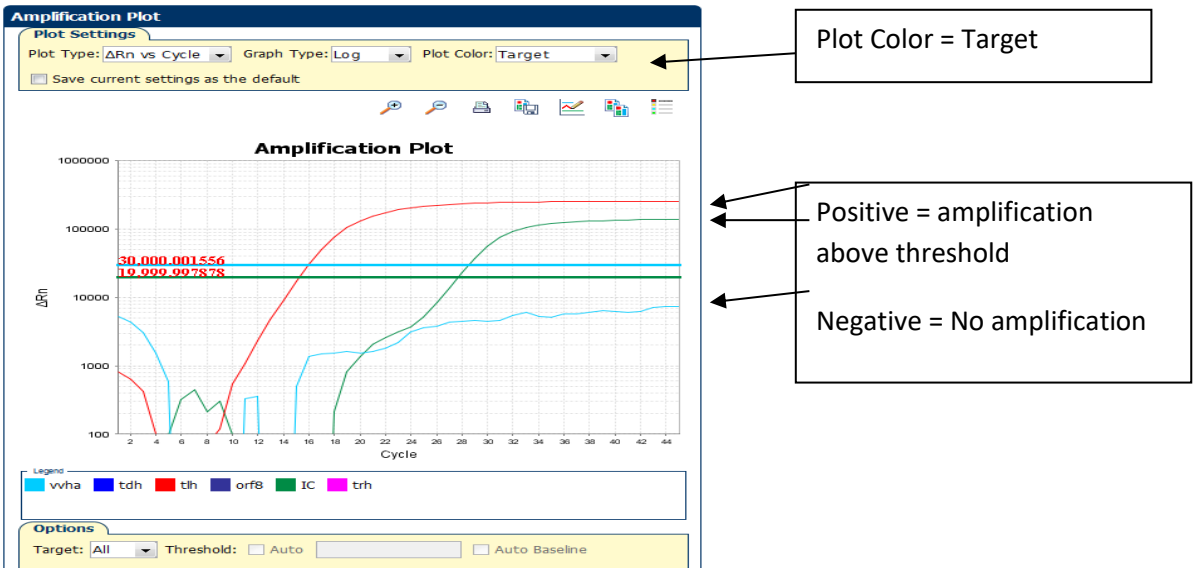
Date of disposal of each sample is recorded on the respective sample worksheet.

12. Interpretation

a. Once run is complete, remove plate from instrument and discard in appropriate waste receptacle.
b. Select “Analysis Settings” on the Amplification Plot screen.
c. Change all thresholds and baseline settings to “manual” and set all thresholds, except trh, to 0.04 and leave baseline settings at 5 to 15. Set trh threshold to 0.08.
d. Apply analysis settings and exit to Amplification Plot screen.
e. Record quality control Ct values on the Vibrio parahaemolyticus Real-Time PCR Ct Value QC worksheet. For all targets record Ct value from the positive mastermix control. Record Ct value for the Internal Control (IC) using the negative mastermix control. All Ct values should be recorded with the threshold set at 0.04 and baseline set at 5 to 15.
f. Select all wells in the plate by clicking in the upper left box of the plate layout.
g. 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.
h. If baseline changes are necessary - view individual wells in the Multicomponent Plot screen. Change baseline settings as needed to exclude early background noise.



i. 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.



12.1 Procedure for Abnormal Results

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

If duplicate results are in agreement, report these results. If the duplicates differ, report the result that is in agreement with the original qPCR run.

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

I. 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.

II. The presence of the *trh* gene in the absence of the *tlh* gene has been documented[i]. 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.

12.2 Interfering Substances

Vibrio alginolyticus possesses a *trh* gene with 98% homology to the *trh* gene in *Vibrio parahaemolyticus*[i]. 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.

13. Calculations

Upon determination of positive reactions, record the number of confirmed positive tubes per dilution series onto the *Vibrio* Sample Worksheet, and generate an MPN index. MPN values (concentration) of each target is derived from the FDA Bacteriological Analytical Manual (BAM) MPN Calculator. This Excel document can be located in Appendix 2 of the FDA BAM. To compute an MPN value, follow the instructions as noted in the FDA BAM MPN Calculator.

Unusual MPN indexes are typically due to 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.

14. Reference Range

Reportable Range

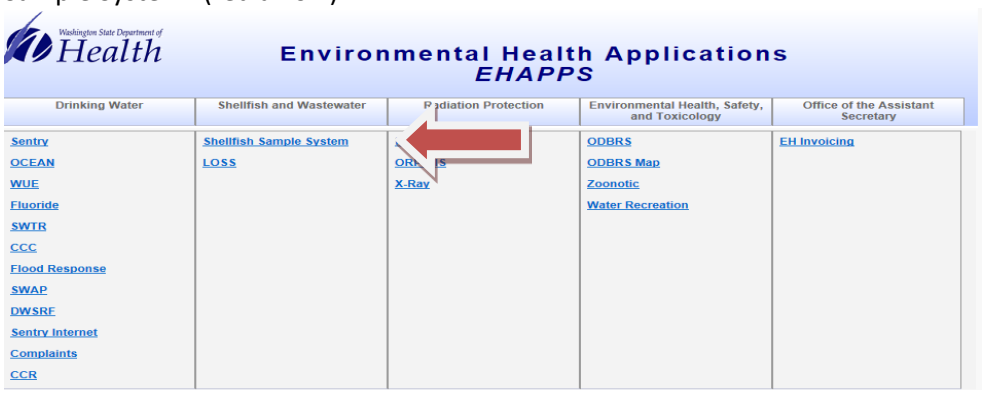
tlh: <0.36 MPN/g to >110,000 MPN/g
tdh: <0.36 MPN/g to >110,000 MPN/g
trh: <0.36 MPN/g to >110,000 MPN/g
ORF8: <0.36 MPN/g to >110,000 MPN/g
vvhA: <0.36 MPN/g to >110,000 MPN/g

15. Reporting Results

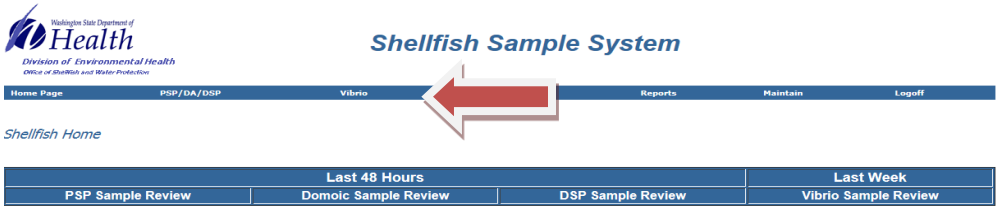
15.1 Environmental Health Applications (EHAPPS) database

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

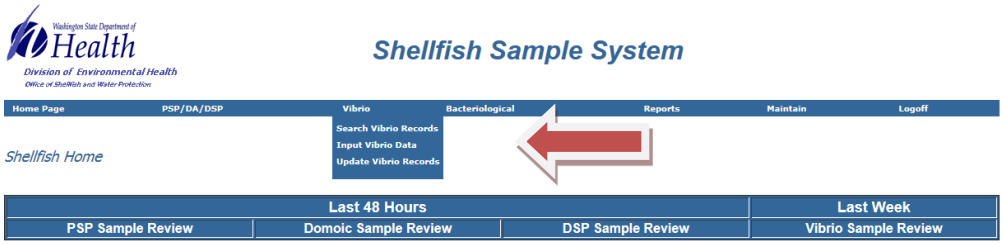
a. After entering web address, find column “Shellfish and Wastewater” and click “Shellfish Sample System” (red arrow)



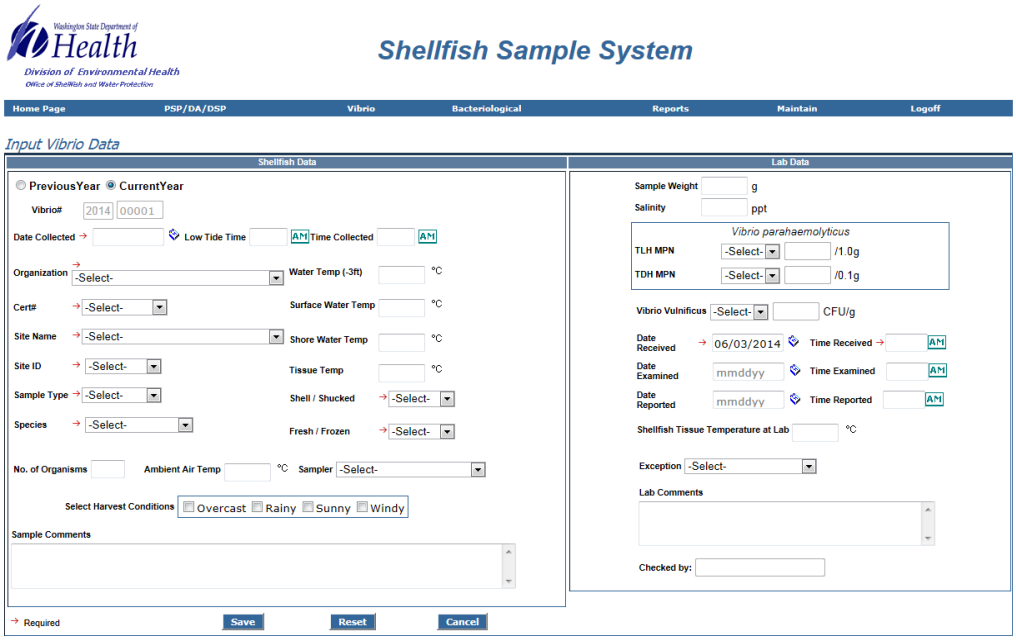
b. Along the top, hover mouse over “Vibrio” (red arrow)



c. Click “Input Vibrio Data” (red arrow)



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



15.2 Notification of Test Results

a. Environmental Health Applications (EHAPPS)

All test results will be entered into the Shellfish Sample System via EHAPPS. Results are reviewed and checked off by the Lead Microbiologist.

b. Email

Test results can be emailed to the Office of Shellfish and Water Protection (OSWP) after Lead Microbiologist approval and signature.

c. Phone

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

15.3 Archiving Results & Retention

a. Filing Results

The Vibrio Testing Worksheet and Sample Submission Form are to be filled in a filing cabinet located within the Food and Shellfish Bacteriology Laboratory.

