Validation Data for Direct plating method for trh

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Specific purpose or intent of the method for use in the NSSP: Seeking approval for this method as an approved limited use method that can be used as appropriate for PHP validation and verification testing, as well as environmental testing such as that which may be required for the re-opening of growing areas closed due to illness.

Validation Criteria Data: For evaluation of all validation criteria below, PHP oysters were obtained in the best effort to find samples free of the target organism. A different lot of PHP oysters was used for each sample. For each sample, a minimum of 10 animals were used to prepare a homogenate. The homogenate was then aliquoted and appropriate aliquots spiked with a *tdh+/trh+ Vibrio parahaemolyticus* (unless otherwise noted), while one aliquot was left unioculated (sample blank). Spike levels were determined by spread plating dilution of the culture in triplicate onto TSA+2% NaCl. Appropriate aliquots of spiked samples were spread plated onto T1N3 agar and colony lifts hybridized with an alkaline phosphatase-labeled probe specific for *trh*.

1. Accuracy/Trueness: Using the data from Table 1, the average of plate counts was 3.80 log and the average from DNA probe was 3.62 log. The *Accuracy/Trueness of the method is 95%.*

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

Sample	Plate Count (log CFU)	Probe Result (log CFU/g)
1-2X	5.18	4.76
2-2X	5.18	4.65
3-4X	3.15	2.90
4-4X	3.15	2.85
5-6X	1.23	1.48
6-6X	1.23	1.00
7-2X	5.76	5.59
8-2X	5.76	5.64
9-4X	3.68	3.59
10-4X	3.68	3.72

2. Measurement Uncertainty: Using the data from Table 1 above, *measurement uncertainty is 0.11*.

3. Precision: Using the data from Table 2, there *was no significant difference between the plate counts and the values generated with DNA probe* (p=0.58). The difference in variance is not significant (p=0.48) for any platform/gene target combination.

4. Recovery: The average of plate counts was 3.40 log the average (adjusted for sample blanks) from DNA probe was 3.65 log. Using this data, the *Recovery of the methods was determined to be 107% on both platforms for both gene targets.*

Table 2. Data for determination of Precision and Recovery			
Sample	**Aliquot**	**Plate Count (log CFU)**	**Probe Result (log CFU/g)**
1	**Blank**	N/A	<1.00
1	**2X**	5.18	4.76
1	**2Z**	5.18	5.38
1	**4X**	3.18	2.48
1	**4Z**	3.18	2.85
1	**6X**	1.18	<1.00
1	**6Z**	1.18	1.00
3	**Blank**	N/A	<1.00
3	**2X**	5.15	4.65
3	**2Z**	5.15	4.76
3	**4X**	3.15	2.90
3	**4Z**	3.15	3.04
3	**6X**	1.15	<1.00
3	**6Z**	1.15	1.78
5	**Blank**	N/A	1.85
5	**2X**	5.23	3.54
5	**2Z**	5.23	3.84
5	**4X**	3.23	2.70
5	**4Z**	3.23	3.00
5	**6X**	1.23	1.48
5	**6Z**	1.23	1.30
7	**Blank**	N/A	1.00
7	**2X**	5.76	5.59
7	**2Z**	5.76	5.23
7	**4X**	3.76	3.57
7	**4Z**	3.76	3.64
7	**6X**	1.76	1.78
7	**6Z**	1.76	1.7
9	**Blank**	N/A	<1.00
9	**2X**	5.68	5.25
9	**2Z**	5.68	5.11
9	**4X**	3.68	3.59
9	**4Z**	3.68	3.54
9	**6X**	1.68	2.94
9	**6Z**	1.68	1.48

5. Specificity: Samples were prepared as above and the interfering organism was spiked at an ~4 log higher concentration than *Vibrio parahaemolyticus*. Using the data from Table 3, the average *Specificity of the method is 1.38,* which is within the 95% confidence interval of the method (0.44) from 1.

Table 3. Data for determination of Specificity.	
	Probe Result

	(log CFU/g)	
Sample	**Spiked with Vp only**	**Spiked with Vp and Vv**
6-Blank	<1.00	---
6-6T	1.60	1.30
6-6U	1.30	<1.00
6-6W	1.48	1.48
6-6X	1.00	1.00
6-6Z	1.48	1.48

6. Working and Linear Range: Based on the data presented in Table 4, there is a significant correlation between the plate counts and CFU values by DNA probe ($p<0.001$). The *correlation coefficient is 0.96*, demonstrating the linearity of the method.

