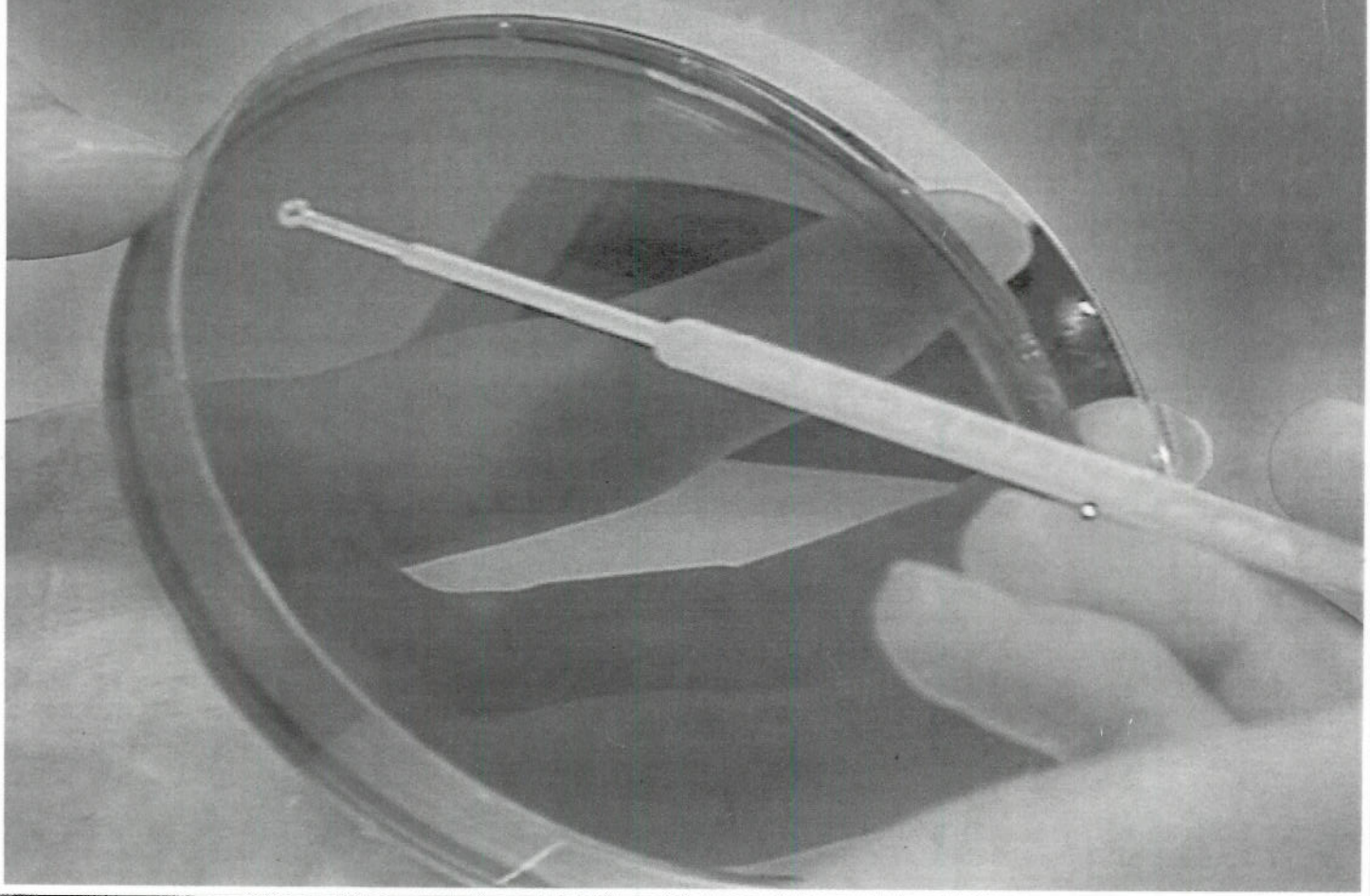
Direct Plating Procedure for the Enumeration of Total and Pathogenic *Vibrio parahaemolyticus* in Oyster Meats



DIRECT PLATING PROCEDURE FOR THE ENUMERATION OF TOTAL AND PATHOGENIC *Vibrio parahaemolyticus* IN OYSTER MEATS

Protocol developed by
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INTRODUCTION

At the 1999 Interstate Shellfish Sanitation Conference, Issue 98-107 was adopted as an Interim Guidance Document to assist states in taking a proactive approach to *Vibrio parahaemolyticus* in oysters. Methodology to support this issue is based on direct plating of homogenized oyster tissue onto a nutrient medium and performing colony lifts to transfer the colonies to a filter that can be tested by DNA gene probes to detect total (*tlh* gene) and pathogenic (*tdh* gene) *V. parahaemolyticus*. The methods described herein are designed to enumerate *V. parahaemolyticus* in oysters at harvest. However, in the hands of a trained analyst, these procedures may be applicable for research on a variety of seafoods.