All other documents (i.e. Mastermix worksheet, PCR Plate Map, MP96 Plate Map, Sample Tracking worksheet, etc.) are to be scanned and uploaded into the Scanned Testing Documents folder under the appropriate year on the PHL P: Drive server. All scanned documents for a given day can be saved under this folder as the date (MMDDYY).

For example:

P:\EHSPHL\PHL\MICRO\FOODLAB\Vibrio\2019 Vp Season\Scanned Testing Documents\060119

All uploaded testing documents will be reviewed by the Lead Microbiologist prior to discarding any hard copies.

b. Retention

Reports and results for samples tested will be archived according to the Department of Health Records Retention Schedule.

EHAPPS database is maintained by the Office of Shellfish and Water Protection.

16. References

[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.

[ii]Dauphin L. A. et. al. Evaluation of Automated and Manual Commercial DNA Extraction Methods for Recovery of *Brucella* DNA from suspensions and spiked swabs. 2009. JCM Vol. 47, No. 12. p. 3920-3926.

[iii]The American Public Health Association, Inc. *Recommended Procedures for the Examination of Sea Water and Shellfish*. 4th ed., 1970.

17. Appendix

Appendix A- Primers and Probes

VIBRIO PARAHAEMOLYTICUS

TRH
Primers
Trh 627F
ATA CCT TTT CCT TCT CCW GGT TC
Trh 731b R
TTG TCC AGT AGT CAT CAA CGA TTG
Trh Glov R
TTG TCC AAT AGT CCT CCA CAA TTG
(Ward) Probe-- Trh P
FAM TAT TTG TYG TTA GAA ATA CAA CAA T **MGBNFQ**

(WA PHL *Vibrio* Internal Control)
Primers
WA IC F
GGC GAA GCG AAT CTG GAA A
WA IC R
GGT GTA GTT GTG CGT GTA ATA TGA GA
Probe-- WA PHL ICP
VIC CGT AAG ACA ATC TGA TAG TAG T **MGBNFQ**

Orf8
Primers
Orf8 F TCA CCT GAG GAC GCA GTT ACG
Orf8 R TTC AAT TGT AGA ACC GCC AGC TA
Orf8 Probe
NED TCC TGC TGT ACT TTT AG **MGBNFQ**

TLH (69 bp amplicon)
Primers
Tlh-F CCG CTG ACA ATC GCT TCT C
Tlh-R TTT GAT CTG GCT GCA TTG CT
Tlh probe
FAM ACC ACA CGA TCT GGA GCA ACG ACG **MGBNFQ**

TDH (94 bp amplicon)
Primers
TDH-F 2013 TAT CCA TGT TGG CTG CAT TC
TDH-R 2013 CGA ACA ACA AAC AAT ATC TCA TCA GA
TDH Probe
VIC TGT CCC TTT TCC TGC CCC CGG MGBNFQ

VIBRIO VULNIFICUS

VVHA (79 bp amplicon)
vvha-F GAT CGT TGT TTG ACC GTA AAC G
vvha-R TGC TAA GTT CGC ACC ACA CTG T
vvha Probe
NED-CAA AAC GCT CAC AGT CG-**MGB probe**

Appendix B- Internal Control Plasmid

The *Vibrio parahaemolyticus* and *Vibrio vulnificus* enumeration and detection through MPN and real-time PCR assay utilizes an exogenous internal control (WA IC). This plasmid is added to the during DNA extraction for the detection of matrix inhibition or other assay failures. The 73bp fragment can be synthesized and clones into a pIDTSMART-AMP plasmid by Intergrated DNA Technologies (IDT), Ref ID: 88772700.

Sequence:
GGCGAAGCGAATCTGGAAAACGTAAGACAATCTGATAGTAGTATATTTCTCATATTACACGCACAACTAC
ACC

Additionally, the Invitrogen OneShot Top10 Chemically Competent Cells and QIAGEN Plasmid Midi Kit can be used collectively to manufacture and purify additional plasmid DNA.

Protocol for Transforming Chemically Competent Cells.

This section provides a procedure to transform Invitrogen One Shot TOP10 chemically compentent E.coli via regular Chemical transformation protocol, as described by the manufacturer's instructions.

Step-by-step Procedure:

1. Prepare a plasmid dilution by transferring 1 µl of 40mM Plasmid (stock) into 3µl of molecular water. Briefly place on ice.

2. Thaw, on ice, one 50 µL vial of One Shot® cells for each ligation/transformation.

3. Pipet 1µl of plasmid dilution (from step 1) directly into the vial of competent cells and mix by tapping gently. Do not mix by pipetting up and down. The remaining ligation mixture(s) can be stored at −20°C.

4. Incubate the vial(s) on ice for 30 minutes.

5. Incubate for exactly 30 seconds in the 42°C water bath. Do not mix or shake.

6. Remove vial(s) from the 42°C bath and place them on ice.

7. Add 250 µL of pre-warmed (room temperature) S.O.C medium to each vial. S.O.C is a rich medium; sterile technique must be practiced to avoid contamination.

8. Place the vial(s) in a microcentrifuge rack on its side and secure with tape to avoid loss of the vial(s). Shake the vial(s) at 37°C for exactly 1 hour at 225 rpm in a shaking incubator.

9. Spread 100µL from each transformation vial on separate, labeled LB agar plates. The remaining transformation mix may be stored at 4°C and plated out the next day, if desired.

10. Invert the plate(s) and incubate at 37°C overnight.

11. Select isolated colonies and pick to LB medium with ampicillin.

Protocol for Plasmid DNA Purification using QIAGEN Plasmid Midi Kit

This protocol is designed for preparation of up to 100µg of high or low copy plasmid DNA using the QIAGEN Plasmid Midi Kit. Consult the manufactures instructions for additional information.

Before starting:

Prepare Buffer P1 according to step 5
Prepare Buffer P2
Prepare buffer P3

Step-by-step Procedure:

1. Pick up to 2 colonies per plate from a freshly streaked selective plate and inoculate a starter culture of 5 ml LB medium containing the appropriate selective antibiotic. Incubate for approx. 8 hr. at 37°C with vigorous shaking (approx. 300 rpm).

Use a tube or flask with a volume of at least 4 times the volume of the culture.

2. Dilute the starter culture 1/500 to 1/1000 into selective LB medium. For high-copy plasmids, in a 250ml flask inoculate 25 ml medium with 50 µl of starter 8hr culture. Grow at
37°C for 12–16 h with vigorous shaking (approx. 300 rpm). Either change shaking incubator platform or attach flask holder to allow for vigorous shaking.

Use a flask or vessel with a volume of at least 4 times the volume of the culture. The culture should reach a cell density of approximately 3–4 x 109 cells per milliliter, which typically corresponds to a pellet wet weight of approximately 3 g/liter medium.

3. Harvest the bacterial cells by centrifugation at 6000 x *g* for 15 min at 4°C. Transfer to 50ml centrifuge tubes (falcon or equivalent). Decant the supernatant and retain the pellet.
If you wish to stop the protocol and continue later, freeze the cell pellets at –20°C.

4. Resuspend the bacterial pellet in 4 ml Buffer P1. Vortex in Falcon tubes until no clumps are visible.

For efficient lysis, it is important to use a vessel that is large enough to allow complete mixing of the lysis buffers. Ensure that RNase A has been added to Buffer P1.
If LyseBlue reagent has been added to Buffer P1, vigorously shake the buffer bottle before use to ensure LyseBlue particles are completely resuspended. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

5. Add 4 ml Buffer P2, mix thoroughly by vigorously inverting the sealed tube 4–6 times, and incubate at room temperature (15–25°C) for 5 min. Total volume is now 8ml.

