Proposal Subject:	Male-specific Coliphage Method for Quahogs (M. mercenaria)
Specific NSSP Guide Reference:	NSSP Guide Section IV Guidance Documents Chapter II Growing Areas .11 Approved Limited Use Methods for Microbiological Testing
Text of Proposal/ Requested Action	This submission presents the 'Male-specific Coliphage method for Quahogs (M. mercenaria)' for consideration as an approved limited use method for microbiological testing. At the 2009 ISSC, the 'Modified Double Agar Overlay Method for Determining Male-specific Coliphage in Soft-shelled Clams and American Oysters' was accepted as an approved limited use method for microbiological testing for re-opening growing areas after emergency closures due to sewage spills. SLV work with quahogs has demonstrated comparable performance characteristics as with soft-shelled clams and American oysters. The requested action is to include quahogs in the footnote for MSC along with soft-shelled clams and American oysters in NSSP Guide Section IV Guidance Documents Chapter II Growing Areas .11 Approved Limited Use Methods for Microbiological Testing.
Public Health Significance:	The MSC method for quahogs was used recently by the State of New Jersey to re-open growing areas after the devastating effects of Superstorm Sandy. Increasingly, enumeration of male-specific coliphage (MSC) in soft-shelled clams, American oysters, and quahogs is needed in the NSSP to assess *viral* contamination in molluscan shellfish harvested from growing areas where fecal coliform levels in both water quality and shellfish meats may be misleading. MSC is a specialized indicator of *viral* sewage contamination, which is substantially more meaningful than fecal coliform or E. coli in evaluating the safety of shellstock harvested from growing areas potentially impacted by treated and partially treated wastewater.
Cost Information (if available):	This method for the enumeration of male-specific coliphage in soft-shelled clams, American oysters, and quahogs is inexpensive, easy to perform, and rapid, providing results within 24 hours. The cost of laboratory glassware, plastic-ware, agars, and reagents is approximately $25 per shellfish sample. In a well-equipped laboratory, the method requires 6 hours of time from initiating host to pouring plates. Hands on technician time to perform this test is significantly less on the order of 1-4 hours per test depending upon how many tests are done per day. The most expensive piece of equipment is a refrigerated centrifuge plus rotor, which costs approximately $12,000. There are no special skill sets required beyond those required to operate a state-approved shellfish laboratory under the NSSP.
Action by 2013 Laboratory Methods Review and Quality Assurance Committee	Recommended adoption of this method for use in detecting MSC in hard clams and direct the Executive Office to amend the table at Section IV. Chapter 2 @ .11 to add Quahogs to footnote #1
Action by 2013 Task Force I	Recommended adoption of Laboratory Method Review and Quality Assurance Committee recommendation on Proposal 13-120.
Action by 2013 General Assembly	Adopted recommendation of Task Force I on Proposal 11-320.

Action by FDA Concurred with Conference action on Proposal 13-120.
May 5, 2014

Proposal for Task Force Consideration at the Interstate Shellfish Sanitation Conference 2013 Biennial Meeting	☒ **Growing Area** ☐ **Harvesting/Handling/Distribution** ☐ **Administrative**
Submitter:	Thomas Howell
Affiliation:	Spinney Creek Shellfish, Inc.
Address:	27 Howell Drive Eliot, ME, 03903
Phone: **Fax:** **Email:**	(207) 439-2719 (207) 439-7643 tlhowell@spinneycreek.com
Proposal Subject:	Male-specific Coliphage Method for Quahogs (M. mercenaria)
Specific NSSP Guide Reference:	NSSP Guide Section IV Guidance Documents Chapter II Growing Areas .11 Approved Limited Use Methods for Microbiological Testing
Text of Proposal/ Requested Action	This submission presents the 'Male-specific Coliphage method for Quahogs (M. mercenaria)' for consideration as an approved limited use method for microbiological testing. At the 2009 ISSC, the 'Modified Double Agar Overlay Method for Determining Male-specific Coliphage in Soft-shelled Clams and American Oysters' was accepted as an approved limited use method for microbiological testing for re-opening growing areas after emergency closures due to sewage spills. SLV work with quahogs has demonstrated comparable performance characteristics as with soft-shelled clams and American oysters. The requested action is to include quahogs in the footnote for MSC along with soft-shelled clams and American oysters in NSSP Guide Section IV Guidance Documents Chapter II Growing Areas .11 Approved Limited Use Methods for Microbiological Testing.
Public Health Significance:	The MSC method for quahogs was used recently by the State of New Jersey to re-open growing areas after the devastating effects of Superstorm Sandy. Increasingly, enumeration of male-specific coliphage (MSC) in soft-shelled clams, American oysters, and quahogs is needed in the NSSP to assess *viral* contamination in molluscan shellfish harvested from growing areas where fecal coliform levels in both water quality and shellfish meats may be misleading. MSC is a specialized indicator of *viral* sewage contamination, which is substantially more meaningful than fecal coliform or E. coli in evaluating the safety of shellstock harvested from growing areas potentially impacted by treated and partially treated wastewater.
Cost Information (if available):	This method for the enumeration of male-specific coliphage in soft-shelled clams, American oysters, and quahogs is inexpensive, easy to perform, and rapid, providing results within 24 hours. The cost of laboratory glassware, plastic-ware, agars, and reagents is approximately $25 per shellfish sample. In a well-equipped laboratory, the method requires 6 hours of time from initiating host to pouring plates. Hands on technician time to perform this test is significantly less on the order of 1-4 hours per test depending upon how many tests are done per day. The most expensive piece of equipment is a refrigerated centrifuge plus rotor, which costs approximately $12,000. There are no special skill sets required beyond those required to operate a state-approved shellfish laboratory under the NSSP.

Method Application and Single Lab Validation Checklist For Acceptance of a Method for Use in the NSSP

The purpose of single laboratory validation in the National Shellfish Sanitation Program (NSSP) is to ensure that the analytical method under consideration for adoption by the NSSP is fit for its intended use in the Program. A Checklist has been developed which explores and articulates the need for the method in the NSSP; provides an itemized list of method documentation requirements; and sets forth the performance characteristics to be tested as part of the overall process of single laboratory validation. For ease in application, the performance characteristics listed under validation criteria on the Checklist have been defined and accompany the Checklist as part of the process of single laboratory validation. Further a generic protocol has been developed that provides the basic framework for integrating the requirements for the single laboratory validation of all analytical methods intended for adoption by the NSSP. Methods submitted to the ISSC LMR Committee for acceptance will require at a minimum 6 months for review from the date of submission.