The following analytical procedures are based on a combination of procedures from the following sources:

1. Direct plating procedure:

   DePaola, A. et al. 1997. Evaluation of an alkaline phosphatase-labeled DNA probe of enumeration of *Vibrio vulnificus* in Gulf Coast oysters. Journal of Microbiological Methods 29:115-120.

2. Identification of total *V. parahaemolyticus* by DNA probe:

   McCarthy, SA., et al. 1999. Evaluation of alkaline phosphatase-and digoxigenin-labelled probes for the detection of thermolabile hemolysin *(tlh)* gene of *V. parahaemolyticus*. Letters in Applied Microbiology, 28:66-70

3. Detection of pathogenic *V. parahaemolyticus*:

   McCarthy, S.A. et al. 1999. Comparison of PCR and DNA hybridization methods for detection of the *tdh* gene in *V. parahaemolyticus*. Abstracts of the 99th General Meeting of the American Society for Microbiology, p. 512.

A) EQUIPMENT AND MATERIALS

1. Autoclave for sterilizing media
2. Incubator, 35°C ±1°C
3. Balance, Top loading, 0.01 g sensitivity
4. Water bath, shaking, heating to 42°C and 54°C±0.1°C
5. Orbital shaker, small, for use at room temperature
6. Pipetter, single channel, variable volume, 2-20 µl
7. Pipetter, single channel, variable volume, 20-200 µl
8. Tips for pipetter
9. Microwave oven, 1000 watt
10. Blender and blender cups
11. Brushes for scrubbing shellfish
12. Shucking knives
13. Blender and sterile blender jars
14. Sterile culture dishes, 100x15 mm, glass or plastic
15. Tweezers for handling filters
16. Sterile, bent glass or plastic spreader rods
17. Whatman #541 filters, 85 mm (filters this size may be hand cut from larger diameter filters or ordered as a special cut from the manufacturer)
18. Fine tip waterproof marker (black)
19. Culture dish lids, glass, 100x15mm

20. "Washing" container, wide mouth jar, plastic with straight side, 500 ml, screw cap (Nalgene 2117-0500)
21. Plastic Whirl-Pak± bags (4.5"x9") (NASCO B00736WA)
22. Culture dishes, glass, 100x20 mm
23. Control cultures: *tdh+ V. parahaemolyticus*
    tdh- V. parahaemolyticus
    Vibrio vulnificus

B) MEDIA AND REAGENTS

1. Alkaline Peptone Water (APW) or
2. Phosphate Buffered Saline (PBS)
3. T1N3 Agar Plates
4. TCBS Agar Plates
5. T1N1 Agar Slants
6. Lysis Solution (0.5M NaOH 1.5M NaCl)
7. Ammonium acetate buffer (2M)
8. Standard Saline Citrate (SSC) Solution (20x)
9. 5x SSC
10. 3x SSC
11. 1x SSC
12. Stock Proteinase K (ProK) Solution
13. Hybridization Buffer
14. 1xSSC/SDS solution
15. 3xSSC/SDS
16. NBT/BCIP solution
17. Alkaline phosphatase labeled *tlh* gene probe
18. Alkaline phosphatase labeled *tdh* gene probe

C) COLLECTION OF SHELLFISH FOR *VIBRIO PARAHAEMOLYTICUS* ANALYSIS

1. Plan collection of shellfish so that analysis will be initiated in the same day as harvest. If this cannot be done, shellfish must be held at a temperature of less than 10°C, but not frozen, and analysis initiated within 36 hrs of harvest.

2. Shellfish may be harvested by dredging or tonging. Immediately after harvest, shellfish should be culled and rinsed to remove excess mud. For each sample, place 13 to 15 shellfish in a plastic bag, label bag, and place oysters into an insulated chest that has a false bottom and drain to prevent melt water from accumulating and possibly contaminating shellfish. Cover bag of shellfish with sheet of bubble wrap and place a bag containing about 5 pounds of crushed wet ice on top of bubble wrap. Keep the shellfish in the chest until delivered to the analytical laboratory.