Do not vortex, as this will result in shearing of genomic DNA. The lysate should appear viscous. Do not allow the lysis reaction to proceed for more than 5 min. After use, the bottle containing Buffer P2 should be closed immediately to avoid acidification from CO2 in the air.

If LyseBlue has been added to Buffer P1, the cell suspension will turn blue after addition of Buffer P2. Mixing should result in a homogeneously colored suspension.
If the suspension contains localized colorless regions or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.

6. Add 4 ml of chilled Buffer P3, mix immediately and thoroughly by vigorously inverting 4–6 times, and incubate on ice for 15 min. Total volume is now 12ml.

Precipitation is enhanced by using chilled Buffer P3 and incubating on ice. After addition of Buffer P3, a fluffy white material forms and the lysate becomes less viscous. The precipitated material contains genomic DNA, proteins, cell debris, and KDS. The lysate should be mixed thoroughly to ensure even potassium dodecyl sulfate precipitation. If the mixture still appears viscous, more mixing is required to completely neutralize the solution.
If LyseBlue reagent has been used, the suspension should be mixed until all trace of blue has gone and the suspension is colorless. A homogeneous colorless suspension indicates that the SDS has been effectively precipitated.

7. Centrifuge 12 ml volume at ≥20,000 x *g* for 30min at 4°C. Remove supernatant containing plasmid DNA promptly. Use high-speed centrifuge (in BSL3 or equivalent). If BSL3 centrifuge is utilized, all steps there after must take place within the BSL3.

Before loading the centrifuge, the sample should be mixed again. Centrifugation should be performed in non-glass tubes (e.g., polypropylene). After centrifugation the supernatant should be clear.

8. Centrifuge the supernatant again at ≥20,000 x *g* for 15 min at 4°C. Remove supernatant containing plasmid DNA promptly.

This second centrifugation step should be carried out to avoid applying suspended or particulate material to the QIAGEN-tip. Suspended material (causing the sample to appear turbid) can clog the QIAGEN-tip and reduce or eliminate gravity flow.

9. Equilibrate a QIAGEN-tip 100 by applying 4 ml Buffer QBT, and allow the column to empty by gravity flow. Prepare Qiagen-Tip 100 during centrifugation by adding 4ml of Buffer QBT.

Flow of buffer will begin automatically by reduction in surface tension due to the presence of detergent in the equilibration buffer. Allow the QIAGEN-tip to drain completely. QIAGEN-tips can be left unattended, since the flow of buffer will stop when the meniscus reaches the upper frit in the column.

10. Apply the supernatant from step 8 to the QIAGEN-tip immediately after centrifugation and allow it to enter the resin by gravity flow.

The supernatant should be loaded onto the QIAGEN-tip promptly. If it is left too long and becomes cloudy due to further precipitation of protein, it must be centrifuged again or filtered before loading to prevent clogging of the QIAGEN-tip.

11. Wash the QIAGEN-tip twice with 10 ml Buffer QC.

Allow Buffer QC to move through the QIAGEN-tip by gravity flow. The first wash is sufficient to remove contaminants in the majority of plasmid DNA preparations. The second wash is especially necessary when large culture volumes or bacterial strains producing large amounts of carbohydrates are used.

12. Elute DNA with 5 ml Buffer QF into a centrifuge tube.

Collect the eluate in a 15 ml or 50 ml tube (not supplied). Use of polycarbonate centrifuge tubes is not recommended as polycarbonate is not resistant to the alcohol used in subsequent steps. For constructs larger than 45–50 kb, prewarming the elution buffer to 65°C may help to increase yield.

Optional: If you wish to stop the protocol and continue later, store the eluate at 4°C. Storage periods longer than overnight are not recommended.

13. Precipitate DNA by adding 3.5 ml (0.7 volumes) room-temperature isopropanol to the eluted DNA. Mix and centrifuge immediately at ≥15,000 x *g* for 30 min at 4°C. Carefully decant the supernatant.

All solutions should be at room temperature to minimize salt precipitation, although centrifugation is carried out at 4°C to prevent overheating of the sample. Alternatively, disposable conical bottom centrifuge tubes can be used for centrifugation at 5000 x g for 60 min at 4°C. Isopropanol pellets have a glassy appearance and may be more difficult to see than the fluffy, salt-containing pellets that result from ethanol precipitation. Marking the outside of the tube before centrifugation allows the pellet to be more easily located. Isopropanol pellets are also more loosely attached to the side of the tube, and care should be taken when removing the supernatant.

14. Wash DNA pellet with 2 ml of room-temperature 70% ethanol, and centrifuge at ≥15,000 x *g* for 10 min. Carefully decant the supernatant without disturbing the pellet.

Alternatively, disposable conical-bottom centrifuge tubes can be used for centrifugation at 5000 x g for 60 min at 4°C. The 70% ethanol removes precipitated salt and replaces isopropanol with the more volatile ethanol, making the DNA easier to redissolve.

15. Air-dry the pellet for 5–10min, and redissolve the DNA in a suitable volume of buffer (e.g., TE buffer, pH 8.0, or 10 mM Tris·Cl, pH 8.5). Add approx. 100µl of TE Buffer to dissolve the plasmid DNA.

Redissolve the DNA pellet by rinsing the walls to recover the DNA, especially if glass tubes have been used. Pipetting the DNA up and down to promote resuspension may cause shearing and should be avoided. Overdrying the pellet will make the DNA difficult to redissolve. DNA dissolves best under slightly alkaline conditions; it does not easily dissolve in acidic buffers.

16. Plate the Plasmid onto a Blood Agar Plate and incubate for 72hrs. Plate may be checked every 24hrs for growth. If no growth is observed after 72hrs, the plasmid can be removed from the BSL3.

Quantification

1. Determine the concentration of plasmid DNA recovered using the Thermo Scientific NanoDrop instrument (or other spectrophotometer). Further dilutions can be made with TE Buffer to achieve target concentration of 40mM.

2. Additional dilutions should be made according to the *Vibrio parahaemolyticus* and *Vibrio vulnificus* enumeration and detection through MPN and real-time PCR assay protocol.

3. Plasmid dilutions must be run on a PCR Detection System to verify concentration, and ensure the amplification falls within its expected Ct range.

4. 10µl aliquots of 1:100 dilution will serve as freezer stock and be stored at -15°C or below. Working stocks can be prepared by adding 990µl molecular grade water to the freezer stock, and should be stored at 2-8°C. On day of use, dilute working stock an additional 1:100. The final concentration of the final product is 1:100,000.

Validation Data for MPN Real-time PCR for Total and Pathogenic *Vibrio parahaemolyticus*

Name of Method Submitter: Gina Olson, Washington State Department of Health

Specific purpose or intent of the method for use in the NSSP:

Requesting adoption of this method as an approved method for Vibrio enumeration, both *Vibrio parahaemolyticus* (Vp) and *Vibrio vulnificus* (Vv) in oysters. The method may be used in the following applications: PHP validation and verification of product and in management of growing areas through environmental testing and surveillance in order to re-open closed growing areas. This method once approved would provide a high-throughput alternative to the current approved MPN real-time PCR method. In addition, this method would be the only approved MPN real-time PCR method to test for total Vp, pathogenic Vp, and Vv in a single assay.

Validation Criteria Data:

All oyster samples used in this validation were collected from different harvest locations and/or different harvest dates in Washington State. All samples were processed using APHA guidelines for the cleaning and shucking of shellstock. Samples were confirmed negative for the target organism of Vp through the FDA BAM culture-based method and through pcr prior to spiking. Spiking levels were determined by spread plating dilutions onto PCA w/2% NaCl in duplicate and averaging the counts.

Vp strain WA4647 was used to spike all samples for all validation criteria. This strain is positive for *tlh*, *tdh*, and *trh*. All data generated for all three targets was identical and has been presented in a single MPN in all validation criteria tables and data calculations.

The validation data for Vp and Vv is presented separately for clarity and ease in reviewing the data, but this is a single assay and all elements were present during the validation of all organisms.

<u>Assay Design</u>

DNA Isolation: Roche MagnaPure 96 using Roche DNA/Viral Nucleic Acid Small Volume Kit

Real-time PCR Instrument: Applied Biosystems QuantStudio Dx (384-well format)

Mastermix: Life Technologies TaqMan Environmental Master Mix 2.0

Real-Time PCR targets: 2 multiplex reactions

- Multiplex 1: Total Vp (*tlh)*, Vv (*vvhA)*, internal control (IC)
- Multiplex 2 (Vp pathogenicity markers): *tdh*, *trh*, *orf8*

Real-Time PCR parameters:

Denaturation: 95°C for 10 mins
Annealing: 95°C for 15 secs
Extension: 59°C for 1 min
Cycles: 40

1. Accuracy/Trueness & Measurement Uncertainty

Accuracy/Trueness

Purpose/Method

Accuracy/Trueness measures the closeness of agreement between the test results (MPNs) and the accepted reference values (CFUs). This was done by analyzing twenty oyster samples over a range of concentrations (low to high) to determine the MPN. The MPN and CFU data set was converted into logs. The average MPN in logs was divided by the average plate count in logs. This provides an estimate (in percent) of the accuracy/trueness of the method.