Name of the New Method	Male-specific Coliphage for Quahogs (M. Mercenaria)
Name of the Method Developer	Thomas Howell, Spinney Creek Shellfish, Inc.
Developer Contact Information	Spinney Creek Shellfish, Inc. 27 Howel Drive Eliot, ME 03903 (207) 439-2719 tlhowell@spinneycreek.com

Checklist	Y/N	Submitter Comments
A. Need for the New Method		
Clearly define the need for which the method has been developed.	Y	
What is the intended purpose of the method?	Y	
Is there an acknowledged need for this method in the NSSP?	Y	
What type of method? i.e. chemical, molecular, culture, etc.	Y	Culture method for Male-specific Coliphage in Quahogs (M. Mercenaria)

B. Method Documentation		
1. Method documentation includes the following information:		
Method Title	Y	
Method Scope	Y	
References	Y	
Principle	Y	
Any proprietary aspects	N	
Equipment required	Y	
Reagents required	Y	
Sample collection, preservation and storage requirements	Y	

Safety requirements	Y	
Clear and easy to follow step-by-step procedure	Y	
Quality control steps specific for this method	Y	

C. Validation Criteria		
1. Accuracy / Trueness	Y	
2. Measurement uncertainty	Y	
3. Precision characteristics (repeatability)	Y	
4. Recovery	Y	
5. Specificity	NA	
6. Working and Linear ranges	Y	Working Range
7. Limit of detection	Y	
8. Limit of quantitation / Sensitivity	Y	
9. Ruggedness	Y	
10. Matrix effects	NA	Matrix effects were observed and modifications made to the MSC method during SLV work with soft-shelled clams and American oysters in 2008-2009. These same modifications are employed in this mehtod for quahogs. No matrix effects are anticipated
11. Comparability (if intended as a substitute for an established method accepted by the NSSP)	NA	

D. Other Information		
1. Cost of the method	Y	
2. Special technical skills required to perform the method	Y	
3. Special equipment required and associated cost	Y	
4. Abbreviations and acronyms defined	Y	
5. Details of turn around times (time involved to complete the method)	Y	
6. Provide brief overview of the quality systems used in the lab	Y	

Submitters Signature	Date:
Submission of validation data and draft method to committee	Date:
Reviewing members:	
Accepted	Date:

Recommendations for further work	Date:

Comments:

Single Laboratory Validation (SLV) Protocol

For Submission to the Interstate Shellfish Sanitation Conference (ISSC)

For Method Approval

Section A. Justification for New Method

Name of the New Method -	Male-specific Coliphage (MSC) for Quahogs.
Specify the Type of Method -	Culture Method/Double Agar Overlay Method
Name of Method Developer -	Thomas Howell, Spinney Creek Shellfish, Inc.
Developer Contact Information –	Spinney Creek Shellfish, Inc. 27 Howell Drive Eliot, Maine 03903 (207) 439-2719 (207) 439-7643 FAX tlhowell@spinneycreek.com
Date of Submission –	November 8, 2013

Purpose and Intended Use of the Method.

The primary purpose and intended use of this method in the NSSP is for re-opening growing areas after emergency closures due to sewage spills. This method has been used recently to re-open growing areas after the devastating effects of Superstorm Sandy by the State of New Jersey. The method presented in this document is the same as that modified and validated for soft-shelled clams and American oyster at the 2009 ISSC in Manchester, NH. Additionally, this method can be used to verify and optimize viral depuration/relay strategies used to reduce viral contamination in quahogs harvested from growing areas impacted by wastewater treatment plant (WTP) outfall.

Need for the New Method in the NSSP, Noting Any Relationships to Existing Methods.

Fecal coliforms (FC), a bacterial indicator, are used for process validation for conventional depuration processes. In growing areas impacted by moderate or low-level non-point source contamination, conventional depuration methods using FC for process validation are adequate, well proven, and widely accepted by the scientific and public health community. Statistical analysis of FC samples, collected during water quality monitoring, are used to determine growing area classification. Limits on the geometric mean and 90th percentile are considered adequate to protect public health from the risks of viral contamination in areas not impacted by sewage and WTP pollution. However, in growing areas impacted by treated sewage, the relationship between bacterial and viral contamination can be substantially altered by the differential inactivation rates of chlorination and other disinfection methods on bacteria and

viruses. This MSC method is needed in the NSSP to evaluate viral contamination in molluscan shellfish harvested from growing areas where FC levels in both water quality and shellfish meats may be misleading. MSC is a specialized indicator of viral contamination, which is substantially more meaningful than FC in evaluating the safety of shellstock harvested from growing areas potentially impacted by treated and partially treated wastewater. Much work has been done to demonstrate that the MSC method is particularly useful and highly advantageous over FC for evaluating the efficacy of viral depuration and viral relay processes in soft-shelled clams. Continuing work is being conducted to assess the usefullness of this method for evaluating the efficacy of viral depuration and viral relay processes for American oysters and quahogs.

Method Limitations and Potential Indications of Cases Where the Method May Not Be Applicable to Specific Matrix Types.

The MSC method described here has been previously validated for soft-shelled clams and American Oysters and is currently being evaluated for quahogs. Further SLV work is needed to evaluate different matrix types / other species of molluscan shellfish.

Other Comments.

SLV work strongly suggests that this modified MSC method is appropriate (fit for purpose) for applications in Quahogs in addition to Soft-shelled clams and American oysters where a regulatory limit of 50 PFU/100gram has been established.

Section B. Method Documentation

Modified Double Agar Overlay Method
for Determining Male-specific Coliphage
In Soft-shelled Clams, American Oysters, and Quahogs (M. mercenaria)
Nov 2013

This method for determining levels of male-specific coliphage in quahog meat is based on the method described by DeBartolomeis and Cabelli[1,2]. FDA had refined the method for oyster and hard clam meats as described in the workshop instructions, *Male-specific Bacteriophage (MSB) Workshop*, conducted in Gloucester, Massachusetts on March 9-12, 2004[3]. This original FDA (2004) method was submitted as ISSC Proposal 05-114. This method was modified again in 2008-2009 by Spinney Creek Shellfish to improve viral recovery and sensitivity for soft-shelled clams and American oysters.