Table 4. Data for determination of Working and Linear Range, Limit of Detection, and Limit of Quantitation/Sensitivity

Sample	**Aliquot**	**Plate Count (log CFU)**	**Probe Result (log CFU/g)**
1	**1X**	6.18	5.36
1	**1Z**	6.18	6.18
1	**2X**	5.18	4.76
1	**2Z**	5.18	5.38
1	**4X**	3.18	2.48
1	**4Z**	3.18	2.85
1	**6X**	1.18	<1.00
1	**6Z**	1.18	1.00
1	**7X**	0.18	<1.00
1	**7X**	0.18	<1.00
3	**1X**	6.15	6.29
3	**1Z**	6.15	6.09
3	**2X**	5.15	4.65
3	**2Z**	5.15	4.76
3	**4X**	3.15	2.90
3	**4Z**	3.15	3.04
3	**6X**	1.15	<1.00
3	**6Z**	1.15	1.78
3	**7X**	0.15	1.00
3	**7Z**	0.15	<1.00
5	**1X**	6.23	5.57
5	**1Z**	6.23	5.64
5	**2X**	5.23	3.54
5	**2Z**	5.23	3.84
5	**4X**	3.23	2.70
5	**4Z**	3.23	3.00
5	**6X**	1.23	1.48

5	**6Z**	1.23	1.30
5	**7X**	0.23	1.30
5	**7Z**	0.23	1.48
7	**1X**	6.76	6.68
7	**1Z**	6.76	6.37
7	**2X**	5.76	5.59
7	**2Z**	5.76	5.23
7	**4X**	3.76	3.57
7	**4Z**	3.76	3.64
7	**6X**	1.76	1.78
7	**6Z**	1.76	1.70
7	**7X**	0.76	1.00
7	**7Z**	0.76	<1.00
9	**1X**	6.68	6.44
9	**1Z**	6.68	4.70
9	**2X**	5.68	5.25
9	**2Z**	5.68	5.11
9	**4X**	3.68	3.59
9	**4Z**	3.68	3.54
9	**6X**	1.68	2.94
9	**6Z**	1.68	1.48
9	**7X**	0.68	<1.00
9	**7Z**	0.68	<1.00

7. Limit of Detection: The *Limit of Detection of the method is 10 CFU/g*. This is reliant upon the amount of sample (0.1g) that can be tested by the spread plate method.

8. Limit of Quantification/ Sensitivity: The limit of quantification/sensitivity is also reliant upon the amount of sample that can be tested.

9. Ruggednes: Replicate spiked aliquots from each sample were processed with different batches of media/ lots of reagents at the same time. Different samples were processed on different days. Using the data in Table 5, there was *no significant difference (p=0.94) between batches/lots* of media and reagents.

Table 5. Data for determination of Ruggedness.

Sample	**Probe Result (log CFU/g)**	
	Replicate 1 (X)	**Replicate 2 (Z)**
2	4.78	4.88
4	2.85	2.78

6	1.00	1.48
8	5.64	5.73
10	3.72	3.57

10. ***Matrix Effects:*** Effects of oyster matrix on the performance of the method was taken into consideration in testing all of the above criteria by using the sample blank.

11. Additional Data: *Inclusivity/Exclusivity*. Control filters with the isolates listed below were prepared and tested as outlined above. All isolates. All isolates gave the expected reaction, demonstrating 100% *Inclusivity/Exclusivity*.

Species	**Number of Strains Tested**	**Number *trh*-positive**
V. parahaemolyticus[†]	43	43
*V. parahaemolyticus**	39	0
V. cholerae	25	0
V. vulnificus	13	0
V. metschnikovii	12	0
V. fluvialis	6	0
V. hollisae	5	0
V. algenolyticus	2	0
Salmonella spp.	20	0
Listeria spp.	20	0
Other non-*Vibrio* species	15	0

[†] *V. parahaemolyticus* strains previously determined to be *trh*-positive.

***** *V. parahaemolyticus* strains previously determined to be *trh*-negative.

Step-by-step procedure including equipment, reagents and safety requirements necessary to run the method:

1. Special Equipment, Media, and Reagents
   1.1. Special Equipment and Materials Required
      1.1.1.Shaking water bath(s) (42°C and 54°C)
      1.1.2.Orbital shaker

1.1.3. Microwave
1.1.4. Plastic tubs with lids (300-500 ml capacity)
1.1.5. Whatman 541 filters, 85mm
1.1.6. Sterile spread rods
1.1.7. Sterile inoculating loops
1.1.8. Sterile toothpicks
1.1.9. Whirl-Pak bags (4.5”x9”)

1.2. Media and Reagents
1.2.1. Alkaline peptone water (APW)
1.2.2. Phosphate buffered saline (PBS)
1.2.3. Thiosulfate citrate bile salts sucrose (TCBS) agar
1.2.4. T_1N_3 agar
1.2.5. Lysis solution
1.2.6. 2M ammonium acetate
1.2.7. 20X SSC and 1X SSC
1.2.8. 1X SSC/SDS
1.2.9. Proteinase K
1.2.10. Hybridization solution
1.2.11. NBT/BCIP tablets
1.2.12. AP-labeled DNA probes (DNA Technology)

