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

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 N/A- Not Applicable

Check the applicable analytical methods:

	Preparation of Samples for the Alkaline Phosphatase Probe Method: Direct Plating [PART III]
	Preparation of Samples for the Alkaline Phosphatase Probe Method: APW Enrichment and Colony Isolation [PART III]
	Alkaline Phosphatase Probe Hybridization [PART III] - **Note: Temperature ranges for hybridization can be expanded with an appropriate study as described in the method.**

PART I – QUALITY ASSURANCE				
CODE	**REF**		**ITEM**	
		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.
				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 Vibrio 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 meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2	In state/county laboratories, the analyst(s) meets 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 or equivalent in microbiology, biology or equivalent discipline with at least two (2) years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4	In commercial laboratories, the analyst(s) must have at least a high school diploma and shall have at least three (3) 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 is fewer than 15 colonies for a 15 minute exposure and 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 or equivalent combination electrode free from Ag/AgCl or contains an ion exchange barrier preventing passage of Ag ions into the solution 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.
K	4		1.4.4	The pH meter is calibrated daily or with each use. Results are recorded and records maintained.

K	6		1.4.5	A minimum of two (2) standard buffer solutions is used to calibrate the pH meter. The first must be 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, 17		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, 15		1.4.7	The balances used provide a sensitivity of at least 0.01 g at the weights of use for direct plating and 0.1 g for MPN.
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 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.
C	**12, 15**		**1.4.10**	**Refrigerator temperatures in which AP-probes are stored are maintained between 2 and 8 ºC.**
K	1		1.4.11	The temperature of general purpose refrigerators, those not containing AP-probes, are maintained between 0 and 4 ºC.
C	**2**		**1.4.12**	**Freezer temperatures are maintained at -15 ºC or below.**
K	6		1.4.13	Freezer temperature is monitored at least once daily on workdays. Results are recorded and records maintained.
C	**12**		**1.4.14**	**The temperature of the incubator is maintained at 35 ± 2.0 ºC.**
C	**6**		**1.4.15**	**Working thermometers used in the air incubators are graduated in at least 0.5 ºC increments.**
K	5, 8		1.4.16	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.
C	**6**		**1.4.17**	**Temperature of the water bath is maintained appropriately under all loading conditions.**
C	**5**		**1.4.18**	**Working thermometers used in the water bath are graduated in at least 0.1 ºC increments.**
K	4, 6		1.4.19	Air incubator/water bath temperatures are taken twice daily on workdays. Results are recorded and records maintained.
C	**3**		**1.4.20**	**All working thermometers are appropriately immersed.**
C	**5**		**1.4.21**	**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	**5, 6**		**1.4.22**	**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 ºC (54 ºC for *Vibrio parahaemolyticus* and 55 ºC for *Vibrio vulnificus*). These calibration records (certificates of calibration) are maintained.**
K	3		1.4.23	Standards thermometers are checked annually for accuracy by ice point determination. Results are recorded and maintained. Date of most recent determination: _____

C	**5**		**1.4.24**	**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 at least an accuracy of ±0.05 ºC are used as the laboratory standards thermometer (*Circle the thermometer type used*).**
K	3, 8		1.4.25	The accuracy of working thermometers is checked annually against the standards thermometer either at the temperatures at which they are used or by ice point determination. Results are recorded and records maintained.
O	8		1.4.26	Appropriate pipet aids are available and used to inoculate samples.
K	7		1.4.27	Micropipettors are calibrated annually and checked for accuracy quarterly at volumes of use. Results are recorded and records maintained.
		1.5 Labware and Glassware Washing		
K	5		1.5.1	Utensils and containers 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.
O	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.
C	**5**		**1.5.5**	**Pipettes used to inoculate the sample deliver accurate aliquots, have unbroken tips and are appropriately graduated. Pipettes larger than 10 mL are not used to deliver 1 mL aliquots; nor, are pipettes larger than 1.1 mL used to deliver 0.1 mL aliquots.**
K	5		1.5.6	In washing reusable pipets, glassware and labware, a succession of at least three (3) fresh water rinses plus a final rinse of deionized water is used to thoroughly rinse off all detergent.
C	**8**		**1.5.7**	**An alkaline or acidic detergent is used for washing glassware/labware.**
C	**6**		**1.5.8**	**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) 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 are maintained.
C	**19, 20, 21**		**1.6.3**	**The autoclave provides sterilization conditions suitable to the load contents. Sterilization temperature range may be 119ºC - 124ºC as determined by the lab's equipment Quality Assurance Verification Testing and recommended practices from the media manufacturer. Sterilization is determined for each load using a working maximum registering thermometer, or an appropriate working temperature monitoring device.**