Results

The average of the plate count CFUs was 2.88 log. The average of MPNs was 3.17 log. Accuracy/Trueness was found to be 109.94%. Results can be found below in Table 1.

Measurement Uncertainty

Purpose/Method

Measurement uncertainty expresses the range of values around the measured result within which the true value is expected to lie. To determine this parameter, twenty oyster samples spiked with a range of concentrations were analyzed. The MPN and CFU data set was converted into logs and the MPN result was subtracted from the CFU result for each sample. A 95% confidence interval was calculated from the difference. This confidence interval represents the measurement uncertainty of the methods.

Results

The measurement uncertainty was determined via 95% CI (0.23, 0.50), resulting in a measurement uncertainty of 0.27. Results can be found below in Table 1.

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

Sample	Plate Count, log(CFUs)	MPN, log(MPN/g)
1	0.89	0.62
2	0.77	1.36
3	0.85	0.96
4	2.85	2.62
5	2.32	3.36
6	2.32	3.17
7	4.18	5.04
8	4.18	4.66
9	0.36	0.63

10	4.36	4.66
11	2.04	2.62
12	2.45	2.36
13	3.52	3.36
14	4.69	5.04
15	4.69	5.04
16	1.66	1.62
17	2.57	2.96
18	3.60	3.59
19	4.69	5.04
20	4.69	4.66

2. Ruggedness

Purpose/Method

The amount of analyte recovered should be consistent between different lots of media/reagents. Ruggedness tests the impact of different lots used to process samples on the final result. This was done by testing ten oyster samples spiked at a range of concentrations in duplicate. The first replicate was performed using “Lot 1” media/reagents and the second was performed using “Lot 2.” To determine if the method was sufficiently rugged to withstand the types of changes anticipated to occur in routine use, a two-sided t-test was utilized on log-transformed data with a significance level (α) of 0.05. There should be no significant difference between Lot 1 and Lot 2 samples.

Results

Using data from Table 2, there was no significant difference (p=0.64) between different lots of media and reagents.

Table 2. Data for Determination of Ruggedness

Sample	Replicate 1, log(MPN/g)	Replicate 2, log(MPN/g)
1	0.62	0.96
2	1.36	1.36
3	0.96	0.96
4	2.96	2.62

5	3.36	3.63
6	2.96	3.18
7	5.04	5.34
8	5.34	4.66
9	0.62	1.62
10	5.04	4.66

3. Precision & Recovery

Precision

Purpose/Method

The difference between the methods results (MPNs) and the reference values (CFUs) should be consistent between different samples and also when detecting varying concentrations of measurand. The precision of the method tests the consistency of the difference between the CFU's found on plates and the MPN values. This was done by testing ten oyster samples at low, medium, and high concentrations in duplicate to determine the MPN. The MPN and CFU data sets were converted into log values. Each MPN was compared to its associate CFU value. A nested ANOVA was then performed, with variance components being the sample, and concentrations within the samples (low, medium, and high), and then the error. The ANOVA component of interest was that comparing the concentrations within the samples to the determinations (or error).

Results

The difference between CFUs and MPNs can be found below in Table 3. The data shows that there are no significant differences between the concentrations in samples and the determinations within concentrations via a nested ANOVA (p=0.08). Additionally, the variance of the method does not exceed the known variance of a 3-tube MPN (p=0.21).

Table 3: Data for determining the Precision

Sample	Concentration	Difference	log(CFUs)	log(MPNs)
1	low	-0.72	-1.17	-0.44
1	low	-1.13	-1.17	-0.04
1	med	-1.40	1.96	3.36
1	med	-1.00	1.96	2.96
1	high	0.13	4.69	4.56

1	high	-0.65	4.69	5.34
2	low	-0.68	-0.51	0.18
2	low	0.24	-0.51	-0.75
2	med	0.08	2.45	2.36
2	med	-0.42	2.45	2.87
2	high	-0.35	4.69	5.04
2	high	0.03	4.69	4.66
3	low	-0.18	-0.92	-0.74
3	low	-0.18	-0.92	-0.74
3	med	-0.45	2.52	2.96
3	med	-0.11	2.52	2.62
3	high	-0.35	4.69	5.04
3	high	-0.35	4.69	5.04
4	low	-0.08	-0.21	-0.13
4	low	-0.57	-0.21	0.36
4	med	-0.13	2.49	2.62
4	med	-0.13	2.49	2.62
4	high	-0.35	4.69	5.04
4	high	0.03	4.69	4.66
5	low	0.07	-0.15	-0.21
5	low	0.30	-0.15	-0.44
5	med	0.13	2.49	2.36
5	med	0.13	2.49	2.36
5	high	-0.35	4.69	5.04
5	high	0.03	4.69	4.66
6	low	-0.24	-0.28	-0.04

6	low	0.17	-0.28	-0.44
6	med	-0.30	2.66	2.96
6	med	0.30	2.66	2.36
6	high	-0.35	4.69	5.04
6	high	-0.35	4.69	5.04
7	low	0.34	-0.41	-0.74
7	low	0.34	-0.41	-0.74
7	med	-0.40	2.57	2.96
7	med	-0.40	2.57	2.96
7	high	0.03	4.69	4.66
7	high	-0.65	4.69	5.34
8	low	-0.12	-0.57	-0.44
8	low	-0.44	-0.57	-0.13
8	med	-0.02	2.60	2.62
8	med	-0.02	2.60	2.62
8	high	0.03	4.69	4.66
8	high	0.03	4.69	4.66
9	low	0.12	-0.33	-0.44
9	low	-0.20	-0.33	-0.13
9	med	-0.12	2.52	2.62
9	med	-0.45	2.52	2.96
9	high	-0.35	4.69	5.04
9	high	-0.35	4.69	5.04
10	low	0.03	-0.48	-0.51
10	low	0.03	-0.48	-0.51
10	med	-0.29	2.67	2.96

10	med	-0.96	2.67	3.63
10	high	0.03	4.69	4.66
10	high	0.03	4.69	4.66

Recovery

Purpose/Method

The amount of analyte recovered should be consistent both between different samples and also when detecting varying concentrations of measurand. The recovery of the method tests the consistency of the analyte recovered via MPNs as compared to the CFUs found on plates. This was done by testing ten oyster samples at low, medium, and high concentrations in duplicate to determine the MPN. The MPN and CFU data set was converted to logs. Each duplicated MPN was averaged and then compared to its associate CFU, in logs. A single-factor ANOVA was then used to compare the recovery at the three concentrations.

Results

The difference between CFUs and MPNs can be found below in Table 4. The recovery across all samples and concentrations was found to be 109.71%. There was not found to be significant differences in the recovery at the various concentrations (p=0.56).

Table 4: Data for the determination of Recovery

Sample	Concentration	Ave log(CFUs) per Conc.	Ave log(MPN) per Conc.
1	low	-1.17	-0.24
	med	1.96	3.16
	high	4.69	4.95
2	low	-0.51	-0.28
	med	2.45	2.62
	high	4.69	4.85
3	low	-0.92	-0.75
	med	2.52	2.79
	high	4.69	5.04
4	low	-0.21	0.12
	med	2.49	2.62
	high	4.69	4.85
5	low	-0.15	-0.33
	med	2.49	2.36
	high	4.69	4.85
6	low	-0.28	-0.24
	med	2.66	2.66
	high	4.69	5.04

7	low	-0.41	-0.75
	med	2.57	2.96
	high	4.69	5.00
8	low	-0.57	-0.29
	med	2.6	2.62
	high	4.69	4.66
9	low	-0.33	-0.29
	med	2.52	2.79
	high	4.69	5.04
10	low	-0.48	-0.51
	med	2.67	3.3
	high	4.69	4.66

4. Specificity

Purpose/Method

The method should only detect the analyte of interest, even in the presence of interfering organisms. Specificity refers to the ability of the method to measure only the target organism. One matrix sample was divided into three aliquots. One aliquot was spiked with a low but determinable level of *Vibrio parahaemolyticus* (*Vp*). The other two aliquots were spiked with the same level of *Vp* as the first, but were also spiked with a high level of potential interfering organisms. One aliquot received a high level of *Vibrio vulnificus (Vv)* and the other received *Vibrio alginolyticus* (*Va*). Five replicates were performed. Each of the replicates was analyzed by taking the average log MPN and calculating the Specificity Index (SI). A paired t-test was used to determine if the average specificity index obtained from the five replicates differed from 1 (significance level = 0.05).

Results

Using the data from Table 5, the average specificity index was 0.88 when in the presence of *Vv* and 0.98 in the presence of *Va*. These values are not significantly different than 1 *(p*=0.12 for *Vv, p*=0.69 for *Va*).