Modification of the FDA (2004) Method

Spinney Creek Shellfish, Inc. (SCS) further refined these procedures for soft-shelled clam and oyster meat in 2006. In this work and in parallel work conducted by Mercuria Cumbo of the Maine Department of Marine Resources, it was observed that the extraction protocol was inadequate. The supernatant produced when soft-shelled clams and some oysters were processed was opaque and creamy while the pellet was loose and indistinct. Subsequent re-washing of the pellets in growth broth, re-processing, and re-plating showed significant levels of MSC left in the pellet, indicating poor recovery. The problem was solved by; eluting the shellfish meats with growth broth (2:1), and increasing the blending time to 180 seconds. This modification, based on EU methodology (ISO 10705-4), resulted in a clear supernatant and a distinct, firm pellet. Further experimentation and subsequent validation work confirmed that this elution approach works very well. SLV validation work conducted by (SCS) in 2009 resulted in further modification of the method to increase the limit of quantitation/sensitivity (LOQ). This increase in LOQ was achieved by plating an increased amount of supernatant (25ml) and using 10 plates. This same modified method is used for quahogs in the SLV application.

A. Apparatus and Materials.

Equipment and Materials for Collection and Transport of Shellfish Samples:
4 mil plastic bags
Labels
Cooler
Gel Packs
Temperature Control Blank

Laboratory Equipment:
Centrifuge with rotor for 50 ml conical (or larger) tubes, 9000 x *g* performance capability, 4°C
Water bath, 50-52°C
Air Incubator, 35-37°C
Balance
Stir plate and magnetic stirring bars, sterile

Mini vortexer
Blender
Autoclave, 121°C
Refrigerator, 0–4° C
Freezer, -20°C
Thermometers, range -20–121°C
pH meter
Erlenmeyer flasks, 1 L and 2 L
Graduated cylinders, 100 ml, 500 ml and 1000 ml
600ml and 3000ml beaker
500 ml jars, autoclavable with caps
Inoculating loops (3 mm in diameter or 10 FL volume)
Bacti-cinerator
Sterile swabs
Sterile, disposable filters, 0.22 or 0.45 µm pore size
Syringes, sterile disposable; 5, 10 or 20 ml
Scrub brushes, sterile
Knives, sterile
Blender jars, sterile
Sterile plastic cups 250 ml
Pipets- 2ml, 5 ml, 10 ml
Pipet-aid
Micro-Pipettors, 100 µL, 200 µL, 1000 µL, 2500 µL
Micro-Pipet tips 200 µL, 1000 µL, 2500 µL
Pipetor Stand
Centrifuge tubes, sterile disposable 50 ml or larger
Petri dishes, sterile disposable 100 x 15 mm
Petri dish racks
Test tubes 16 x 100 mm (for soft agar)
Test tubes 16 x 150 mm, with screw caps
Test tube racks--size to accommodate tubes
Freezer vials, sterile 30 ml with screw caps
Baskets with tops to hold freezer vials
Parafilm tape
Aluminum foil

Reagents:
Reagent water
Glycerol- sterile
Ethanol, 70% or laboratory disinfectant
Calcium chloride, 1M
Mineral oil

Antibiotic stocks:
Ampicillin sodium salt (Sigma A9518)
Streptomycin sulfate (Sigma S6501)
Streptomycin and Ampicillin stock solutions (50 µg/ml each). Note: Antibiotics must always be added to liquids and media after these have been autoclaved and cooled.

Media:
Bottom Agar
DS Soft Agar
Growth Broth

Bacterial Host Strain:
E.coli F_{amp} . *E. coli* HS(pFamp)RR (selected by Dr. Victor J. Cabelli, University of Rhode Island, Kingston, RI, USA, frozen stock ATCC # 700891).

MSC (Coliphage) Stock:
Type Strain - MS2, ATCC # 15597

B. Media Composition.

Bottom Agar:

Tryptone	10.0 g
Dextrose	1.0 g
NaCl	5.0 g
Agar	15.0 g
DI water	990 ml
Final pH	6.7 ± 0.2 at 25°C

1. With gentle mixing, add all the components to 990 ml of dH_2O in a 2000 ml flask. Dissolve, heat until clear and boiling started.
2. Sterilize at 121°C ± 2°C for 15 minutes.
3. Temper to 50°C in the water bath.
4. Add 5 ml of Streptomycin sulfate/Ampicillin solution, aseptically to the flask (50 µg/ml each in final) and mix. Transfer to 2 – 500ml sterile jars (easier to pour plates from jars).
5. Pipet (or pour) 15 ml aliquots aseptically into sterile 100 x 15 mm Petri dishes and allow the agar to harden. Tip Petri dish lids off slightly to reduce condensation.
6. Store bottom agar plates inverted at 4°C and warm to room temperature for 1 hour before use.
7. Plates stored sealed at 4°C can be used up to 6 weeks.

Streptomycin sulfate/Ampicillin Solution:
1. Dissolve 0.5g of streptomycin sulfate and 0.5g of ampicillin in 50 ml of dH_2O with a sterile 100 ml graduated cylinder in sterile 600 ml beaker with sterile stir bar.
2. Stir for 2 to 3 minutes, no heat.
3. Filter through sterile 0.22 µm filter.
4. Store in 5 ml aliquots in sterile 30 ml capped freezer vials at -20°C for up to one year. Label and date.
5. Allow to come to room temperature before adding and mixing in tempered bottom agar at 50°C.

DS Soft Agar:

Tryptone	10.0 g
Dextrose	1.0 g
NaCl	5.0 g
1M $CaCl_2$	0.5 ml
Agar	7.0 g
DI water	500 ml
Final pH	6.7 ± 0.2

1. With gentle mixing, add all the components to 500 ml of dH_2O in a 1000 ml flask.
2. Bring flask contents to a boil.
3. Dispense in 2.5 ml aliquots into 16 x 100 ml tubes, cover and freeze (-20°C) for up to three months.
4. Sterilize prior to use at 121°C ± 2°C for 15 minutes, then temper to 50-52°C for no longer than 2 hours

1M $CaCl_2$ Solution:

1. Add 11.1 g of $CaCl_2$ anhydrous (FW 111.0, Dihydrate FW 147) to 100 ml
2. dH_2O in a screw top bottle and dissolve or use prepared from VWR.
3. Sterilize by autoclaving at 121°C for 15 minutes.
4. Store up to three months at 4°C.
5. Use at room temperature.

