**PUBLIC HEALTH SERVICE**
**U.S. FOOD AND DRUG ADMINISTRATION**
**OFFICE OF FOOD SAFETY**
**SHELLFISH AND AQUACULTURE POLICY BRANCH**
**5001 CAMPUS DRIVE**
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**SHELLFISH LABORATORY EVALUATION CHECKLIST**

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

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| **LABORATORY REPRESENTED BY:** | **TITLE:** |
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| **LABORATORY EVALUATION OFFICER:** | **SHELLFISH SPECIALIST:** |
| **OTHER OFFICIALS PRESENT:** | **TITLE:** |
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**Items which do not conform are noted by: Conformity it noted by a "√"**

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

Check the applicable analytical methods:

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|---|---|
| | Alkaline Phosphatase Probe Method for *Vibrio vulnificus* detection in Oysters [PART III] |
| | Alkaline Phosphatase Probe Method for *Vibrio parahaemolyticus* detection in Oysters [PART II] |

| **PART I – Quality Assurance** | | | |
|---|---|---|---|
| | | **ITEM** | |
| **CODE** | **REF** | | |
| | | **1.1 Quality Assurance (QA) Plan** | |
| K | 4, 6 | 1.1.1 Written Quality Assurance 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 the *Vibrio* portion of the FDA Shellfish proficiency testing program annually. Specify the program(s): _____ |
| **C** | **2** | | **1.1.4 The Laboratory has and implements a plan to address poor, questionable or unsatisfactory performance in proficiency tests.** |
| | | **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 is maintained. |
| 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 at least 0.1 pH units |
| K | 9 | | 1.4.2 The pH electrodes being used consist of a pH half cell and reference half cell double junction combination electrode, single junction combination electrode or triode. If a single junction electrode is used, it is free of silver/silver chloride or contains an ion exchange barrier to prevent passage of silver ions into the solution (Circle the type of electrode used). |
| 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*). |

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| 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. |
| K | 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, 16 | | 1.4.7 The balances used provide a sensitivity of at least 0.01g at the weights of use. |
| K | 6 | | 1.4.8 Balance calibrations are checked monthly according to manufacturer' specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance calibrations are 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 2 and 8°C. |
| **C** | 1, 7 | | **1.4.11 Freezer temperature is maintained at -20°C or below.** |
| K | 6, 7 | | 1.4.12 Freezer temperature is monitored at least once daily on workdays. Results are recorded and records maintained. |
| **C** | **13, 17** | | **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, 18** | | **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) possessing the appropriate level of accuracy for the intended monitoring application.** |
| **C** | **6, 13, 16** | | **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, 35, 42, 54 and/or 55°C (54°C for *Vibrio parahaemolyticus* and 55°C for *Vibrio vulnificus*). These calibration records (certificates of calibration) are maintained.** |
| K | 3, 5 | | 1.4.20 Standard thermometers are checked annually for accuracy by ice point determination. Any changes are incorporated into all the other calibrated temperature points on the thermometer. These results are recorded and maintained<br>Date of most recent determination:_____ |
| **C** | **2, 18** | | **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 |

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| | | | standards thermometer at the temperature(s) of use. Results for the in-use temperature checks 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 at appropriate volumes used annually and checked for accuracy quarterly. Results are recorded and records maintained. |
| K | 5 | | 1.4.25 Pipets used to inoculate samples and prepare reagents deliver accurate aliquots and are tested for accuracy with each new lot received. |
| | | | **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 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 of preparation is used to ensure the appropriate volumes of diluent. |
| 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** |
| 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 including calibration of temperature gauges. |
| **C** | **6, 18** | | **1.6.3 The autoclave provides a sterilizing temperature of 121± 2°C as determined for each load using a calibrated gauge, sensor or thermometer. This measurement is verified weekly with an external maximum registering working thermometer or data logger (if not routinely used). 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 | 2, 4, 18 | | 1.6.4 An autoclave standards thermometer (data logger) 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 for mercury autoclave standards thermometers but not required as this allows for in-house checks (by steam point) of the thermometer's accuracy at 121°C . |
| K | 2, 10, 18 | | 1.6.5 The autoclave standards thermometer (data logger) is checked every five years for accuracy at either 121°C by a qualified calibration laboratory or in-house at 100°C (mercury thermometer only), the steam point if the (mercury) thermometer has been previously calibrated by a qualified calibration laboratory at this temperature. |