D) SAMPLE PREPARATION AND CULTURE

Note: **T1N3 plates** to be used in this section must be dried in an inverted position with lids partly open in a 35°C incubator for 30 to 60 min. before use. This will permit the sample to be completely absorbed into the medium and prevent colonies spreading.

1. Verify that the temperature of the shellfish when received at the laboratory is < 10°C by checking the internal temperature of one or more animals. Using a knife, pop the hinge of the animal and insert a temperature probe into the meat. If temperature is > 10°C do not initiate analysis of sample. Investigate reason for high temperature and take corrective action. [Note: If shellfish have been harvested within 3 h. of examination, sufficient cooling time may not have elapsed and shellfish may be > 10°C. Consider such shellfish as acceptable for use.]

2. Wash shellfish with a stiff brush under cold running tap water to remove mud and debris. Place shellfish on absorbent paper to drain.

   Note: The remaining steps in this section must be carried out without delay.

3. Shuck 10 to 14 shellfish (200 to 250 g meat and shell liquor) into a sterile blender jar. Add an equal weight of sterile **phosphate buffered saline (PBS)** or **alkaline peptone water (APW).**

4. Blend for 90 to 120 sec on high speed. This produces a 1:1 shellfish:diluent homogenate. (Figure 1)

5. Prepare a 1:10 shellfish:diluent homogenate by weight (20 g homogenate and 80 g **PBS** or **APW**).

6. Using a balance having a sensitivity of 0.01 g, weigh 0.2±0.01 g of 1:1 shellfish:diluent homogenate onto the surface of each of four (4) dried **T1N3 plates.** Immediately spread the sample over the surface of the plate with a sterile spreading rod and continue spreading each plate until all liquid is absorbed. All 4 plates may be spread with the same rod. Label these plates as inoculated with 0.1 g shellfish.

Colonies developing on one of these plates will be probed for the *tlh* gene. Two plates will be replicate plates to be probed for the *tdh* gene. The forth plate is for archiving.

7. Place 100 µl of the 1:10 shellfish:diluent homogenate onto the surface of two dried **T1N3 plates**. Immediately spread the sample over the surface of each plate with a sterile spreading rod. Label this plate as inoculated with 0.01 g shellfish.

8. Incubate all **T1N3 plates** at 35°C overnight (16 to18 hr).

E) PREPARATION OF COLONY LIFTS

Note: One of the filters inoculated with the 0.1 g shellfish homogenate and one filter with the 0.01 gram shellfish homogenate will be run using the *tlh* gene probe to determine total *V. parahaemolyticus* counts. Two of the filters (replicates) inoculated with 0.1 g shellfish homogenate will be run using the *tdh* gene probe to determine the pathogenic *V. parahaemolyticus* count. The two remaining filters should be archived and developed later if needed.

1. Mark the sample number, sample volume and test (*tlh* or *tdh*) on the edge of an 85 mm Whatman #541 filter paper disk with a fine point permanent marker. Place the filter, labeled side down, on the surface of the **T1N3 plate** with colonies. Use a spreading rod to press the filter directly against the agar surface to insure colonies are transferred. Filters may be lifted as soon as filter is wet or may remain on the plate for up to 30 min.

2. Place 1 ml of **lysis solution** in the center of an inverted 100x15 mm glass petri dish lid (one for each filter to be lysed). Remove the #541 filter with colonies from the **T1N3** plate and place colony side up over the lysis solution. Position filter to exclude all air bubbles between the filter and the glass. The process is intended to wet the entire filter with the lysis solution. Do not let the lysis solution run over the surface of the filter.

   Note: See **section H** procedure for handling of **T1N3** plates for recovery of ***tdh***+ cultures.

3. Place glass petri dishes (maximum of 6) with filters in microwave and heat on full power (1000 watts or less) for 30 sec/filter. Filters should be completely dry, but not brown. If needed, heating time may be extended.

4. Into a clean plastic "washing" container, place 4 ml of **ammonium acetate buffer** for each filter to be neutralized. Add the filters one at a time insuring that each filter is saturated before adding the next. Let filters remain in buffer for 5 min. at room temperature with swirling on the orbital shaker.