Growth Broth:

Tryptone	10.0 g
Dextrose	1.0 g
NaCl	5.0 g
DI water	1000 ml

1. With gentle mixing, add all the components to 1000 ml of dH_2O water in a 2000 ml flask.
2. Dissolve and dispense into sterile screw top containers.
3. Sterilize at 121°C ± 2°C for 15 minutes.
4. Store for up to three months at 4°C.

Storage Slants: Tryptic Soy Agar.

C. Storage and Propagation of Host Strain, E. coli F_{amp}.

Storage:

1. Lab stock culture – Frozen at – 80°C indefinitely (most desirable method) in broth culture containing 10% glycerol under no selective pressure. Selective pressure is reapplied when the culture is retrieved, by streaking onto Bottom Agar plates containing the two antibiotics.
2. Long-term working stock culture – Grown tryptic soy agar slant with sterile mineral oil overlay under no selective pressure and stored at room temperature in the dark for up to 2 years.
3. Long-term working stock – 6-hour grown tryptic soy agar slant and deep stab with sterile mineral oil overlay containing the two antibiotics, Ampicillin and Streptomycin (least desirable method).

4. Short-term working stock culture - Grown Bottom Agar streak plate stored at 4°C up to 3 weeks.
5. Short-term working stock culture – Grown in Growth broth and used within 6-12 hours (same day).

Glycerol Solution, 10%: Add 9 ml of distilled water to 1 ml of undiluted glycerol. Autoclave resulting 10% glycerol solution at 121°C for 15 minutes and use at room temperature. For storage, add 1/5th volume of 10% glycerol solution, let stand for 30 minutes, dispense 1 ml aliquots in 2 ml cryo-vials and store at -70 to –80°C (best) or at –20°C.

Propagation:
1. Vortex to aerate 10 ml of Growth Broth medium tempered to 35 – 37°C just prior to inoculation.
2. Transfer host strain to Growth Broth using sterile swab to collect material from several colonies off grown Bottom Agar streak plate and warmed to room temperature.
3. Gently shake to mix, then incubate at 35–37°C for 4-6 hours (turbidity=10^7cells/ml; O.D at 540nm=0.4).
4. Once turbidity is observed, use of the host strain broth culture (log-phased growth) may commence

(following initial inoculation and mixing, do not shake or mix the host strain broth culture).

D. Control Plates.
1. Negative Control - Add 2.5 ml of Growth Broth and 0.2 ml host to the 2.5 ml DS Soft Agar tube.
2. Positive Control - Make serial dilutions using growth broth of the concentrated MS2 control (to grow approximately 50-100 PFU per 2.5 ml), and add 2.5 ml of appropriate MS2 dilution and 0.2 ml of host to 2.5 ml DS Soft agar.

E. MSC Density Determinations in Soft Shelled Clam, American Oyster, and Quahog Tissues.

Sample Requirements. Samples of shellstock and shucked meats are held under dry refrigerated conditions at 1–4°C. Samples must be comprised of a representative number of animals (12 to 15). Samples are analyzed within 24 hours of collection. Animals with broken shells or animals that appear dead are discarded. Sample collection bags must be properly identified with lot #, date and time of collection, collection location and collector's initials.

Preparation of Shellfish for Analysis. Using soap and water, analyst's hands are thoroughly scrubbed and rinsed. Using a sterile brush, shells of whole animals are scrubbed under running potable water to remove loose material from the shells. Shellfish then are placed on a clean paper towel or in an open weave basket to dry. Scrubbed, drying animals should not come in contact with each other. Once the shells of washed shellfish are dry, analysts wash their hands thoroughly with soap and water, then rinse their hands with 70% alcohol and allow to air dry. Shellfish are shucked and the meats and liquors are saved into a sterile 250 ml cups.

Direct Analytical Technique for Soft Shelled Clams, American Oysters, and Quahogs. For each soft shelled clam, American oyster, or quahog sample, ten (10) Bottom Agar plates and ten (10) 2.5 ml DS Soft Agar tubes are prepared. Use a 4 to 6 h culture of host strain, *E. coli* F_{amp}. Always begin analyses with a negative control (blank) plate and finish analyses with a positive control plate followed by a second negative control plate.

1. Shuck 12 soft shelled clams, American oysters, or quahogs into sterile 250 ml cup, tare and add to sterile blender. To make a 1:2 (wgt:vol) elution with growth broth eluent using twice the volume of the shellfish. Add to blender with sample. Homogenize by blending for 180 seconds at high speed.
2. Immediately weigh 33.0 g of homogenate from each sample into labeled sterile 50 ml centrifuge tubes after blender has stopped before foam separation can occur.
3. Centrifuge each sample for 15 min. @ 9,000-10,000 x g; 4°C.
4. Pipette off and weigh the supernatant in a new sterile 50 ml centrifuge tube.
5. Allow the supernatant to warm to RT (approximately 20-30 minutes).
6. Shake or vortex the supernatant.
7. Gently pipette 200 µL of log phase host strain, *E. coli* HS(pFamp)RR using 200 µL micro pipettor and a 200 µL pipet tip, then pipette 2500 µL aliquot of supernatant using the 2500 µL micro pipettor and a 2500 µL pipet tips, to 2.5 ml DS Soft agar tube (tempered to 52°C).
8. **Once E. coli F_{amp} is added to the mixture do not shake, only gently mix contents by rolling the tube between palms.**
9. Overlay the 5.2 ml onto a Bottom Agar plate containing Streptomycin and Ampicillin (50 g/ml final concentrations). Drag the mixture into a clear area and gently swirl the plates to spread sample and agar mixture.
10. Allow plates to set then inverted and incubated for 16 - 20 hours at 35- 37°C.

Calculations of Results

$$\frac{\text{Total number of MSC (N)}}{\text{Total supernatant plated (25gm)}} \times \frac{\text{Weight of supernatant extracted (Ws)}}{\text{grams of sample used (11gm)}} \times 100 =$$

$$\frac{N}{25\text{ gm}} \times \frac{Ws}{11\text{ gm}} \times 100 = (0.364)(N)(Ws) = \text{PFU of MSC/100 gm}$$

Example: Clam/Oyster plate counts - 13, 23, 12, 16, 12, 18, 17, 21, 19, 17 and 27.5 g supernatant.