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| | | | Date of most recent determination: _____ |
| K | 1, 2 | | 1.6.6 Working autoclave thermometers (data loggers) are checked against the autoclave standards thermometer at 121°C yearly.<br><br>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 biological 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 load to indicate that the load has been sterilized. |
| K | 6 | | 1.6.9 Autoclave sterilization records are maintained which include the length of the sterilization cycle, total heat exposure time (time in to time out) and maximum chamber temperature<br><br>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 | 9 | | 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 | 13 | | 1.6.12 Records of temperature and exposure times are maintained for the operation of the hot-air sterilizing oven. |
| K | 11 | | 1.6.13 Spore strips/suspensions appropriate for use in dry heat are used quarterly to evaluate the biological effectiveness of the sterilization process in the hot-air oven. Results are recorded and records maintained. |
| K | 9 | | 1.6.14 Reusable pipets are stored and sterilized in aluminum or stainless steel containers. |
| K | 9 | | 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.**<br><br>**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 pre-sterilized disposable pipets, pipet tips and microcentrifuge tubes is determined with each lot received. Results are recorded and records maintained.** |
| K | 13 | | 1.6.19 Spent broth cultures and agar plates are properly decontaminated before disposal. |
| | | **1.7 Media Preparation** | |
| **C** | **19, 29** | | **1.7.1 TCBS is commercially dehydrated and alkaline peptone water (APW), mCPC, T1N3, CC and VVA agars are prepared from the individual components and pH adjusted appropriately.** |
| K | 11 | | 1.7.2 Media components are properly stored. in a cool dry place. |
| K | 11, 19 | | 1.7.3 Media components are labeled with the analyst's initials, date of receipt, and date opened and date of preparation if applicable (dye solutions). |

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| **C** | **2** | | **1.7.4 Caked or expired media or media components are discarded.** |
| **C** | **11** | | **1.7.5 Reagent water is tested monthly and exceeds 0.5 megohms-cm resistance (2 megohms-cm in-line) or is less than 2.0 µSiemens/cm conductivity at 25°C. Results are recorded and the records maintained. (Circle the appropriate water quality descriptor determined)** |
| **C** | **11** | | **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 | 11 | | 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 | 9 19 | | 1.7.8 The volume and concentration of media (APW) in the tube is suitable for the amount of sample inoculated. |
| **C** | **2, 11, 19** | | **1.7.9 The total time of exposure of media broths to autoclave temperatures does not exceed 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 when the medium is made from its individual components.**<br><br>**Positive *Vibrio parahaemolyticus* productivity control_____**<br><br>**Negative *Vibrio parahaemolyticus* productivity control _____**<br><br>**Positive *Vibrio vulnificus* productivity control _____**<br><br>**Negative *Vibrio vulnificus* productivity control _____** |
| **C** | **11** | | **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 | 9 | | 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 | 13 | | 1.8.2 Stored media are labeled with the storage expiration date. or sterilization date. |
| K | 9 | | 1.8.3 Storage of prepared culture media at room temperature does not exceed 7 days. |
| K | 2, 11 19 | | 1.8.4 Storage of prepared broth media with loose fitting closures and prepared plates stored in sealed plastic bags or containers, to minimize evaporation, does not exceed 1 month. |
| K | 35 | | 1.8.5 Refrigerated prepared plates are dried inverted before use to permit the sample to be completely absorbed into the medium to prevent colony spreading. |
| K | 2,17 | | 1.8.6 All prepared broth media stored under refrigeration is warmed to room temperature prior to use, at temperatures that do not exceed the medium's incubation temperature. |
| | | | **PART II – Oyster Samples** |