5. Decant the **ammonium acetate buffer** from the "washing" container. Add 10 ml of **lx SSC** solution per filter. Swirl container for 1 to 2 minutes. Decant the liquid and rinse a second time by adding 10 ml of **1x SSC** per filter to the container and swirling for 1 to 2 minutes. Decant solution.

6. Continue with probing of filters or place filters, colony side up, onto absorbent paper and allow to air dry at room temperature. Once dry, filters can be stored indefinitely in plastic bags until ready to probe.

F) PROBING THE COLONY LIFTS

(Use Attachment 1, Quick Reference and Checklist, to insure that all steps are completed.)

Note: Filters being probed for *tdh* and *tlh* genes can be combined in steps 1, 2, 6, 7, and 8. However, care must be taken to insure that the gene probes are not mixed and that the filters are processed with the correct probe.

1. Into the "washing" container, add 10 ml of **lxSSC** and 20 **µl stock ProK** for each filter to be treated. Warm to 42°C and add filters one at a time to insure that each is saturated with the solution before the next is added. Incubate with shaking (~50 spm) in a water bath at 42°C for 30 min.

2. Rinse filters 3 times in **lxSSC** (10 ml/filter) for 10 min at room temperature in the "washing" container with shaking on the orbital shaker at ~125 rpm. Filters can be dried, as above, at this point and stored indefinitely or you can proceed with hybridization.

Note: In steps 3 through 5 it is critical that the temperature be maintained at a constant 54°C. Check the temperature of your water bath with a certified thermometer to insure correct temperature.

3. Place 1 to 5 filters marked *tlh* in a plastic (Whirl Pack 4.5"x9") bag marked to receive the *tlh* gene probe and filters marked *tdh* in bag marked to receive the *tdh* gene probe. Add a control strip to each bag (see section G). Add 10 ml of **hybridizing buffer** to each bag. Weight corner of bags so that they remain completely submerged, but are free to move with shaking. Soak filters for 30 min. at 54°C with shaking (~50 spm).

4. Pour buffer from the bag and add 10 ml of fresh pre-warmed **hybridizing buffer**. Hold the bag so that buffer pools on one side. Add 5 picamoles of **probe** and mix into buffer by massaging the bag gently. Close the bag while excluding air bubbles. Weight the bags so that they remain completely submerged but are free to move with shaking. Incubate 1 hr in the water bath at 54°C with shaking (~50 spm).

5. Remove filters from bags, placing those probed with *tlh* and t*dh* into separate "washing" containers.
   a. Rinse *tlh* filters 2 times with **1xSSC/SDS** (10 ml filter) for 10 min in bath at 54°C with shaking.
   b. Rinse *tdh* filters 2 times with **3xSSC/SDS** (10 ml filter) for 10 min in bath at 54°C with shaking.

6. Filters can now be combined into one "washing" container. Rinse 5 times for 5 min. in **1x SSC** (10ml/filter) at room temperature with shaking on the orbital shaker.

7. Into a glass petri dish (100x20 mm), add 20ml of **NBT/BCIP solution** and add up to 5 filters. Incubate with shaking at room temperature or at 35°C for faster results. Cover to omit light during incubation. Check development of positive control on the control strip every half hour. Full development is usually complete in 1to 2 hours.

8. Stop the development reaction when control filters are developed by placing filters into a "washing" container with distilled or deionized water (10 ml/filter). Rinse 3 times for 10 min. each time.

9. Place filters on absorbent paper in the dark to dry. Count and record the number of colony blots that develop a bluish-gray or dark brown color. Colony blots that are colorless, yellow, gray or light brown are negative. Filters should be stored in the dark to prevent color change. Filters may be photocopied or scanned into a computer to produce a permanent record.

10. Express results in colony forming units (CFU) per gram of oysters in sample. Each colony on a 0.1 g filter represents 10 CFU/g and each colony on a 0.01 g filters represents 100 CFU/g

G) CONTROL STRIPS

Control strips are prepared using *V. vulnificus*, a *tdh*- strain of *V. parahaemolyticus*, and a *tdh*+ strain of *V. parahaemolyticus*. These strains are spot inoculated in multiple lines on a T1N3 plate and incubated overnight. Colony lifts are made from the plate and the filters are lysed. The filters are cut into strips so that each strip contains all of the three controls. Control strips can be mass-produced, dried and stored for later use. A control strip should be used in each bag of filters being probed. The expected reactions from the controls are as follows.