Table 5. Data for Determination of Specificity

Replicate	***Vp* only, log(MPN/g)**	***Vp* + *Vv*, log(MPN/g)**	***Vp* + *Va*, log(MPN/g)**
1	1.96	2.36	2.36
2	1.62	1.58	2.36
3	1.62	1.96	1.96
4	2.36	2.36	1.96
5	2.17	2.96	1.62

5. Linear Range, Limit of Detection & Limit of Quantification/Sensitivity

Linear Range

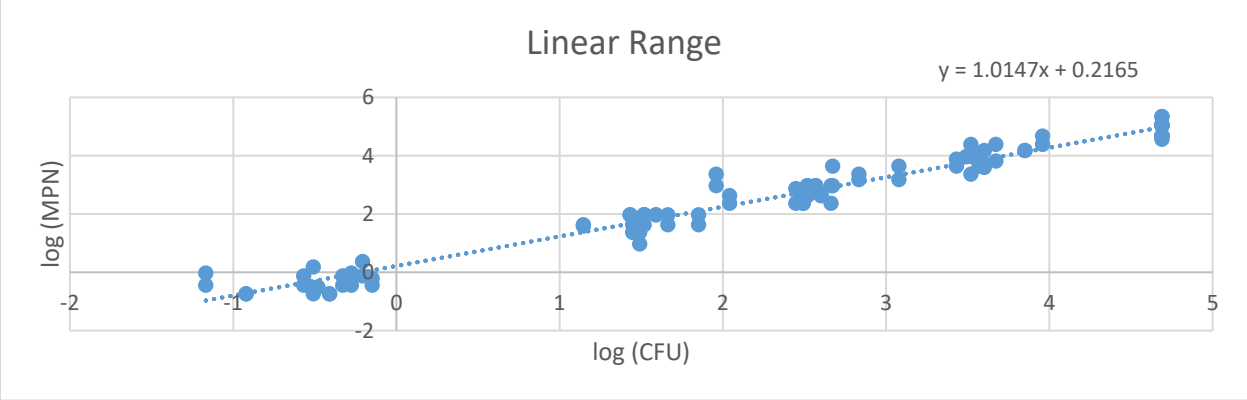
Purpose/Method

The MPN value found should directly correlate to the concentration of analyte within the sample, within the working range of the method. Thus, as the concentration increases, the MPN value should also increase in a linear fashion. Ten Oyster samples were tested at 5 concentration levels, in duplicate. Each MPN was compared to its associate CFU, found by plate count. The relationship between the log(MPN) and log(CFU) was then found by obtaining the correlation coefficient by performing a linear regression with log(CFU) as the independent variable and log(MPN) as the dependent variable.

Results

The relationship between the MPNs and CFUs can be seen in Figure 1 below. The relationship between MPNs and CFUs was found to be linear, with a Pearson's *r* of 0.99. The working range used was of concentrations ranging from 10^{-1} to 10^{4} cells/gram.

Figure 1: Graph representing the relationship between the log values of CFUs and MPNS



Limit of Detection

Purpose/Method

The method should be capable of detecting as little as 1 cell/gram of sample, or 0 cells/gram, in log form. Therefore, it must be determined whether the method can detect one cell per gram of sample. The log(MPN) was compared to the log(CFU) of ten oyster samples, spiked at five varying concentrations, in duplicate. This was done by performing a regression analysis on the data and calculating the Limit of Detection by taking the antilog of the intercept. The independent variable was set as log(CFU) and the dependent variable was set as log(MPN).

Results

The Limit of Detection was found to be 1.65 cells. The overall regression standard error, the

95.0% confidence interval was found to be 0.67, which encompasses the Limit of Detection. The 99.99% confidence interval of the intercept was found to be 0.23. These both contain the intercept of 0.22 within the interval.

Table 7: Data for determination of the Limit of Detection

Sample	Concentration	Log(CFU)	Log(MPN)
Sample 1, Rep 1	**10^-1**	-1.17	-0.45
	10^1	2.05	2.63
	10^2	2.96	3.36
	10^3	3.96	4.66
	10^4	4.69	4.66
Sample 1, Rep 2	**10^-1**	-1.17	-0.04
	10^1	2.05	2.36
	10^2	2.96	2.96
	10^3	3.96	4.38
	10^4	4.69	5.34
Sample 2, Rep 1	**10^-1**	-0.51	0.17
	10^1	1.44	1.36
	10^2	2.44	2.36
	10^3	2.83	3.17
	10^4	4.69	5.04

Sample 2, Rep 2	**10^-1**	-0.51	-0.75
	10^1	1.44	1.63
	10^2	2.44	2.87
	10^3	2.83	3.36
	10^4	4.69	4.66
Sample 3, Rep 1	**10^-1**	-0.93	-0.75
	10^1	1.13	1.58
	10^2	2.51	2.96
	10^3	3.51	3.36
	10^4	4.69	5.04
Sample 3, Rep 2	**10^-1**	-0.93	-0.75
	10^1	1.13	1.63
	10^2	2.51	2.63
	10^3	3.51	4.38
	10^4	4.69	5.04
Sample 4, Rep 1	**10^-1**	-0.21	-0.13
	10^1	1.48	0.96
	10^2	2.48	2.63

	10^3	3.07	3.63
	10^4	4.69	5.04
Sample 4, Rep 2	**10^-1**	-0.21	0.36
	10^1	1.48	1.36
	10^2	2.48	2.63
	10^3	3.07	3.17
	10^4	4.69	4.66
Sample 5, Rep 1	**10^-1**	-0.15	-0.21
	10^1	1.85	1.96
	10^2	2.49	2.36
	10^3	3.49	3.97
	10^4	4.69	5.04
Sample 5, Rep 2	**10^-1**	-0.15	-0.45
	10^1	1.85	1.63
	10^2	2.49	2.36
	10^3	3.49	3.97
	10^4	4.69	4.66
Sample 6, Rep 1	**10^-1**	-0.28	-0.04

	10^1	1.66	1.63
	10^2	2.66	2.96
	10^3	3.85	4.17
	10^4	4.69	5.04
Sample 6, Rep 2	**10^-1**	-0.28	-0.45
	10^1	1.66	1.96
	10^2	2.66	2.36
	10^3	3.85	4.17
	10^4	4.69	5.04
Sample 7, Rep 1	**10^-1**	-0.41	-0.75
	10^1	1.59	1.96
	10^2	2.57	2.96
	10^3	3.57	3.97
	10^4	4.69	4.66
Sample 7, Rep 2	**10^-1**	-0.41	-0.75
	10^1	1.59	1.96
	10^2	2.57	2.96
	10^3	3.57	3.63

	10^4	4.69	5.34
Sample 8, Rep 1	**10^-1**	-0.57	-0.45
	10^1	1.43	1.96
	10^2	2.60	2.63
	10^3	3.60	3.59
	10^4	4.69	4.66
Sample 8, Rep 2	**10^-1**	-0.57	-0.13
	10^1	1.43	1.96
	10^2	2.60	2.63
	10^3	3.60	4.17
	10^4	4.69	4.66
Sample 9, Rep 1	**10^-1**	-0.33	-0.45
	10^1	1.51	1.96
	10^2	2.51	2.63
	10^3	3.43	3.63
	10^4	4.69	5.04
Sample 9, Rep 2	**10^-1**	-0.33	-0.13
	10^1	1.51	1.63

	10^2	2.51	2.96
	10^3	3.43	3.87
	10^4	4.69	5.04
Sample 10, Rep 1	**10^-1**	-0.49	-0.52
	10^1	1.51	1.96
	10^2	2.67	2.96
	10^3	3.67	4.38
	10^4	4.69	4.66
Sample 10, Rep 2	**10^-1**	-0.49	-0.52
	10^1	1.51	1.96
	10^2	2.67	3.63
	10^3	3.67	3.80
	10^4	4.69	4.66

Limit of Quantification/Sensitivity

Purpose/Method

The quantifiable limit of the method is bounded by the values defined by a 3-tube MPN. In the case that the Limit of Detection is not significantly different than 1 cell/gram, than the Limit of Quantification can be extrapolated using the FDA BAM MPN Calculator.

Results

As the method starts with a low dilution of 1 gram of sample per tube, use of a 3-tube MPN and corresponding dilution ratios will result in the Limit of Quantification/Sensitivity for the method being 0.36 MPN/gram.

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 MagNAPure 96 instrument and PCR performed using the QuantStudio Dx Real-Time PCR System. The strains that make up the inclusivity panel were obtained from the Center for Disease Control and Prevention (CDC), National Oceanic and Atmospheric Administration (NOAA), American Type Culture Collection (ATCC), or Washington State Public Health Laboratory (WAPHL). The primers and probes utilized in this method have been tested against DNA extracts from the isolates listed in Table 1 below.

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 more challenging due to 2 variations of the *trh* gene. All isolates were confirmed using 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 (Lida et al paper 1997). The *ORF8* pandemic marker was confirmed using the primer set from Myers et al. 2003.

Results

Primer / Probe Sensitivity
Sensitivity= (# of true positives/ (# of true positives + # of false negatives))
tlh sensitivity = 73/73 = **100% *tlh* sensitivity**
tdh sensitivity =33/33 = **100% *tdh* sensitivity**
trh sensitivity = 33/33 = **100% *trh* sensitivity**
ORF8 sensitivity = 24/24 = **100% *ORF8* sensitivity**

The primers and probes utilized in this method for their respective target demonstrates 100% inclusivity. See Table 1 (Inclusivity Panel) for the breakdown of strains, source, targets tested, and its corresponding result.