K	2, 5, 6		1.6.4	An autoclave standards thermometer (or data logger) has been calibrated by a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at 121 °C. If in-house checks for accuracy of the standards thermometer will be conducted at the steam point, calibration of the autoclave standards thermometer at 100 °C is also recommended, but not required.
K	2, 10, 18		1.6.5	The autoclave standards thermometer (or data logger) is checked every five (5) years for accuracy at either 121 °C by a qualified calibration laboratory; or, is checked in-house at the steam point (100 °C) if it has been previously calibrated at both 100 °C and 121 °C. Any change in temperature at the steam point changes the calibrated temperature at 121 °C by the same magnitude. Date of most recent determination: _____
K	2, 8		1.6.6	Working autoclave thermometers (or data loggers) are checked against the autoclave standards thermometer at 121 ºC yearly. Date of last check:_____Method:_____
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, 8		1.6.9	Autoclave sterilization records including the length of sterilization cycle, 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	5, 8		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 ºC.
K	8		1.6.11	Records of temperature and exposure times are maintained for the operation of the hot-air sterilizing oven during use.
K	8		1.6.12	Spore strips/suspensions are used quarterly to evaluate the effectiveness of the sterilization process in the hot-air oven. Results are recorded, and records maintained.
K	5		1.6.13	Reusable pipets are stored and sterilized in aluminum or stainless-steel containers.
K	5		1.6.14	Reusable pipets (in canisters) are sterilized in a hot-air oven at 170 ºC for two (2) hours.
C	**2**		**1.6.15**	**The sterility of reusable pipets is determined with each load sterilized. Results are recorded, and records maintained.**
C	**2**		**1.6.16**	**The sterility of autoclave sterilized disposable pipet tips and microcentrifuge tubes is determined with each load sterilized. Results are recorded, and records maintained.**
C	**2**		**1.6.17**	**The sterility of pre-sterilized disposable pipettes, pipet tips and microcentrifuge tubes is determined with each lot received. Results are recorded, and records maintained.**
K	8		1.6.18	Spent broth cultures and agar plates are decontaminated by autoclaving for at least 30 minutes before conventional disposal.

		1.7 Media and Reagent Preparation		
C	**12, 15**		**1.7.1**	**Media and reagents are prepared from the individual components and pH adjusted appropriately, except in the case of TCBS, which is commercially dehydrated.**
K	1, 5, 8		1.7.2	Dehydrated media, and media and reagent components are properly stored in a cool, clean, dry place.
K	1		1.7.3	Media and components are labeled with the analyst's initials, date of receipt, date opened or date of preparation, if applicable (dye solutions).
C	**1, 2, 7**		**1.7.4**	**Caked or expired media or components are discarded.**
C	**6**		**1.7.5**	**Reagent water is distilled or deionized (*circle appropriate choice*), tested monthly and exceeds 0.5 megohms-cm resistivity (2 megohms-cm in-line) or is less than 2.0 µSiemens/cm conductivity at 25 ºC. (*Circle the appropriate water quality descriptor determined*). Results are recorded and the records maintained.**
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 mg/L). Results are recorded, and records maintained.** **Specify method of determination: _____**
K	6		1.7.7	Reagent water for media and diluent preparation contains <100 CFU/mL as determined monthly using the heterotrophic plate count method. Results are recorded, and records maintained.
K	12		1.7.8	The volume and concentration of media (APW) in the tube is suitable for the amount of sample inoculated.
C	**2**		**1.7.9**	**The total time of exposure of the sugar containing agar VVA to autoclave temperatures does not exceed 45 minutes. Total exposure time of APW and T1N3 agar does not exceed 60 minutes. TCBS, CC and mCPC are not autoclaved.**
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.** **Positive *Vibrio parahaemolyticus* productivity control_____** **Negative *Vibrio parahaemolyticus* productivity control_____** **Positive *Vibrio vulnificus* productivity control_____** **Negative *Vibrio vulnificus* productivity control_____**
C	**6, 12**		**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 and Reagents		
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	2		1.8.2	Stored media are labeled with the storage expiration date or sterilization date.