Culture	*tlh* Probe	*tdh* Probe
V. vulnificus	-	-
tdh- V. parahaemolyticus	+	-
tdh+ V. parahaemolyticus	+	+

H) PROCEDURE FOR ISOLATING *TDH*+ STRAINS FROM T1N3 PLATES INOCULATED DIRECTLY WITH OYSTER HOMOGENATES

The *V. parahaemolyticus* monitoring program uses a direct plating procedure for enumerating total and pathogenic (*tdh*+) *V. parahaemolyticus* in oyster homogenates. Below is a procedure for recovering viable cultures of *tdh*+ *V. parahaemolyticus* in the event of detecting colonies on the Whatman 541 colony lifts that appear to hybridize with the *tdh* probe.

1. In **step E.1**, prior to overlaying plates (T1N3 plates with overnight growth), the 541 Whatman filters designated for use with the *tdh* probe should be marked with a short line near the label on the filter and one on the opposite side of the filter in permanent ink.

2. When the filter is placed on the agar surface for the colony lift, make marks with permanent ink on the petri dish bottom that line up with those on the filter.

3. After the colonies are lifted, leave plates at room temperature for 4 to 6 h to allow partial regrowth of colonies and then store plates in a sealed plastic bag in a refrigerator.

   Note: Attempts to recover colonies should be made as soon as the filters are probed and *tdh*+ colonies are detected, but plates should not be held under refrigeration for more than two weeks.

4. If filters show *tdh*+ colonies, use the filters to locate the appropriate colony (viable cells) on the corresponding plates. The colonies will probably be obscured and possibly mixed with adjacent colonies as a result of the colony lift procedure but viable cells of the target colony should be present in the general area of the colony's original location on the plate.

5. Place the filter, colony side up on a tabletop or preferably a light box. Remove the top from the corresponding plate and place the plate in the inverted position directly over the filter and align the lines on the plate over the lines on the filter.

6. Make a circle (approx. 0.5 cm diameter) on the plate bottom directly over each *tdh* positive spot on the filter. The position of the circles can be confirmed by placing the filter on top of the inverted plate and holding it up to the light to see if the *tdh* positive spot lies within the circle.

7. Touch an inoculating loop or needle to the agar surface within the circled area and streak one or more **TCBS** plates for colony isolation. Incubate overnight at 35°C. From each **TCBS** plate, transfer 5 to 10 typical (green) colonies into APW in separate wells of a 96-well plate. Incubate at 35°C until growth is detected and use a 48- prong replicator to inoculate cultures onto two T1N3 plates. After overnight incubation, perform colony lifts and probe one filter with *tlh* probe and the other with the *tdh* probe.

8. If any wells produce growth that hybridizes with both *tlh* and *tdh* probes, streak APW onto T1N3 to check for purity. Send purified culture on T1N1 slants to GCSL for confirmation and further characterization.

MEDIA AND REAGENTS

(M & R numbers correspond to BAM numbers.)

Note: Distilled or deionized water (d. water) may be used for all media and reagents.

Phosphate Buffered Saline (PBS) [R 59]

NaCl	7.650 g
Na_2HPO_4, anhydrous	0.724 g
KH_2PO_4	0.210 g
d. water	1000 ml

Dissolve ingredients in d. water. Adjust pH to 7.4 (with 1 N NaOH). Autoclave 15 min at 121°C.

Store at room temperature. Discard after 90 days.

Alkaline Peptone Water (APW) [M9]

Peptone	10 g
NaCl	10 g
d. water	1000 ml

Dissolve ingredients. Adjust pH with NaOH so that value after sterilization is 8.5+0.2. Dispense in 100 ml amounts in media bottles and autoclave 15 min at 121C. Store under refrigeration. Discard after 90 days.

T1N3 Agar

Tryptone	10 g
NaCl	30 g
Agar	20 g
d. water	1000 ml

Adjust pH to 7.2 before heating. Autoclave, cool and pour into plates. Immediately after plates solidify, package in plastic bags and store under refrigeration. Discard after 90 days.

Lysis solution (0.5M NaOH 1.5M NaCl)

NaOH	20.0 g
NaCl	87.0 g
d. water	1000 ml

Store room temperature. Discard after 90 days.

Ammonium acetate buffer (2M)

Ammonium acetate	154 g
d. water	1000 ml

Store room temperature. Discard after 90 days.