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| | | **2.1 Sample Handling and Receipt** | |
| **C** | **2, 11** | | **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 | 9, 2 | | 2.1.2 Oyster samples as received are collected in clean, waterproof, puncture resistant containers loosely sealed or are rejected for regulatory analysis. |
| K | 9, 2 | | 2.1.3 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** | **9, 2** | | **2.14 Immediately after collection, samples as received have been are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10°C with ice or cold packs for transport to the laboratory or rejected. Once received, the samples are placed under refrigeration unless processed immediately.** |
| C | **9, 35** | | **2.1.5 If ice is used in sample transport, samples are rejected if melt water has come in contact with the samples.** |
| **C** | **1, 9** | | **2.1.6 Analysis of the samples is initiated as soon as possible after collection, but not to exceed 36h. If processing IQF samples, samples are defrosted under refrigeration for no longer than 36h once removed from the freezer.** |
| | | **2.2 Preparation of Samples for Analysis** | |
| K | 2, 11 | | 2.2.1 Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes prior to use. |
| O | 2 | | 2.2.2 Blades of shucking knives are not corroded. |
| K | 9 | | 2.2.3 The hands of the analyst are thoroughly washed with soap and water 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 | 9 | | 2.2.5 Oysters are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality. |
| K | 9 | | 2.2.6 Oysters are allowed to drain in a clean container or on clean towels prior to opening |
| K | 9, 30 2 | | 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** | **9** | | **2.2.8 Oysters are not shucked through the hinge.** |
| **C** | **9** | | **2.2.9 The contents of the oyster (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.** |
| **C** | **9** | | **2.2.10 A representative sample of at least 12 shellfish is used for analysis.** |
| **C** | **2, 9** | | **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, 16, 17 | | 2.2.12 Either a 1:1 dilution is made at this point, or the sample is homogenized without dilution. If a dilution is made, the sample is weighed to the nearest 0.1 gram 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 at for 60 to 120 seconds until homogenous.** |
| **PART III- Alkaline Phosphatase Probe method for *Vibrio vulnificus* and *Vibrio parahaemolyticus* detection in Oysters** | | | |
| | | **3.1 Preparation of Samples for the Alkaline Phosphatase Probe Method:** | |