K	2		1.8.3	Storage of prepared culture media at room temperature does not exceed seven (7) days.
K	6		1.8.4	Storage under refrigeration of prepared agar plates in sealed plastic bags shall not exceed two (2) weeks.
K	6		1.8.5	Storage under refrigeration of prepared broth media with loose fitting closures shall not exceed one (1) month.
K	6		1.8.6	Storage under refrigeration of prepared broth media and diluent with screw-cap closures shall not exceed three (3) months.
K	12, 15		1.8.7	Refrigerated prepared plates are dried inverted before use to permit the sample to be completely absorbed into the medium to prevent colony spreading, for direct plating.
K	2, 6		1.8.8	All prepared broth media and diluent stored under refrigeration are warmed to room temperature prior to use, at temperatures that do not exceed the medium's incubation temperature.
K	15		1.8.9	Storage at room temperature of Lysis Solution, Ammonium Acetate Buffer, 20XSSC, 1XSSC/SDS, and 3XSSC/SDS for the hybridization procedure shall not exceed three (3) months.
K	15		1.8.10	Storage under refrigeration of Hybridization Buffer for the hybridization procedure shall not exceed one (1) week.
C	**15**		**1.8.11**	**NBT/BCIP solution and 1XSSC for the hybridization procedure should be made fresh the day of use.**
PART II – SHELLFISH SAMPLES				
		2.1 Sample Handling and Receipt		
C	**1, 5, 12, 15**		**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, 15		2.1.2	Shellfish samples are received in clean, waterproof, puncture resistant containers loosely sealed or are rejected for regulatory analysis.
K	1, 5		2.1.3	Samples are received 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, 12, 15**		**2.1.4**	**Immediately after collection, samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10 ºC with ice or cold packs for transport to the laboratory or rejected. Direct contact of the shellfish with ice in the transport container should be avoided. Once received, the samples are placed under refrigeration unless processed immediately.**
K	5, 15		2.1.5	If ice is used in sample transport, samples are rejected if melt water has come in contact with the samples.
C	**15**		**2.1.6**	**Analysis of the samples is initiated as soon as possible after collection, but not to exceed 36 hours. If processing IQF samples, samples are defrosted under refrigeration for no longer than 36 hours 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, 11		2.2.2	Blades of shucking knives are not corroded.
K	5, 11		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, 11		2.2.4	The faucet used for rinsing the shellfish does not contain an aerator.

K	5, 11		2.2.5	Shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
K	5, 11		2.2.6	Shellfish are allowed to drain in a clean container or on clean towels prior to opening.
K	2, 5, 11		2.2.7	Immediately prior to shucking, the hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol, or gloves are donned. The gloves, if worn, are latex, nitrile and/or stainless-steel mesh to protect analyst's hands from injury.
C	**5, 11**		**2.2.8**	**Shellfish are not shucked through the hinge.**
C	**5, 11, 12, 15**		**2.2.9**	**The contents of the shellfish (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
C	**12, 15**		**2.2.10**	**A representative sample of 10 to 14 shellfish is used for analysis.**
C	**2, 11**		**2.2.11**	**The quantity of meat and liquor is sufficient to cover the blender blades or additional shellfish are used in order to ensure sample homogeneity.**
K	5, 12, 13, 15		2.2.12	Either a 1:1 dilution is made, or the sample is homogenized without dilution. 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	12, 14, 15		2.2.13	Sterile phosphate buffered saline (pH 7.4) or alkaline peptone water (APW) is used as the sample diluent. If APW is used, sample analysis is conducted immediately.
C	**12, 15**		**2.2.14**	**Samples are blended for 90-120 seconds until homogenous.**
PART III – ALKALINE PHOSPHATASE PROBE METHOD FOR *VIBRIO VULNIFICUS* AND *VIBRIO PARAHAEMOLYTICUS* DETECTION IN SHELLFISH				
		3.1 Preparation of Samples for the Alkaline Phosphatase Probe Method: Direct Plating		
C	**2, 12, 15**		**3.1.1**	**For oyster samples, two tenths (0.20) of a gram of the initial 1:1 diluted homogenate (or 0.10 g of undiluted homogenate) and/or appropriate dilutions are used as inoculum. Dilutions are made in sterile PBS or APW. If APW is used, time from initial dilution until plating does not exceed 30 minutes.** **For samples other than oysters, 100 µl of the 1:10 dilution and/or subsequent dilutions should be used as inoculum.**
K	12, 15		3.1.2	For analysis of total *V. parahaemolyticus*, at least one (1) T1N3 plate is inoculated to be probed for the *tlh* gene. For pathogenic *V. parahaemolyticus*, at least two (2) T1N3 plates are inoculated to be probed for the *tdh* gene. For analysis of *V. vulnificus*, at least one (1) VVA plate is inoculated to be probed for the *vvhA* gene.
K	12, 15		3.1.3	Sterile cell spreaders are used to spread each inoculum evenly onto the dry T1N3 and/or VVA agar plates.