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

Strain	Source	tlh	tdh	trh	orf8	vvha
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	+	-	+	-	
17803	ATCC	+	-	+		
27519	ATCC	+	-			
27969	ATCC	+				
33844	ATCC	+	+			
33845	ATCC	+	+			
33846	ATCC	+	+			
33847	ATCC	+	+			
35117	ATCC	+	-			
35118	ATCC	+	+			
43996	ATCC	+	+			
49398	ATCC	+	-			
BAA-238	ATCC	+	+		+	
BAA-239	ATCC	+	+		+	
BAA-240	ATCC	+	+		+	
BAA-241	ATCC	+	+		+	
BAA-242	ATCC	+	+		+	
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	+	+	-	+	

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	+	+	-	+	
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	+		+		
06-2410	06-2410 (CDC)					+

06-2450	06-2450 (CDC)					+
07-2405	07-2405 (CDC)					+
08-2468	08-2468 (CDC)					+
08-2470	08-2470 (CDC)					+
08-2472	08-2472 (CDC)					+
08-2485	08-2485 (CDC)					+
1831-81	1831-81 (CDC)					+
2009V-1002	2009V-1002 (CDC)					+
2009V-1055	2009V-1055 (CDC)					+
2010V-1021	2010V-1021 (CDC)					+
209V-1035	209V-1035 (CDC)					+
2431-04	2431-04 (CDC)					+
2473-85	2473-85 (CDC)					+
2492-88	2492-88 (CDC)					+
2809-78	2809-78 (CDC)					+
430-79	430-79 (CDC)					+
AM38622	AM38622 (CDC)					+
AM38623	AM38623 (CDC)					+
27562	27562					+
29307	29307					+
Total Confirmed Isolated		73	33	33	24	21

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 MagNAPure 96 instrument and PCR performed using the QuantStudio Dx Real-Time PCR System. All strains were obtained from the Center for Disease Control and Prevention (CDC) and American Type Culture Collection (ATCC).

Results

Primer / Probe Specificity

Specificity = (# of true negative/ (# of true negatives + # of false positives))

tlh specificity= 49/49 = **100% *tlh* Specificity**
tdh specificity = 49/49 = **100% *tdh* Specificity**
trh specificity = 49/50 = **98% *trh* Specificity**
ORF8 specificity = 49/49 = **100% *ORF8* Specificity**

The tlh, tdh, and ORF8 primers and probes utilized in this method demonstrate 100% exclusivity. The trh primers and probe demonstrate a 98% specificity (see Known Limitations below). See Table 2 (Exclusivity Panel) for the breakdown of strains, source, targets tested, and its corresponding result.

Known limitations and interferences

Vibrio alginolyticus possesses a *trh* gene with 98% homology to the *trh* gene in *Vibrio parahaemolyticus*. Most probable number (MPN) values for *trh* should be reported only if *tlh (V. parahaemolyticus* specific gene) is present in the corresponding tube. Tubes only positive for *trh* should not be accounted for when generating the MPN value. This assay utilizes the Taqman Environmental Mastermix 2.0, which is specifically formulated to detect bacterial pathogens with greater specificity and sensitivity. There are no additional known limitations when using the Taqman Environmental Mastermix 2.0.

Table 2. Exclusivity panel with results by target. Targets not tested or confirmed are blocked out.

Organism	Strain	*tlh*	*tdh*	*trh*	*orf8*	*vvha*
E. coli	ATCC 25922	-	-	-	-	-
G. hollisae	ATCC 33564	-	-	-	-	-
K. pneumoniae	ATCC 33495	-	-	-	-	-
P. aeruginosa	ATCC 33495	-	-	-	-	-
S. aureus	ATCC 10145	-	-	-	-	-
S. sonnei	ATCC 25925	-	-	-	-	-
S. typhimurium	ATCC 9290	-	-	-	-	-
V. aestuarians	ATCC 35048	-	-	-	-	-
V. alginolyticus	ATCC 17749	-	-	-	-	-
V. alginolyticus	S14-048 (Environmental- WA PHL)	-	-	+	-	-
V. algosus	ATCC 14390	-	-	-	-	-
V. campbellii	ATCC 25920	-	-	-	-	-
V. cholerae	ATCC 39050	-	-	-	-	-
V. cinncinatiensis	ATCC 35912	-	-	-	-	-
V. furnissii	ATCC 33813	-	-	-	-	-
V. marinagilis	ATCC 14398	-	-	-	-	-

V. marinofulvus	ATCC 14395	-	-	-	-	-
V. marinovulgaris	ATCC 14394	-	-	-	-	-
V. metschnikovii	ATCC 700040	-	-	-	-	-
V. mimicus	ATCC 33653	-	-	-	-	-
V. natriegens	ATCC 14048	-	-	-	-	-
V. nereis	ATCC 25917	-	-	-	-	-
V. nigripulchritudo	ATCC 27043	-	-	-	-	-
V. ponticus	ATCC 14391	-	-	-	-	-
V. proteolyticus	ATCC 15338	-	-	-	-	-
V. spledidus	ATCC 33789	-	-	-	-	-
V. tubiashii	ATCC 19106	-	-	-	-	-
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	209V-1035 (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	AM38622 (CDC)	-	-	-	-	
V. vulnificus	AM38623 (CDC)	-	-	-	-	
V. vulnificus	27562	-	-	-	-	
V. vulnificus	29307	-	-	-	-	
A. trota	2013V-1197 (CDC)	-	-	-	-	-
A. veronii	N/A (CDC)	-	-	-	-	-
Total Strains	49					

Validation Data for MPN Real-time PCR for *Vibrio vulnificus*

Name of Method Submitter: Gina Olson, Washington State Department of Health

Specific purpose or intent of the method for use in the NSSP:

Requesting adoption of this method as an approved method for Vibrio enumeration, both *Vibrio parahaemolyticus* (Vp) and *Vibrio vulnificus* (Vv) in oysters. The method may be used in the following applications: PHP validation and verification of product and in management of growing areas through environmental testing and surveillance in order to re-open closed growing areas. This method once approved would provide a high-throughput alternative to the current approved MPN real-time PCR method. In addition, this method would be the only approved MPN real-time PCR method to test for total Vp, pathogenic Vp, and Vv in a single assay.

Validation Criteria Data:

All oyster samples used in this validation were collected from different harvest locations and/or different harvest dates in Washington State. All samples were processed using APHA guidelines for the cleaning and shucking of shellstock. Samples were confirmed negative for the target organism of Vv through the FDA BAM culture-based method and through pcr prior to spiking. Spiking levels were determined using a 5-tube MPN dilution series in APW in duplicate (averaging the 2 values).

Vv strain ATCC 29307 was used to spike all samples for all validation criteria. This strain is positive for *vvhA*.

The validation data for Vp and Vv is presented separately for clarity and ease in reviewing the data, but this is a single assay and all elements were present during the validation of all organisms.

<u>Assay Design</u>

DNA Isolation: Roche MagnaPure 96 using Roche DNA/Viral Nucleic Acid Small Volume Kit

Real-time PCR Instrument: Applied Biosystems QuantStudio Dx (384-well format)

Mastermix: Life Technologies TaqMan Environmental Master Mix 2.0

Real-Time PCR targets: 2 multiplex reactions

- Multiplex 1: Total Vp (*tlh)*, Vv (*vvhA)*, internal control (IC)
- Multiplex 2 (Vp pathogenicity markers): *tdh*, *trh*, *orf8*

Real-Time PCR parameters:

Denaturation: 95°C for 10 mins
Annealing: 95°C for 15 secs
Extension: 59°C for 1 min
Cycles: 40

1. Accuracy/Trueness & Measurement Uncertainty

Accuracy/Trueness

Purpose/Method

Accuracy/Trueness measures the closeness of agreement between the test results (MPNs) and the reference results (spiked MPNs without matrix). This was done by analyzing twenty oyster samples over a range of concentrations (low to high) to determine the MPN. The MPN and reference data set was converted into logs. The average MPN in logs was divided by the average reference value in logs. This provides an estimate in percent of the accuracy/trueness of the method.

Results

The average of the reference values was 2.20 log. The average of MPNs was 2.15 log. Accuracy/Trueness was found to be 97.69%. Results can be found below in Table 1.

Measurement Uncertainty

Purpose/Method

Measurement uncertainty expresses the range of values around the measured result within which the true value is expected to lie. To determine this parameter, twenty oyster samples spiked with a range of concentrations were analyzed. Each MPN and reference value was converted into logs and the MPN result was subtracted from the reference result for each sample. A 95% confidence interval was calculated from the difference. This confidence interval represents the measurement uncertainty of the methods.

Results

The measurement uncertainty was determined via 95% CI (0.16, 0.30), resulting in a measurement uncertainty of 0.14. Results can be found below in Table 1.