Result = (0.364)(168MSC)(27.5gm) = 1681 PFU of MSC/100 gm*
**0.364=100/(25 x 11)*

F. Sample Collection and Storage.

1. Record all pertinent information on the collection form.
2. During transportation store samples in a cooler at 0 to 10°C.
3. At laboratory, store samples in a refrigerator at 0 to 4 °C.
4. Maximum holding times for shellfish samples is up to 24 hours.

G. Quality Assurance.

1. Positive and negative control plates are run with MSC analyses each day.
2. Media sterility checks are made per batch and records are maintained.
3. Media log book is maintained (pH, volume, weights of each components, lot numbers, etc.).
4. An intra- and inter-laboratory performance program is developed.
5. Circular zones of clearing (typically 1 to 10 mm in diameter) in lawn of host bacteria after 16- 20 hours of incubation are counted as plaques. (Count the number of plaques on each plate.)
6. MSC determinations are reported as plaque forming unit (PFU) per 100 grams.
7. The desired range for counting is 0 to 100 PFU per plate. If the count exceeds the upper range or if the plaques are not discrete, results should be recorded as "too numerous to count" (TNTC) or >10,000 PFU of MSC/100gm.
8. Temperatures incubators are checked twice daily (at least 4 hours apart) to ensure operation within the stated limits of the method, and results are recorded in a logbook.
9. Check thermometers at least annually against a NIST-certified thermometer.
10. Calibrate the balance monthly using ASTM-certified Class 1 or 2 or NIST Class S reference weights.
11. Laboratory analysts adhere to all applicable quality control requirements set forth in the most recent version of FDA's *Shellfish Laboratory Evaluation Checklist*.
12. Calibration of micro-pipettors needs to be checked quarterly and records kept. Micro-pipettors used for handling MSC control and transferring host cells need to have a barrier tip or be dedicated to the specific use to prevent contamination

H. Safety.

Samples, reference materials, and equipment known or suspected to have Coliphage attached or contained must be sterilized prior to disposal.

I. Technical Terms.

°C - degrees Celsius
µL - microliter
g - gram
L - liter
M - molar
ml - milliliter
rpm - revolutions per minute
Ave. - average
MSC - Male-specific Coliphage, Male-specific Bacteriophage, F+ Bacteriophage
NIST - National Institute of Standards and Technology
PFU - plaque forming units
RT - room temperature
TNTC - too numerous to count
LOD - Limit of Detection
LOQ - Limit of Quantitation
Host Strain - *E.coli* F_{amp} bacteria (*E.coli* HS(pFamp)RR)
Male-specific Coliphage - Viruses that infect coliform bacteria only via the F-pili.
Plaque - Clear circular zones (typically 1 to 10 mm in diameter) in lawn of host cells after incubation.

References:

1. Cabelli, V.J. 1988. Microbial indicator levels in shellfish, water, and sediments from the upper Narragansett Bay conditional shellfish-growing area. Report to the Narragansett Bay Project, Providence, RI.
2. DeBartolomeis, J. and V.J. Cabelli. 1991. Evaluation of an *Escherichia coli* host strain for enumeration of F male-specific Coliphages. Appl. Environ. Microbiol. 57(4):1201-1205.
3. U.S. Food and Drug Administration. 2004. Male-specific Coliphage (MSC) Workshop, conducted in Gloucester, Massachusetts on March 9-12, 2004.

Other Information:

This method for the enumeration of male-specific coliphage in soft-shelled clams, American oysters, and quahogs is inexpensive, easy to perform, and rapid, providing results within 24 hours. The cost of laboratory glassware, plastic-ware, agars, and reagents is approximately $25 per shellfish sample. In a well equipped laboratory, the method requires 6 hours of time from initiating host to pouring plates. Hands on technician time to perform this test is significantly less on the order of 1-4 hours per test depending upon how many tests are done per day. The most expensive piece of equipment is a refrigerated centrifuge plus rotor, which costs approximately $12,000. There are no special skill sets required beyond those required to operate a state-approved shellfish laboratory under the NSSP.

C. Validation Criteria

Preliminary Studies

A master spike determination experiment was run before other SLV work was performed to evaluate the planned routine for the spike determinations. In previous SLV work with soft-shelled clams and oysters, viral clumping was identified as a problem when the master spike was evaluated using growth broth and then compared to determination of MSC levels in the soft-shelled clam and oyster matrix. The spike determination was lower than the spiked samples of clean shellfish suggesting a negative recovery (the spike determinations were underestimating the sample results). The solution was to use clean soft-shelled clam or oyster supernatant and spin down the master spike sample to break up the clumps of MSC. This was sufficient for soft-shelled clam and oyster matrix. However, with quahogs, clean quahog homogenate was superior to both quahog supernatant and soft-shelled clam supernatant in making the spike determination. Preliminary studies of viral recovery as determined by resuspending the pellet in growth broth and re-processing twice showed that the recovery was very high.

As a result of these preliminary studies, two modifications of the SLV procedures used for soft-shelled clams and oysters were needed. First, the independent spike determination was dropped and the replicate plate values were used to calculate the estimated mean spike concentration. This meant that various validiation criteria were plotted against estimated mean spike from the triplicate samples verses an independent spike concentrations. This also required that the recovery be determined by the double re-wash and replate routine to directly evaluate the viral recovery. Because we do not have an independent estimate for the spike, we calculated and used measurement uncertainty for the mean replicate plate value which will give us a range of values for LOQ and LOD rather than a single value. Consequently, the determination of linear range is not possible and working range has been substituted as a validation criteria.

The Determination of LOD, LOQ, and Working Range using the NSSP SOP for the Single Laboratory Validation of Marine Biotoxin and Non-MPN Based Microbiological Methods.

The SOP for the determination of LOQ, LOD, and the Working Range yields a database from which subsets of data can be use to generate other validation criteria. For this LOQ, LOD database ten trials were run for quahogs. Supplemental samples were taken at the low range with a custom low-level master spike because of problems getting determinate results at those low levels. Effort was taken to use different shellstock from a variety of growing areas over a period of time and to utitilize shellstock that had non detectable levels of MSC (no plaques in the 10 plates). Several trial batches of shellstock were held in depuration for several days to weeks prior to the validation trials to ensure no detectable levels of MSC. Table 1 below shows the trial #, growing area, harvest date, and date of analysis for shellstock used during these validation trials.