C	**2**		**3.1.4**	**For *V. parahaemolyticus* analysis, a *tdh*+ *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.**
C	**2**		**3.1.5**	**The process control cultures accompany the samples throughout incubation and hybridization and color development phases of the method. Results are recorded, and records are maintained.**
C	**12, 15**		**3.1.6**	**Inoculated plates are incubated 16-24 hours at 35 ± 2 ºC. All plates are used for colony lifts and hybridization, except for those with confluent growth.**
		3.2 Preparation of Samples for the Alkaline Phosphatase Probe Method: APW Enrichment and Colony Isolation		
K	11, 12		3.2.1	Sterile phosphate buffered saline (PBS) is used as the sample diluent.
C	**12**		**3.2.2**	**The 1:10 dilution is prepared gravimetrically with sterile PBS. All successive dilutions are prepared volumetrically.**
C	**12, 16**		**3.2.3**	**Appropriate sample dilutions are inoculated into sterile APW.** **Specify dilution(s) used:_____** **Specify number of tubes per dilution:_____**
C	**2**		**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 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.**
C	**2**		**3.2.5**	**The process control cultures accompany the samples throughout incubation, isolation and confirmation. Results are recorded, and records are maintained.**
C	**12**		**3.2.6**	**Inoculated APW enrichment tubes are incubated at 35 ± 2.0 ºC.**
C	**12**		**3.2.7**	**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.**
K	12		3.2.8	A loopful from the top one (1) cm of APW tubes showing growth is streaked onto TCBS for *V. parahaemolyticus* and mCPC or CC agars for *V. vulnificus* isolation.
C	**12**		**3.2.9**	**TCBS plates are incubated at 35 ± 2 ºC and mCPC or CC plates are incubated at 35-40 ºC for 18-24 hours.**

C	**12**		**3.2.10**	**Presumptive colonies are selected meeting these phenotypic characteristics:** ***V. parahaemolyticus* appear on TCBS agar as round, opaque, green or bluish colonies, two (2) to three (3) mm in diameter. Interfering large, opaque and yellow colonies are avoided.** ***V. vulnificus* appear on mCPC or CC agar as round, flat, opaque, yellow colonies, one (1) to two (2) mm in diameter. Typical positives have "fried egg" appearance. Purple/blue colonies are avoided.**
C	**12**		**3.2.11**	**A sterile 96-well microtiter plate is filled with 100 µl/well of APW. Presumptive vibrios are picked from a selective agar plate using a sterile toothpick or wood transfer stick to individual wells. The plate is incubated 3-5 hours or overnight at 35 ± 2 °C. A 48-prong replicator is used to replicate/transfer isolates in the wells to an agar plate (T1N3 for *V. parahaemolyticus* and VVA for *V. vulnificus*).**
C	**12**		**3.2.12**	**Plates are incubated at 35 ± 2 ºC for 18-24 hours.**
		3.3 Alkaline Phosphatase Probe Hybridization: Filter Preparation		
C	**12, 15**		**3.3.1**	**VVA/T1N3 plates are overlaid with labeled (sample number, dilution) #541 Whatman filters for one (1) to 30 minutes.**
K	12, 15		3.3.2	Filters are transferred with colony side up to a plastic or glass Petri dish lid containing one (1) ml of lysis solution to wet the filter.
C	**12, 15**		**3.3.3**	**Filters are microwaved to dryness, but not brown. Microwave for 15-30 seconds/filter, depending on the wattage of the microwave. Additional heating cycles may be required.**
K	12, 15		3.3.4	Filters are neutralized for five (5) minutes in an appropriate vessel or container with ammonium acetate (4 ml/filter) on a shaker at room temperature.
C	**12, 15**		**3.3.5**	**#541 Whatman filters are rinsed two (2) times in 1X SSC buffer (10 ml/filter) for 1-2 minutes. Filters may be air dried and stored at this point.**
C	**12, 15**		**3.3.6**	**Up to 30 filters are incubated in proteinase K solution (10 ml/filter) for 30 minutes at 42 °C with shaking (~50 rpm).**
K	12, 15		3.3.7	Filters are rinsed three (3) times in 1X SSC (10 ml/filter) for 10 minutes at room temperature with shaking at 50-125 rpm. Filters may be air dried and stored at this point.
		3.4 Alkaline Phosphatase Probe Hybridization: Hybridization		
C	**12, 15**		**3.4.1**	**For total *V. parahaemolyticus* (*tlh*), the 5'AP-labeled probe 5'aa agc gga tta tgc aga agc act g 3' is used.** **For pathogenic *V. parahaemolyticus* (*tdh*), the 5'AP-labeled probe 5'gg ttc tat tcc aag taa aat gta ttt g 3' is used.** **For *V. vulnificus* (*vvhA*), the 5'AP-labelled probe 5'ga gct gtc acg gca gtt gga acc a 3' is used.**
C	**12, 15**		**3.4.2**	**Probes are stored in the refrigerator and are not frozen.**
K	12, 15		3.4.3	A maximum of five (5) filters to be hybridized with the same probe are added to a plastic bag.