2. Outlined Procedure
2.1. Preparation of shellfish
2.1.1. Hands of examiner must be scrubbed thoroughly with soap and potable water; latex or nitrile gloves should be worn while cleaning oysters.
2.1.2. Scrape off growth and loose material from shell, and scrub shell stock with sterile stiff brush under running water.
2.1.3. Place clean shellstock on clean towels or absorbent paper.
2.1.4. Change gloves and brushes between samples.
2.1.5. Protective chain mail glove can be used under a latex glove; outer gloves should be changed between samples.
2.1.6. Tare a sterile blender.
2.1.7. Using a sterile oyster knife, insert the point between the shells on the ventral side, about ¼ the distance from the hinge to the bill; alternately, knife can be inserted after making small opening with sterile bone cutting forceps.
2.1.8. Cut adductor muscle from upper flat shell and pry the shell wide enough to drain shell liquor into the blender.
2.1.9. The upper shell can then be pried loose at hinge and discarded.
2.1.10. The whole animal (including adductor muscle) should be transferred to the sterile blender after severing the adductor muscle connection to the lower shell.
2.1.11. A minimum of 12 animals or 200g is required.
2.1.12. Blend without adding diluent for 60-120 sec at 14,000 rpm.

2.2. Preparation of spread plates
2.2.1. Prepare 10-fold serial dilutions of shellfish homogenate in PBS
2.2.2. Inoculate 100µl of appropriate dilutions onto pre-dried T_1N_3 agar plates
2.2.3. Spread inoculum gently into agar until completely absorbed
2.2.4. Invert plates and incubate at 30-37°C overnight.

2.2.5. Alternately, this method can be utilized with suspect isolates replicated to T_1N_3 agar from 96 well plates obtained from a standard MPN method

2.3. DNA Probe Colony Hybridization

2.4. Alkaline phosphatase-labeled oligonucleotide probes (AP-*tlh*, AP-*tdh*, and AP-*trh*) can be stored in the refrigerator (4°C) for 1-2 years; do not freeze.

2.5. Filter Preparation

2.5.1. Label #541 Whatman filters with sample number, date, analyst initials, and probe to be hybridized with (*tlh, tdh,* or *trh*). Make sure orientation of filter is noted so that positive spots can be correlated to the appropriate well in the microtiter plate. A dot near the A1 well is sufficient.

2.5.2. Place each filter label-side down on appropriate T_1N_3 plate; apply gentle pressure to ensure contact with each colony. Allow labeled filter to sit at RT for 1-30 min. Transfer each filter with colony-side up to a plastic or glass petri dish lid containing 1 ml of lysis solution.

2.5.3. Microwave filters in petri dishes (full power) for 15-20 sec/filter depending on wattage of microwave; rotate dishes with filters and repeat microwaving. Filters should be hot and almost completely dry but not brown.

2.5.4. Transfer filters to a plastic wash container (up to 30 filters can be combined in one container) and neutralize with ammonium acetate (4 ml/filter) for 5 min on shaker at RT.

2.5.5. Decant ammonium acetate and rinse filters 2 times with 1X SSC buffer (10 ml/filter), for 2 min each time. (Filters can be air dried and stored at this point.)

2.6. Proteinase K (proK) treatment

2.6.1. Prepare proK solution (this is made by adding 10 ml/filter of 1X SSC and 20 µl/filter of proK stock solution) for the appropriate number of filters. Place filters (up to 30) in plastic wash container of proK solution. Incubate for 30 min in a 42°C water bath with shaking (50 rpm) to destroy naturally occurring alkaline-phosphatase and digest bacterial protein.

2.6.2. Decant proK solution. Rinse filter 3 times in 1X SSC (10 ml/filter) for 10 min at RT with shaking at 50 rpm. (Filters can be air dried by placing on paper towels and stored when completely dry.)

2.7. Hybridization

2.7.1. Place up to 5 proK-treated filters (either dried or straight from treatment) in a Whirl-Pak bag. Add 10 ml of pre-warmed hybridization buffer and close bag to exclude air. Avoid trapping air bubbles. Incubate filters for 30 min at 54°C in a shaking (50 rpm) water bath.

2.7.2. Pour off buffer from bag and add 10 ml fresh pre-warmed buffer/bag. Add probe (final conc. is 0.5 pmol/ml) to bag with filters. Reseal bag, excluding air, and incubate 1 h in a 54°C water bath with shaking. The temperature is critical for hybridization and washing steps.

2.7.3. Remove filters from hybridization bags and place in plastic wash container(s).

2.7.4. Add 10 ml/filter 1X SSC/1% SDS . Incubate in a 54°C water bath with shaking for 10 min. Repeat wash a second time.

2.7.5. Rinse filter 5 times for 5 min each in 1X SSC at RT on an orbital shaker, 100 rpm.

2.8. Color development

2.8.1. In petri dish, add 20 ml of NBT/BCIP solution. Add filters (5 or fewer) to dish and incubate with gentle shaking at 35-37°C; cover to omit light. Check development of positive control every 30 min.

2.8.2. Transfer filters to a plastic wash container and add tap water (10 ml/filter). Rinse filters at RT with shaking for 10 min. Repeat rinse 2 additional times to stop color development. Do not expose filters to light as they will continue to develop. Consider purple or brown spots positive.