Table 1

Trial #	Growing Area	Harvest Date	Date of Analysis
Quahogs			
1	CT268, CT	12/17/13	1/8/13
2	CT268, CT	12/17/13	1/15/13
3	Hog Island, VA	12/24/13	1/21/13
4	Hog Island, VA	12/24/13	1/29/13
5	CT431, CT	1/28/13	2/4/13
5A	Barnegat Bay, NJ	1/16/13	2/13/13
5B	Barnegat Bay, NJ	2/4/13	2/18/13
5C	Barnegat Bay, NJ	2/4/13	2/18/13
6A	Barnegat Bay, NJ	2/4/13	2/18/13
6B	CT115, CT	2/7/13	2/27/13
6C	Hog Island, VA	2/21/13	3/4/13
6D	Hog Island, VA	2/21/13	3/5/13
6E	Hog Island, VA	2/21/13	3/6/13
6F	Hog Island, VA	2/21/13	3/7/13
7	New Inlet, VA	3/7/13	3/12/13
8	New Inlet, VA	3/7/13	3/19/13
9	Spinney Creek, ME	3/21/13	3/27/13
10	Spinney Creek, ME	3/21/13	4/3/13

For each of the 10 validation trials, 12-15 shellfish were homogenized in a 2:1 eluate of growth broth to shellfish meat in accordance with the method described above. The homogenate was evenly distributed to 5 sterile beakers with Spinplus magnetic stir bars, tared and weighed. A master spike solution was prepared in growth broth and was varied in concentration during the trials. The master spike solution was on the order of 10^3 MSC/ml. Four subsequent serial dilutions were made for each trial from the master spike at a 3:1 dilutions. This represented different spike concentrations over the working range of the method. The 5 beakers were spiked with spike concentration 1 through 5 and three aliquots of 33 grams each were taken from each

of the 5 beakers which were actively stirred to prevent separation. In this way, 3 true replicates were generated at each of the 5 spike concentrations. The 5 sets of 3 aliquots were processed and plated according to the method description above. Supplement trials 5A-5C and 6A-6F were performed using a low-level spike that was made to get some additional low-level replicates.

Table 2 below show the estimated mean spike and tabulated MSC replicate plate concentrations results in units of PFU of MSC/100gm. RSD is relative standard deviation.

Table 2 – Tabulated Results of the Quahog Validation Trials

Trial #	Estimated Mean Spike value (PFU/100gm)	MSC Repicate Plate Concentrations (PFU/100gm)	Log of Replicate MSC Plates	RSD
1	17788	17729	4.249	0.0092
		16213	4.210	
		19421	4.288	
	5105	4501	3.653	0.0233
		4479	3.651	
		6335	3.802	
	1976	2373	3.375	0.0220
		1795	3.254	
		1761	3.246	
	452	389	2.590	0.0229
		454	2.657	
		514	2.711	
	68	97	1.987	0.0975
		43	1.633	
		65	1.813	
2	21724	21470	4.332	0.0042
		20971	4.322	
		22731	4.357	
	4277	4650	3.667	0.0099
		4234	3.627	
		3946	3.596	
	1298	1188	3.075	0.0109
		1321	3.121	
		1384	3.141	
	414	399	2.601	0.0180
		377	2.576	

		465	2.667	
	97	54	1.732	0.1010
		119	2.076	
		119	2.076	

3	10470	9360	3.971	0.0103
		11149	4.047	
		10900	4.037	
	2890	2671	3.427	0.0088
		3060	3.486	
		2939	3.468	
	871	743	2.871	0.0285
		800	2.903	
		1069	3.029	
	225	230	2.362	0.0178
		244	2.387	
		202	2.305	
	51	77	1.886	0.1700
		55	1.740	
		22	1.342	

4	10255	10203	4.009	0.0065
		10899	4.037	
		9664	3.985	
	2397	2500	3.398	0.0073
		2446	3.388	
		2245	3.351	
	1000	879	2.944	0.0160
		1035	3.015	
		1085	3.035	
	301	279	2.446	0.0126
		322	2.508	
		302	2.480	
	50	54	1.732	0.0336
		54	1.732	
		43	1.633	

5	6056	6257	3.796	0.0034
		5997	3.778	
		5914	3.772	
	1539	1534	3.186	0.0168
		1352	3.131	
		1731	3.238	
	476	515	2.712	0.0321
		539	2.732	
		375	2.574	
	103	121	2.083	0.0348
		88	1.944	
		99	1.996	
5A	61	43	1.633	0.0875
		53	1.724	
		86	1.934	
	60	94	1.973	0.3136
		74	1.869	
		11	1.041	
5B	52	21	1.322	0.1836
		83	1.919	
		52	1.716	
5C	59	42	1.623	0.1147
		93	1.968	
		42	1.623	
	62	72	1.857	0.1317
		31	1.491	
		83	1.919	

6A	57	64	1.806	0.0581
		43	1.633	
		65	1.813	
6B	79	75	1.875	0.1180
		118	2.072	
		43	1.633	
6C	36	53	1.724	0.1257
		22	1.342	
		32	1.505	
6D	17	22	1.342	0.1786
		11	1.041	

	15	22	1.342	0.1522
		11	1.041	
		11	1.041	
6E	22	32	1.505	0.1815
		11	1.041	
		22	1.342	
	18	22	1.342	0.1399
		22	1.342	
		11	1.041	
6F	32	43	1.633	0.1260
		21	1.322	
		21	1.322	
	21	21	1.322	0.1811
		11	1.041	
		32	1.505	

7	8295	9036	3.956	0.0088
		8103	3.909	
		7745	3.889	
	1914	2141	3.331	0.0187
		1627	3.211	
		1974	3.295	
	528	549	2.740	0.0147
		474	2.676	
		562	2.750	
	108	151	2.179	0.0750
		97	1.987	
		76	1.881	
	18	22	1.342	0.1399
		22	1.342	
		11	1.041	

8	6885	7515	3.876	0.0091
		6430	3.808	
		6710	3.827	
	1700	1883	3.275	0.0132
		1552	3.191	
		1664	3.221	
	464	491	2.691	0.0091
		439	2.642	