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| | | | **Direct Plating** |
| K | 13, 16 | | 3.1.1 If direct plating, use sterile cell spreaders are used to spread inoculum evenly onto three dry T1N3 agar plates for the analysis of *Vibrio parahaemolyticus*. |
| **C** | **13, 16** | | **3.1.2 Two tenths (0.2) of a gram of the initial 1:1 diluted oyster homogenate (or 0.1 g of undiluted homogenate) is used as inoculum; one is used to probe for the total (*tlh*) gene and the two remaining are replicate plates used to probe for the pathogenic (*tdh*) gene.** |
| **C** | **13** | | **3.1.3 Inoculated T1N3 plates are incubated 18-24 h at 35 ±2° C. All plates are used for colony lifts and hybridization, except for those with confluent growth.** |
| **C** | **2, 13** | | **3.1.4 A tdh+ *V. parahaemolyticus* culture diluted to <$10^3$ per ml is used as a positive process control. A *V. vulnificus* culture is used as a negative process control. The process control cultures accompany the samples throughout incubation, and hybridization and color development phases of the method. Results are recorded and are maintained.** |
| | | | **3.2 APW Enrichment** |
| K | 13 | | 3.2.1 Sterile phosphate buffered saline (PBS) is used as the sample diluent. |
| **C** | **13, 16, 17** | | **3.2.2 The 1:10 dilution is prepared gravimetrically with sterile PBS. All successive dilutions are prepared volumetrically.**<br><br>**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 sterile PBS. If the homogenate was not diluted, the 1:10 dilution is prepared by adding 10g of sample homogenate to 90 mL of sterile PBS.** |
| **C** | **14** | | **3.2.3 Appropriate sample dilutions are inoculated into sterile APW.**<br><br>**Specify dilution(s) used** _____<br>**Specify number of tubes per dilution** _____ |
| **C** | **2, 16** | | **3.2.4 For *V. parahaemolyticus* analysis, a tdh+ *V. parahaemolyticus* culture diluted to <$10^3$ per ml is used as a positive process control. A *V. vulnificus* culture is used as a negative process control.**<br><br>**For *V. vulnificus* analysis, a *V. vulnificus* culture diluted to <$10^3$ per ml is used as a positive process control. A *V. parahaemolyticus* culture is used as a negative process control. The process control cultures accompany the samples throughout incubation, isolation, and confirmation. Results are recorded and records are maintained.** |
| **C** | **13** | | **3.2.5 Inoculated APW enrichment tubes are incubated at 35±2°C.** |
| **C** | **13** | | **3.2.6 Tubes are read after 18 – 24 hours of incubation. Clear tubes are negative. Turbid tubes are positive. Positive tubes are confirmed as *Vibrio parahaemolyticus* or *Vibrio vulnificus* as appropriate.** |
| | | | **3.3 Colony Isolation** |
| K | 13 | | 3.3.1 A loopful from the top 1 cm of APW tubes showing growth is streaked onto TCBS for *V. parahaemolyticus* and mCPC or CC agars for *V. vulnificus* isolation |
| **C** | **13, 15** | | **3.3.2 TCBS plates are incubated at 35 ±2°C and mCPC or CC plates are incubated at 35-40°C for 18-24 hours.** |
| **C** | **13** | | **3.3.4 Presumptive colonies are selected meeting these phenotypic** |

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| | | | **characteristics:**<br><br>***V. parahaemolyticus* appear on TCBS agar as round, opaque, green or bluish colonies, 2 to 3 mm in diameter. Interfering large, opaque, and yellow colonies are avoided.**<br><br>***V. vulnificus*: appear on mCPC or CC agar, colonies are as round, flat, opaque, yellow colonies, and 1 to 2 mm in diameter. Typical positives have a "fried egg" appearance. Purple/blue colonies are avoided.** |
| **C** | **13, 16** | | **3.3.5 Colonies are picked and spotted on VVA (*V. vulnificus*) or T1N3 (*V. parahaemolyticus*). For storage and/or ease of replication, colonies are inoculated into a 48 or 96 well plate with APW and incubated for at least 4 and no more than 24 hrs prior to transfer to agar plates.** |
| | | **3.4 Filter preparation.** | |
| **C** | **13, 16** | | **3.4.1 VVA/T1N3 plates are overlaid with labeled (sample number, dilution) #541 Whatman filters (90 mm) for 1 to 30 min.** |
| K | 13, 16, 17 | | 3.4.2 Filters are transferred with colony side up to a plastic or glass Petri dish lid containing 1 ml of lysis solution to wet the filter. |
| **C** | **13, 16, 17** | | **3.4.3 Filters are microwaved in a vessel or tray for 15-20 sec/filter depending on the wattage of the microwave; filters are dry but not scorched or burned.** |
| K | 13, 16, 17 | | 3.4.4 Filters are neutralized 5 min. in a vessel with ammonium acetate (4 ml/filter) on a shaker at room temperature. |
| **C** | **13** | | **3.4.5 #541 Whatman filters are briefly rinsed 2 times in 1X SSC buffer (10 ml/filter).** |
| **C** | **13, 16, 17** | | **3.4.6 Up to 30 filters are incubated in proteinase K solution (10 ml/filter) for 30 min at 42°C. May be conducted in an environmental chamber with shaking (50 rpm) or a water bath.** |
| K | 13 | | 3.4.7 Filters are rinsed 3 times in 1X SSC (10 /filter) for 10 min at room temperature with shaking, at 50 rpm. |
| | | **3.5 Hybridization. (May be conducted in an environmental chamber with shaking or a water bath)** | |
| **C** | **13** | | **3.5.1 For *V parahaemolyticus*, the thermolabile hemolysin (*tlh*), AP-labelled probe 5'Xaa agc gga tta tgc aga agc act g 3' is used. For the thermostable direct hemolysin (*tdh*), the AP-labelled probe 5'Xgg ttc tat tcc aag taa aat gta ttt g 3' is used. For *V. vulnificus*, the cytolysin gene (*vvhA*), AP- labelled probe 5'; Xga gct gtc acg gca gtt gga acc a 3' is used.** |
| **C** | **13** | | **3.5.2 Probes are stored in the refrigerator, not frozen.** |
| **C** | **13, 16** | | **3.5.3 Filters are presoaked in hybridization buffer for 30 min at 54±0.5°C for *V. parahaemolyticus* or 55±0.5°C for *V. vulnificus.* A maximum of 5 filters with 10ml of buffer is used per bag. Up to 20 filters at a time with buffer at the ratio of 10ml per 5 filters can be combined into a vessel of appropriate size to ensure the solution covers the filters.** |
| **C** | **13, 16, 17** | | **3.5.4 10 ml fresh pre-warmed buffer per 5 filters is added. Probe (final conc. of 0.5 pmol/ml) is quickly added to bag or vessel with filters and incubated 1-1.5 h at 54±0.5°C for *Vibrio parahaemolyticus* of 55±0.5°C for *Vibrio vulnificus*.** |
| **C** | **13** | | **3.5.5 Filters are rinsed 2 times for 10 min each in 1X SSC - 1% SDS (for tlh and *Vibrio vulnificus*) or 3X SSC - 1% SDS (for tdh) (10 ml/filter) at 54°±0.5°C C for *Vibrio parahaemolyticus* or 55±0.5°C for *Vibrio vulnificus.*** |