Standard Saline Citrate (SSC) Solution [R77]

20xSSC

NaCl	175.4 g
Sodium Citrate. $2H_20$	88.2 g
d. water	1000 ml

Dissolve in 800 ml d. water and adjust to pH 7 with 10 N NaOH. Bring to volume of 1000 ml. Store at room temperature. Discard after 90 days.

5x SSC

20X SSC	25 ml
d. water	75 ml

3x SSC

20X SSC	150 ml
d. water	850 ml

1xSSC

20X SSC	50 ml
d. water	950 ml

Dilute prior to use. Discard lx SSC after 1 day.

Stock Proteinase K (ProK) solution

Add 5ml distilled water to 100 mg bottle of proteinase K (P 6556, Sigma Chemical). The stock ProK solution will contain 20mg/ml. Divide into 200 µl amounts and store frozen at -20C.

Hybridization Buffer

Bovine Serum Albumin (BSA) (Fraction V Powder)	0.5 g
Sodium Dodecyl sulfate (SDS) (Sodium Lauryl Sulfate)	1.0 g
Polyvinylpyrrolidone (PVP-360)	0.5 g
5x SSC	100.0 ml

Store at 4°C for no longer then 1 week. Warm to 54°C before use.

1XSSC/SDS solution

Sodium Dodecyl Sulfate (SDS)	10.0 g
1xSSC	1.0 liter

Store at room temperature. Discard after 90 days.

3SSC/SDS solution

Sodium Dodecyl Sulfate (SDS)	10.0 g
3xSSC	1.0 liter

Store at room temperature. Discard after 90 days.

NBT/BCIP solution

Just before use, dissolve 2 NBT/BCIP ready-to-use tablets (Boehringer Mannheim, Cat. No. 1697471) in 20 ml of d. water in a glass petri dish. Discard after one use.

TLH-L AP-probe

Probe sequence is 5' XAA AGC GGA TTA TGC AGA AGC ACT G 3' where X = alkaline phosphatase conjugated 5' Amine-C6. Probe available from DNA Technology A/S, Forskerparkern/Science Park Aarhs, Gustav Wieds Vej 10 A, DK-8000 Aarhuys C. Denmark; Phone +45 87 32 30 00; Fax +45 87 32 30 11; E-mail Oligo@DNA-technology.dk. Probes vary in strength and a volume equal to 5 picomoles must be calculated for each batch. Store probes under refrigeration. DO NOT FREEZE. Shelf life is unknown, but exceeds one year.

TDH-M2 AP- probe

Probe sequence is 5' XGG TTC TAT TCC AAG TAA AAT GTA TTT G 3' where X = alkaline phosphatase conjugated 5' Amine-C6. Probe available from DNA Technology A/S, Forskerparkern/Science Park Aarhs, Gustav Wieds Vej 10 A, DK-8000 Aarhuys C. Denmark; Phone +45 87 32 30 00; Fax +45 87 32 30 11; E-mail Oligo@DNA-technology.dk. Probes vary in strength and a volume equal to 5 picomoles must be calculated for each batch. Store probes under refrigeration. DO NOT FREEZE. Shelf life is unknown, but exceeds one year.

Calculation of probe amount

Data sheets provided with each batch of probe will show the concentration of the probe in nanomoles (nmol) and the total volume of probe shipped. Enter those values into the following equation:

(µl probe equal to 5 picomoles) = (volume in µl x 5) ÷ (concentration in nmol x 1000)