C	**12, 15**		**3.4.4**	**Filters are presoaked in 10-15 ml of hybridization buffer for 30 minutes at 54-± 0.1 ºC for *V. parahaemolyticus* (*tlh* and *tdh*) or 55 ± 0.1 °C for *V. vulnificus* with shaking.**
C	**12, 15**		**3.4.5**	**Used buffer is discarded and 10 ml of fresh pre-warmed buffer per bag is added. Probe (final concentration of 0.5 pmol/ml) is quickly added to each bag and incubated for 1 hour at 54 ± 0.1 ºC for *Vibrio parahaemolyticus* or 55 ± 0.1 ºC for *Vibrio vulnificus* with shaking.**
K	15		3.4.6	Filters are removed from the bag(s) and transferred to an appropriate vessel or container. Up to 30 filters hybridized with the same probe can be combined.
C	**12, 15**		**3.4.7**	**Filters are rinsed two (2) times for 10 minutes each in 1X SSC – 1% SDS (for tlh and *Vibrio vulnificus*) or 3X SSC – 1% SDS (for tdh) (10 ml/filter) at 54 ± 0.1 ºC for *Vibrio parahaemolyticus* or 55 ± 0.1 ºC for *Vibrio vulnificus* with shaking.**
K	12, 15		3.4.8	Filters are rinsed five (5) times for five (5) minutes each in 1X SSC (10 ml/filter) at room temperature with shaking.
		3.5 Alkaline Phosphatase Probe Hybridization: Color development		
C	**12, 15**		**3.5.1**	**In a petri dish containing 20 ml of NBT/BCIP solution, filters (5 or fewer) are added and incubated with gentle shaking at room temperature, or at 35 ºC for faster results. The petri dish is kept covered to omit light.**
K	12, 15		3.5.2	Color development of the positive control is checked every 30 minutes. Reaction time varies.
K	12, 15		3.5.3	Filters are rinsed in tap or deionized/distilled water (10 ml/filter) three (3) times for 10 minutes each to stop color development.
C	**12, 15**		**3.5.4**	**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.**
		3.6 Alkaline Phosphatase Probe Hybridization: Computation of Results		
C	**12, 15**		**3.6.1**	**For direct plating, probe-positive colonies are counted and multiplied by the plated dilution factor of the sample to determine the concentration. Note that filter colonies must correspond to colonies visible on the agar plate.**
K	15		3.6.2	For direct plating, results are reported as CFU/g of sample.
C	**12**		**3.6.3**	**For APW enrichment, upon identification of probe-positive colonies refer to the original positive APW dilutions and record MPN value as derived in Appendix 2 of the FDA Bacteriological Analytical Manual (BAM).**
K	12, 16		3.6.4	For APW enrichments, results are reported as MPN/g of sample or pass/fail in the case of PHP samples.

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LABORATORY:	DATE OF EVALUATION:

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
ALKALINE PHOSPHATASE PROBE 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 (C), Key (K), and Other (O) Nonconformities in Parts I-III	 _____ _____ _____

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

1. **Does Not Conform Status**: The Alkaline Phosphatase Probe 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 Alkaline Phosphatase Probe Microbiological component of this laboratory is determined to be provisionally conforming to NSSP requirements if the number of critical nonconformities is **≥ 1 but ≤ 3**.

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:_____

LEO Signature: _____ Date:_____

NSSP Form *9 – Alkaline Phosphatase Probe Checklist*, Rev June 2024