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| K | 13 | | 3.5.6 Filters are rinsed 5 times for 5 min each in 1X SSC (10 ml/filter) at room temperature with shaking, at100 rpm. |
| | | **3.6 Color development** | |
| **C** | **13, 16, 17** | | **3.6.1 In petri dish or suitable vessel, containing 20 ml of NBT/BCIP solution filters (5 or fewer) are added to the petri dish/container and incubated with gentle shaking at room temperature, or at 35°C for faster results. The petri dish/container is kept covered to omit light. Color development of the positive control is checked every 30 minutes. Reaction time varies.** |
| K | 13 | | 3.6.2 Rinse in tap water (10 mL/filter) 3 times for 10 min each to stop color development. |
| **C** | **2, 13, 16** | | **3.6.3 Reactions of test sample colonies are compared to the positive and negative process control cultures. Positive reactions appear as purple or brown spots, yellow spots are considered negative reactions. Filters are stored in the dark.** |
| **C** | **13** | | **3.6.4 Store probes in the refrigerator; do not freeze.** |
| | | **3.7 Computation of Results** | |
| **C** | **13, 16, 17** | | **3.7.1 For direct plating, upon identification of *Vibrio parahaemolyticus* and/or *Vibrio vulnificus*, positive colonies are counted and multiplied by the use dilution factor of the sample to determine the concentration.** |
| K | 16 | | 3.7.2 For direct plating, results are reported as CFU/g of sample. |
| **C** | **13, 19** | | **3.7.3 For APW enrichment, upon identification of *Vibrio parahaemolyticus* and/or *Vibrio vulnificus*, refer to the original positive APW dilutions and record MPN value as derived from the calculator in Appendix 2 of the FDA Bacteriological Analytical Manual (BAM).** |
| K | 13, 16, 17 | | 3.7.4 For APW enrichments, results are reported as MPN/g of sample. |

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| **LABORATORY:** | **DATE of EVALUATION:** |
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**SHELLFISH LABORATORY EVALUATION CHECKLIST**

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