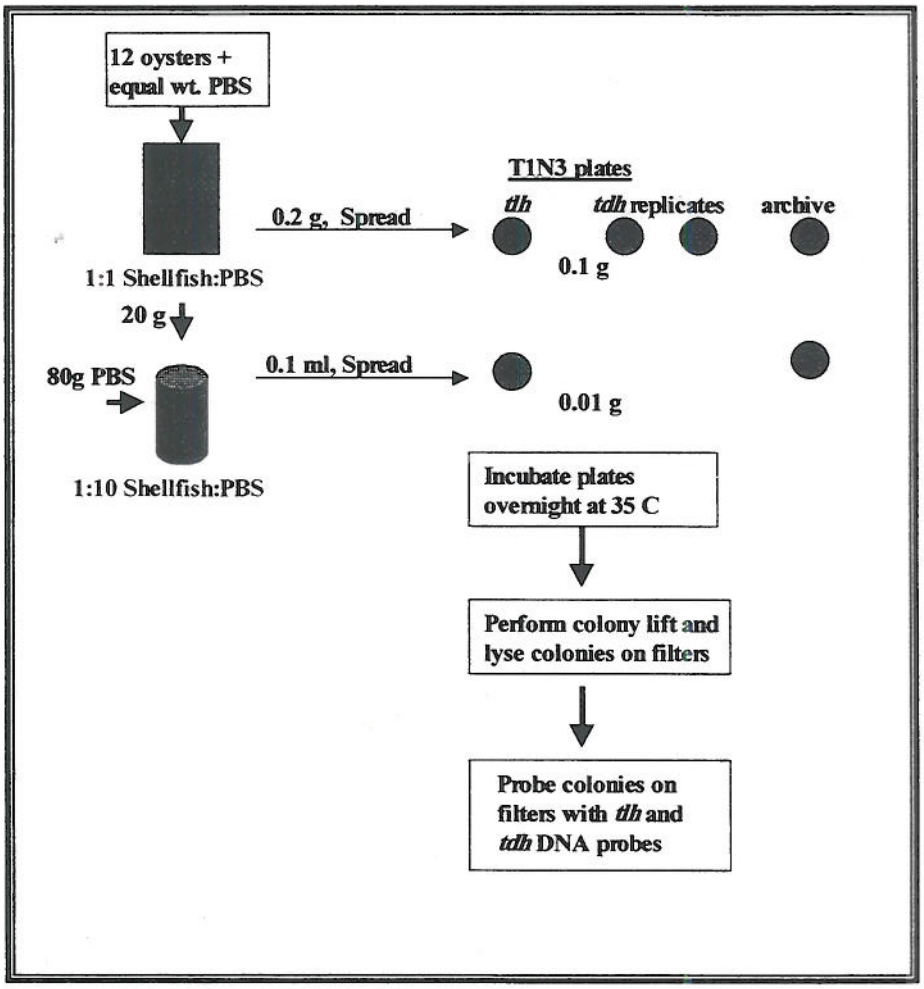
Attachment 1:

Quick Reference and Checklist for AP-DNA Probe Development

Steps in Developing AP-DNA Probe	Temp	Time	Date				
Pro K treatment (10 ml 1xSSC & 20 µl stock Pro K per filter)	42C	30 min					
Rinse 1x SSC buffer (10 ml/filter)	RT	10 min					
Rinse 1x SSC buffer (10 ml/filter)	RT	10 min					
Rinse 1x SSC buffer (10 ml/filter)	RT	10 min					
Hybridizing buffer (10 ml/bag)	54 C	30 min					
Hybridizing buffer (10 ml/bag) & Probe	54 C	60 min					
FOR tlh PROBE							
Rinse w 1xSSC/SDS (10 ml/filter)	54 C	10 min					
Rinse w 1xSSC/SDS (10 ml/filter)	54 C	10 min					
FOR tdh PROBE							
Rinse w 3xSSC/SDS (10 ml/filter)	54 C	10 min					
Rinse w 3xSSC/SDS (10 ml/filter)	54 C	10 min					
Rinse 1x SSC Buffer (10 ml/filter)	RT	5 min					
Rinse 1x SSC Buffer (10 ml/filter)	RT	5 min					
Rinse 1x SSC Buffer (10 ml/filter)	RT	5 min					
Rinse 1x SSC Buffer (10 ml/filter)	RT	5 min					
Rinse 1x SSC Buffer (10 ml/filter)	RT	5 min					
NBT/BCIP (20 ml/5 filters)	RT or 35C	60 to 120 min					
Rinse d. water (10 ml/filter)	RT	10 min					
Rinse d. water (10 ml/filter)	RT	10 min					
Rinse d. water (10 ml/filter)	RT	10 min					
Dry filters							

RT = Room Temperature (~25°C)
Use X marks to indicate step has been completed.

Figure 1.


12 oysters + equal wt. PBS
1:1 Shellfish:PBS
20 g
80g PBS
1:10 Shellfish:PBS
0.2 g, Spread
0.1 ml, Spread
T1N3 plates
tlh
tdh replicates
archive
0.1 g
0.01 g
Incubate plates overnight at 35 C
Perform colony lift and lyse colonies on filters
Probe colonies on filters with tlh and tdh DNA probes

The Interstate Shellfish Sanitation Conference (ISSC) promotes the discussion and exchange of shellfish safety information. For more information on shellfish harvesting, please contact your local state shellfish control agency, the ISSC, or the U.S. Food and Drug Administration.

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