		462	2.665	
	86	75	1.875	0.0278
		96	1.982	
		86	1.934	
	21	11	1.041	0.1811
		21	1.322	
		32	1.505	

9	6341	6672	3.824	0.0051
		6149	3.789	
		6203	3.793	
	1633	1594	3.202	0.0126
		1802	3.256	
		1502	3.177	
	437	392	2.593	0.0167
		480	2.681	
		438	2.641	
	87	141	2.149	0.1165
		54	1.732	
		65	1.813	
	18	11	1.041	0.1399
		22	1.342	
		22	1.342	

10	6468	6969	3.843	0.0076
		6174	3.791	
		6260	3.797	
	1356	1766	3.247	0.0349
		1106	3.044	
		1196	3.078	
	517	474	2.676	0.0223
		603	2.780	
		474	2.676	
	82	75	1.875	0.0337
		75	1.875	
		97	1.987	

	36	43	1.633	0.2544
		11	1.041	
		54	1.732	

To precisely determine the LOD and LOQ, it is necessary to convert the data to log coefficient of variation and log estimated mean spike and to run the log linear regression. Graphs 1 show this log linear regression from the quahog data. The LOQ of the method may be found at the point of intersection of the log estimated mean spike and the log coefficient of variation of –1.0 (or its antilog, 10%). The LOD may be found at the point of intersection of the log estimated mean spike and the log coefficient of variation of –0.477 (or its antilog of, 33%). Taking the antilog of the spike concentrations at these points of intersection gives the LOQ and LOD, respectively. Graph 1 indicates the LOQ and LOD for the quahogs to be 43 PFU/100gm and 4 PFU/100gm, respectively. Table 3 shows the results of the log linear regression.

Graph 1 - The LOQ and LOD of Quahogs.

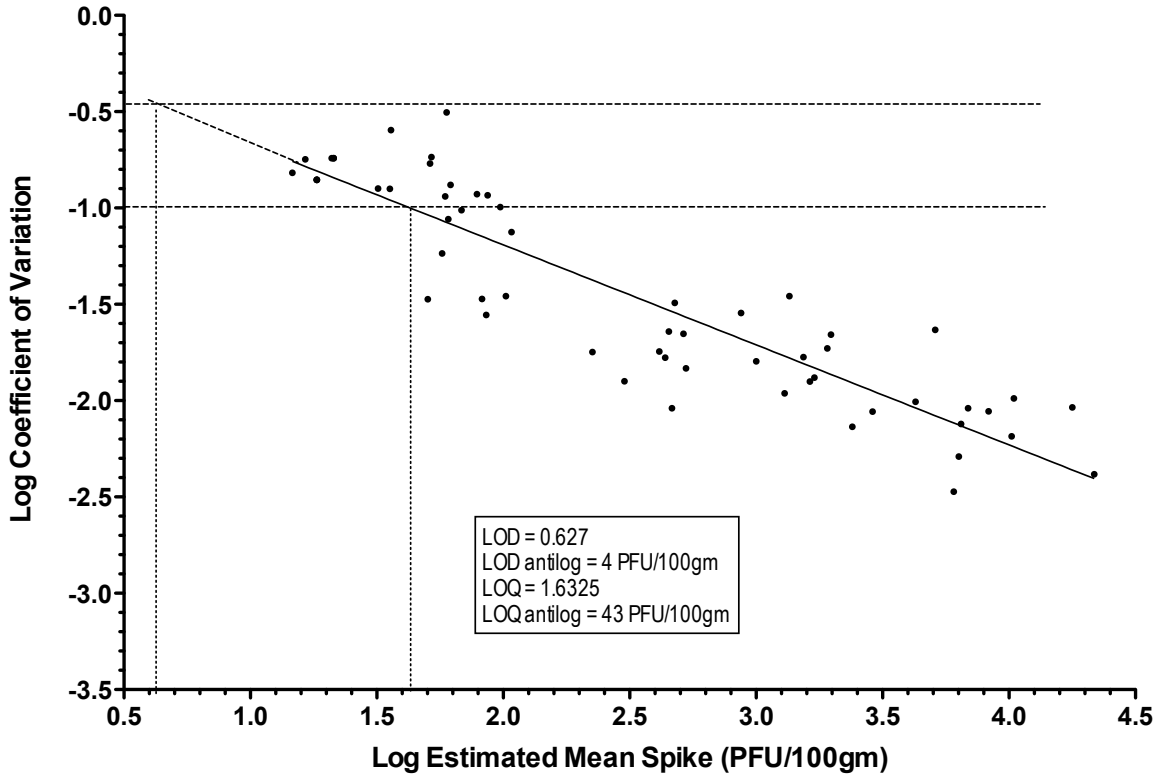


Table 3 – Results of the Log Linear Regression and Calculation of LOQ and LOD

Best-fit values	
Slope	-0.5193 ± 0.03312
Y-intercept when X=0.0	-0.1524 ± 0.08902
X-intercept when Y=0.0	-0.2934
1/slope	-1.926
95% Confidence Intervals	
Slope	-0.5857 to -0.4529
Y-intercept when X=0.0	-0.3308 to 0.02605
X-intercept when Y=0.0	-0.7250 to 0.04479
Goodness of Fit	
R square	0.8145
Sy.x	0.2352
Is slope significantly non-zero?	
F	245.8
DFn, DFd	1.000, 56.00
P value	<0.0001
Deviation from zero?	Significant

LOQ = Antilog [-1.926 (-1.0 + 0.1524)] = 42.90
LOD = Antilog [-1.926 (-0.478 + 0.1524)] = 4.25

Measurement Uncertainty

In this SLV, an independent estimate of spike concentration was not used. Therefore, the LOQ and LOD had to be determined as a range of values determined as the measurement uncertainty. Measurement Uncertainty was determined by subtracting the log replicate plate values from the log estimated mean spike, then calculating the 95% confidence limits of the mean difference. Table 4 shows these statistics from the quahogs.