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

Sample	Reference MPN, log(MPN/g)	MPN, log(MPN/g)
1	0.11	-0.44
2	0.11	-0.04
3	0.11	0.36
4	2.11	1.96
5	2.11	2.36
6	2.20	1.87

7	2.20	2.36
8	4.08	3.97
9	4.08	4.38
10	4.08	3.88
11	0.23	-0.04
12	1.18	1.18
13	2.30	1.96
14	3.15	3.36
15	4.30	4.38
16	0.49	0.36
17	1.32	0.96
18	2.08	2.62
19	3.45	3.36
20	4.30	4.18

2. Ruggedness

Purpose/Method

The amount of analyte recovered should be consistent between different lots of media/reagents. Ruggedness tests the impact of different lots used to process samples on the final result. This was done by testing ten oyster samples spiked at a range of concentrations in duplicate. One replicate was performed using “Lot 1” media/reagents and one replicate was performed using “Lot 2.” To determine if the method was sufficiently rugged to withstand the types of changes anticipated to occur in routine use, a two-sided t-test was utilized on log-transformed data with a significance level (α) of 0.05. There should be no significant difference between Lot 1 and Lot 2 samples.

Results

Using data from Table 2, there was no significant difference (p=0.37) between different lots of media and reagents.

Table 2. Data for Determination of Ruggedness

Sample	Replicate 1, log(MPN/g)	Replicate 2, log(MPN/g)
1	-0.44	0.17
2	-0.04	0.36
3	0.36	0.36
4	1.96	1.96
5	2.36	1.87
6	1.87	1.96
7	2.36	2.36
8	3.97	3.88
9	4.38	4.66
10	3.88	3.97

3. Precision & Recovery

Precision

Purpose/Method

The difference between the methods results (MPNs) and the reference values should be consistent both between different samples and also when detecting varying concentrations of measurand. The precision of the method tests the consistency of the difference between the reference values and the MPN values found in spiked matrix. This was done by testing ten oyster samples at low, medium, and high concentrations in duplicate to determine the MPN. The MPN and reference data sets were converted into log values. Each MPN was compared to its associate reference value. A nested ANOVA was then performed, with variance components being the sample, and concentrations within the samples (low, medium, and high), and then the error. The ANOVA component of interest was that comparing the concentrations within the samples to the determinations (or error).

Results

The difference between reference values and MPNs can be found below in Table 3. The data shows that there are no significant differences between the concentrations in samples and the determinations within concentrations via a nested ANOVA (p=0.69). Additionally, the variance of the method does not exceed the known variance of a 3-tube MPN (p=0.16).

Table 3: Data for determining the Precision

Sample	Concentration	Difference	log(reference MPNs)	log(MPNs)
1	low	0.27	0.23	-0.04
1	low	-0.40	0.23	0.63
1	med	-0.55	3.41	3.97
1	med	0.45	3.41	2.96
1	high	-0.24	4.41	4.66
1	high	-0.24	4.41	4.66
2	low	-0.19	0.18	0.36
2	low	0.21	0.18	-0.04
2	med	0.06	3.23	3.17
2	med	-0.13	3.23	3.36
2	high	0.26	4.23	3.97
2	high	0.06	4.23	4.17
3	low	0.27	0.13	-0.14
3	low	0.17	0.13	-0.04
3	med	0.21	3.18	2.96
3	med	-0.28	3.18	3.46
3	high	-0.20	4.18	4.38
3	high	0.21	4.18	3.97
4	low	0.23	0.29	0.06
4	low	0.73	0.29	-0.44
4	med	-0.23	3.13	3.36
4	med	0.17	3.13	2.96

4	high	-0.20	4.13	4.33
4	high	-0.53	4.13	4.66
5	low	-0.13	0.18	0.31
5	low	-0.45	0.18	0.63
5	med	-0.07	3.29	3.36
5	med	-0.07	3.29	3.36
5	high	-0.09	4.29	4.38
5	high	0.32	4.29	3.97
6	low	0.13	0.49	0.36
6	low	0.53	0.49	-0.04
6	med	-0.14	3.49	3.63
6	med	0.32	3.49	3.17
6	high	0.00	4.18	4.17
6	high	0.00	4.18	4.17
7	low	-0.05	0.31	0.36
7	low	0.35	0.31	-0.04
7	med	-0.05	3.31	3.36
7	med	0.35	3.31	2.96
7	high	0.34	4.31	3.97
7	high	0.34	4.31	3.97
8	low	-0.28	0.08	0.36
8	low	0.22	0.08	-0.14
8	med	0.12	3.08	2.96
8	med	-0.28	3.08	3.36
8	high	-0.25	4.08	4.33

8	high	-0.09	4.08	4.17
9	low	0.27	0.44	0.17
9	low	0.12	0.44	0.32
9	med	0.08	3.44	3.36
9	med	0.27	3.44	3.17
9	high	0.06	4.44	4.38
9	high	0.27	4.44	4.17
10	low	0.13	0.30	0.17
10	low	0.34	0.30	-0.04
10	med	-0.06	3.30	3.36
10	med	0.67	3.30	2.63
10	high	0.13	4.30	4.17
10	high	0.33	4.30	3.97

Recovery

Purpose/Method

The amount of analyte recovered should be consistent both between different samples and also when detecting varying concentrations of measurand. The recovery of the method tests the consistency of the analyte recovered via MPNs as compared to the reference values. This was done by testing ten oyster samples at low, medium, and high concentrations in duplicate to determine the MPN. Each MPN and reference value was converted to logs. Each duplicated MPN was averaged and then compared to its associate reference value, in logs. A single-factor ANOVA was then used to compare the recovery at the three concentrations.

Results

The difference between reference values and MPNs can be found below in Table 4. The recovery across all samples and concentrations was found to be 97.44%. There was not found to be significant differences in the recovery at the various concentrations (p=0.49).

Table 4: Data for the determination of Recovery

Sample	Concentration	Avg log(Reference) per Conc.	Avg log(MPN) per Conc.
1	low	0.23	0.30
	med	3.41	3.47
	high	4.41	4.66
2	low	0.18	0.16
	med	3.23	3.27
	high	4.23	4.07
3	low	0.13	-0.09
	med	3.18	3.21
	high	4.18	4.17
4	low	0.29	-0.19
	med	3.13	3.16
	high	4.13	4.49
5	low	0.18	0.47
	med	3.29	3.36
	high	4.29	4.17
6	low	0.49	0.16
	med	3.49	3.40
	high	4.18	4.17
7	low	0.31	0.16
	med	3.31	3.16
	high	4.31	3.97
8	low	0.08	0.11
	med	3.08	3.16
	high	4.08	4.25
9	low	0.44	0.24
	med	3.44	3.27
	high	4.44	4.28
10	low	0.30	0.07
	med	3.30	3.00
	high	4.30	4.07

4. Specificity

Purpose/Method

The method should only detect the analyte of interest, even in the presence of interfering

organisms. Specificity refers to the ability of the method to measure only the target organism. One matrix sample was divided into two aliquots. One aliquot was spiked with a low but determinable level of *Vibrio vulnificus* (*Vv*). The other aliquot was spiked with the same level of *Vv* as the first, but also spiked with a high level of potential interfering *Vibrio parahaemolyticus (Vp)*. Five replicates were performed. Each of the replicates was analyzed by taking the average log MPN and calculating the Specificity Index (SI). A paired *t*-test was used to determine if the average specificity index obtained from the five replicates differed from 1 (significance level = 0.05).

Results
Using the data from Table 5, the average specificity index was 0.99 when in the presence of *Vp.* These values are not significantly different than 1 (p=0.74).

Table 5. Data for Determination of Specificity

Replicate	*Vv* only, log(MPN/g)	*Vp* + *Vv*, log(MPN/g)
1	1.63	1.96
2	2.32	2.17
3	1.96	1.63
4	1.96	2.36
5	2.17	2.17

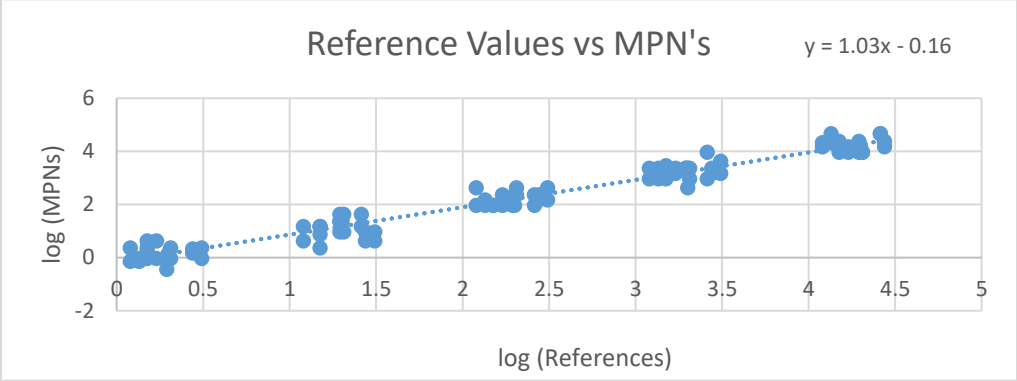
5. Linear Range, Limit of Detection & Limit of Quantification/Sensitivity

Linear Range

Purpose/Method
The MPN value found should directly correlate to the concentration of analyte within the sample, within the working range of the method. Thus, as the concentration increases, the MPN value should also increase in a linear fashion. Ten Oyster samples were tested at 5 concentration levels, in duplicate. Each MPN was compared to its associate reference value. The relationship between the log(MPN) and log(reference) was then found by obtaining the correlation coefficient by performing a linear regression with log(reference) as the independent variable and log(MPN) as the dependent variable.

Results
The relationship between the MPNs and reference values can be seen in Figure 1 below. The relationship between MPNs and reference values was found to be linear, with a Pearson's *r* of 0.98. The working range used was of concentrations ranging from 10^0 to 10^4 cells/gram.

Figure 1: Graph representing the relationship between the log of reference values and MPNS



Limit of Detection

Purpose/Method

The method should be capable of detecting as little as 1 cell/gram of sample, or 0 cells/gram, in log form. Therefore, it must be determined whether the method can detect one cell per gram of sample. The log(MPN) was compared to the log(reference) of ten oyster samples, spiked at five varying concentrations, in duplicate. This was done by performing a regression analysis on the data and calculating the Limit of Detection by taking the antilog of the intercept. The independent variable was set as log(reference) and the dependent variable was set as log(MPN).

Results

The Limit of Detection was found to be 0.68 cells. The overall regression 95.0% confidence interval was found to be 0.58, which encompasses the Limit of Detection. The 99.99% confidence interval of the intercept was found to be 0.23. These both contain the intercept value of -0.16 within the interval.

Table 7: Data for determination of the Limit of Detection

Sample	Concentration	Log(reference)	Log(MPN)
Sample 1, Rep 1	**10^0**	0.23	-0.04
	10^1	1.41	1.17
	10^2	2.41	1.96

	10^3	3.41	3.97
	10^4	4.41	4.66
Sample 1, Rep 2	**10^0**	0.23	0.63
	10^1	1.41	1.63
	10^2	2.41	2.36
	10^3	3.41	2.96
	10^4	4.41	4.66
Sample 2, Rep 1	**10^0**	0.18	0.36
	10^1	1.18	1.17
	10^2	2.23	1.96
	10^3	3.23	3.17
	10^4	4.23	3.97
Sample 2, Rep 2	**10^0**	0.18	-0.04
	10^1	1.18	0.87
	10^2	2.23	2.36
	10^3	3.23	3.36
	10^4	4.23	4.17

Sample 3, Rep 1	**10^0**	0.13	-0.14
	10^1	1.29	0.96
	10^2	2.29	1.96
	10^3	3.18	2.96
	10^4	4.18	4.38
Sample 3, Rep 2	**10^0**	0.13	-0.04
	10^1	1.29	1.36
	10^2	2.29	2.17
	10^3	3.18	3.46
	10^4	4.18	3.97
Sample 4, Rep 1	**10^0**	0.29	0.06
	10^1	1.29	1.36
	10^2	2.13	2.17
	10^3	3.13	3.36
	10^4	4.13	4.33
Sample 4, Rep 2	**10^0**	0.29	-0.44
	10^1	1.29	1.63

	10^2	2.13	1.96
	10^3	3.13	2.96
	10^4	4.13	4.66
Sample 5, Rep 1	**10^0**	0.18	0.31
	10^1	1.18	0.36
	10^2	2.18	1.96
	10^3	3.29	3.36
	10^4	4.29	4.38
Sample 5, Rep 2	**10^0**	0.18	0.63
	10^1	1.18	1.17
	10^2	2.18	1.96
	10^3	3.29	3.36
	10^4	4.29	3.97
Sample 6, Rep 1	**10^0**	0.49	0.36
	10^0	1.49	0.63
	10^2	2.49	2.63
	10^3	3.49	3.63

	10^4	4.18	4.17
Sample 6, Rep 2	**10^0**	0.49	-0.04
	10^1	1.49	0.96
	10^2	2.49	2.17
	10^3	3.49	3.17
	10^4	4.18	4.17
Sample 7, Rep 1	**10^0**	0.31	0.36
	10^1	1.31	0.96
	10^2	2.31	2.36
	10^3	3.31	3.36
	10^4	4.31	3.97
Sample 7, Rep 2	**10^0**	0.31	-0.04
	10^1	1.31	1.63
	10^2	2.31	2.63
	10^3	3.31	2.96
	10^4	4.31	3.97
Sample 8, Rep 1	**10^0**	0.08	0.36

	10^1	1.08	1.17
	10^2	2.08	2.63
	10^3	3.08	2.96
	10^4	4.08	4.33
Sample 8, Rep 2	**10^0**	0.08	-0.14
	10^1	1.08	0.63
	10^2	2.08	1.96
	10^3	3.08	3.36
	10^4	4.08	4.17
Sample 9, Rep 1	**10^0**	0.44	0.17
	10^1	1.44	0.96
	10^2	2.44	2.17
	10^3	3.44	3.36
	10^4	4.44	4.38
Sample 9, Rep 2	**10^0**	0.44	0.32
	10^1	1.44	0.63
	10^2	2.44	2.36

	10^3	3.44	3.17
	10^4	4.44	4.17
Sample 10, Rep 1	**10^0**	0.30	0.17
	10^1	1.30	1.36
	10^2	2.30	1.96
	10^3	3.30	3.36
	10^4	4.30	4.17
Sample 10, Rep 2	**10^0**	0.30	-0.04
	10^1	1.30	1.36
	10^2	2.30	2.36
	10^3	3.30	2.63
	10^4	4.30	3.97

Limit of Quantification/Sensitivity

Purpose/Method

The quantifiable limit of the method is bounded by the values defined by a 3-tube MPN. In the case that the Limit of Detection is not significantly different than 1 cell/gram, than the Limit of Quantification can be extrapolated using the FDA BAM MPN Calculator.

Results

As the method starts with a low dilution of 1 gram of sample per tube, use of a 3-tube MPN and corresponding dilution ratios will result in the Limit of Quantification/Sensitivity for the method being 0.36 MPN/gram.

Inclusivity

Purpose

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

Method

Vibrio vulnificus (*Vv*) strains were grown in APW for 18-24 hours. DNA was extracted using the MagNAPure 96 instrument and PCR performed using the QuantStudio Dx Real-Time PCR System. The strains that make up the inclusivity panel were obtained from the Center for Disease Control and Prevention (CDC). The primers and probes utilized in this method have been tested against DNA extracts from the isolates listed in Table 1 below. The *vvhA* marker was either confirmed by the CDC or ATCC paperwork.

Results

Primer / Probe Sensitivity
Sensitivity= (# of true positives/ (# of true positives + # of false negatives))
vvhA sensitivity = 21/21 = **100% *vvhA* sensitivity**

The primers and probes utilized in this method demonstrates 100% inclusivity. See Table 1 (Inclusivity Panel) for the breakdown of strains, source, targets tested, and its corresponding result.

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

Strain	Source	tlh	tdh	trh	orf8	vvha
06-2410	06-2410 (CDC)					+
06-2450	06-2450 (CDC)					+
07-2405	07-2405 (CDC)					+
08-2468	08-2468 (CDC)					+
08-2470	08-2470 (CDC)					+
08-2472	08-2472 (CDC)					+
08-2485	08-2485 (CDC)					+
1831-81	1831-81 (CDC)					+
2009V-1002	2009V-1002 (CDC)					+
2009V-1055	2009V-1055 (CDC)					+
2010V-1021	2010V-1021 (CDC)					+
209V-1035	209V-1035 (CDC)					+
2431-04	2431-04 (CDC)					+
2473-85	2473-85 (CDC)					+
2492-88	2492-88 (CDC)					+

2809-78	2809-78 (CDC)					+
430-79	430-79 (CDC)					+
AM38622	AM38622 (CDC)					+
AM38623	AM38623 (CDC)					+
27562	27562					+
29307	29307					+
Total Confirmed Isolated		0	0	0	0	21

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 MagNAPure 96 instrument and PCR performed using the QuantStudio Dx Real-Time PCR System. All strains were obtained from the Center for Disease Control and Prevention (CDC) and American Type Culture Collection (ATCC).

Results

Primer / Probe Specificity

Specificity = (# of true negative/ (# of true negatives + # of false positives))

vvhA specificity = 28/28 = **100% *vvhA* Specificity**

The vvhA primers and probes utilized in this method demonstrate 100% exclusivity. None of the exclusivity panel had detection of *vvhA*. See Table 2 (Exclusivity Panel) for the breakdown of strains, source, targets tested, and its corresponding result.

Table 2. Exclusivity panel with results by target. Targets not tested or confirmed are blocked out.

Organism	ATCC #	*tlh*	*tdh*	*trh*	*orf8*	*vvha*
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. spledidus	33789	-	-	-	-	-
V. tubiashii	19106	-	-	-	-	-
A. trota	2013V-1197 (CDC)	-	-	-	-	-
A. veronii	N/A (CDC)	-	-	-	-	-
Total Strains	28					