Table 4 – Measurement Uncertainty for Quahogs.

		antilog
Number of values	172	
Mean	0.0178	**1.042**
Std. Deviation	0.288	
Std. Error	0.009816	
Lower 95% CI of mean	-0.00158	**0.996**
Upper 95% CI of mean	0.03718	**1.089**

From the regression, the LOQ intercept of -1.0 on the y-axix (log coefficient of variation) of Graph 1 and Table 3 equals 1.63248 on the x-axis (log estimated mean spike). The LOD intercept at -.0478 on the y-axix of Graph 1 and Table 3 equals 0.62711 on the x-axis. Substracting the lower limit of the measurement uncertainty log value -0.00158 from the LOD log value of 0.62711 equals 0.6287. The antilog of which is the lower limit of 4.25 for LOD.

Adding the upper limit of the measurement uncertainty log value of 0.03718 to the LOD log value of 0.62711 equals 0.66429. The antilog of which is the upper limit of 4.62 for LOD. Substracting the lower limit of the measurement uncertainty log value -0.00158 from the LOQ log value of 1.63248 equals 1.6341. The antilog of which is the lower limit of 43.06 for LOQ. Adding the upper limit of the measurement uncertainty log value of 0.03718 to the LOD log value of 1.63248 equal 1.6697. The antilog of which is the upper limit of 46.74 for LOQ.

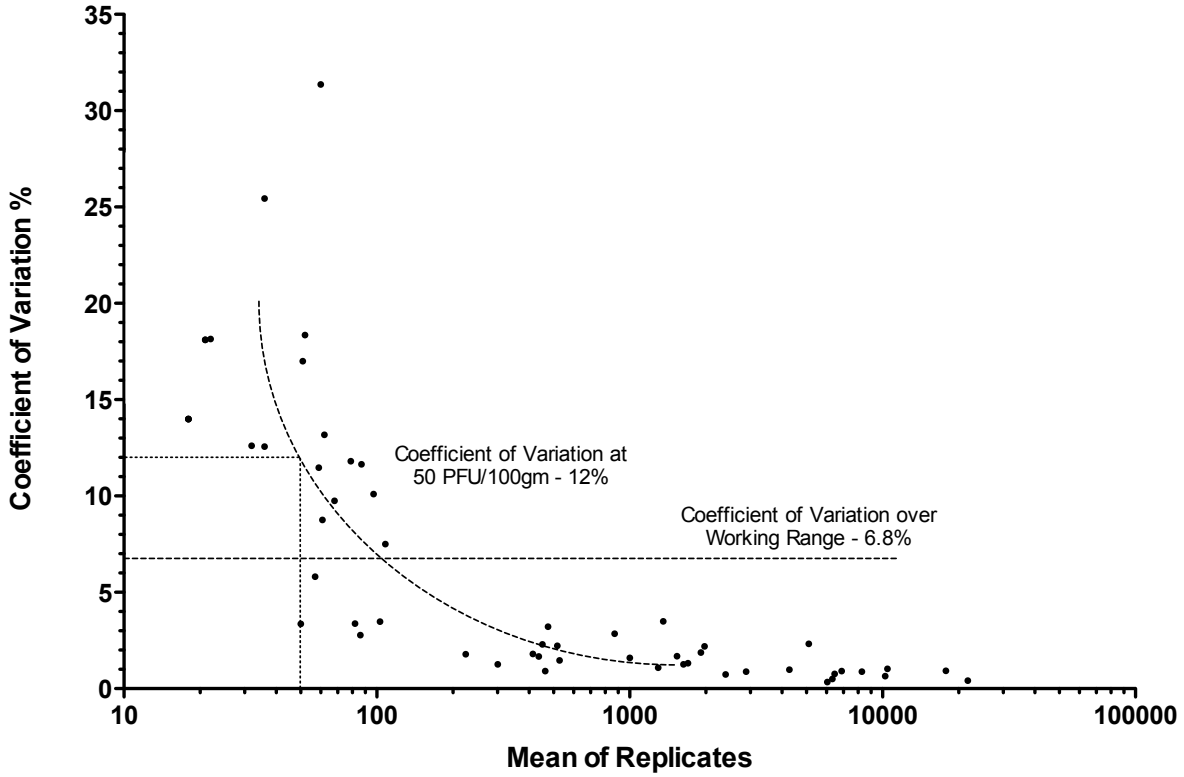
In summary, the LOD for quahogs ranges from 4.25 to 4.65 PFU/100gram. The LOQ for quahogs ranges from 43.06 to 46.74. As a result, a conservative estimate for the LOD and LOQ for quahogs was chosen to be 5 and 47 PFU/100gm, respectively. The upper working range is estimated to be approximately 200 PFU per plate or 20,000 PFU/100gm. In summary, the method has a working range of 5 PFU/100gm to 20,000PFU/100gm for quahogs. This method is fit for purpose with respect to a regulatory level of 50 PFU/100gm as the LOQ is less than the regulatory level.

Data Summary: Quahogs
Working range of the method as implemented 5 to 20,000 PFU/100gm
The limit of detection of the method as implemented 5 PFU/100gm
The limit of quantitation/sensitivity of the method as implemented 47 PFU/100gm

The Determination of Accuracy/Trueness is based upon the NSSP SOP for the Single Laboratory Validation of Marine Biotoxin and Non-MPN Based Microbiological Methods using the more robust databases acquired from the determination of the LOQ/LOD/Linear Range. Because we do not have an independant estimate of spike concerntration in this SLV, The Accuracy/Trueness can not be calculated.

The Determination of the Precision and Recovery is based upon the NSSP SOP for the Single Laboratory Validation of Marine Biotoxin and Non-MPN Based Microbiological Methods using the more robust data set acquired from the determination of the LOQ/LOD/Linear Range. To examine the precision over the working range of the method, a simple graphical approach was followed. The coefficients of variation were determined from the log transformed replicate data (50 sets of three true replicates) and were plotted verses the mean of the triplicate results (non log transformed data). The results are shown in Graph 2 for quahogs.

Graph 2 - Coefficient of Variability (%) of Replicates verses Mean of Replicate for Quahogs.



In Graph 2 above, the coefficient of variation at 50PFU/100gm level was determined graphically (approximately 12% for Quahogs) and shows the precision at this regulatory point. As expected, the precision decreases as the LOQ and LOD are approached. The mean, minimum, and maximum coefficient of variations as determined over the working range for quahogs appear in Table 5 below.

Table 5 – Mean, Minimum, and Maximum Coefficient of Variation over the Working Ranges.

Average Coefficient of Variation = 6.81%
Minimum Coefficient of Variation = 0.34%
Maximum Coefficient of Variation = 31%

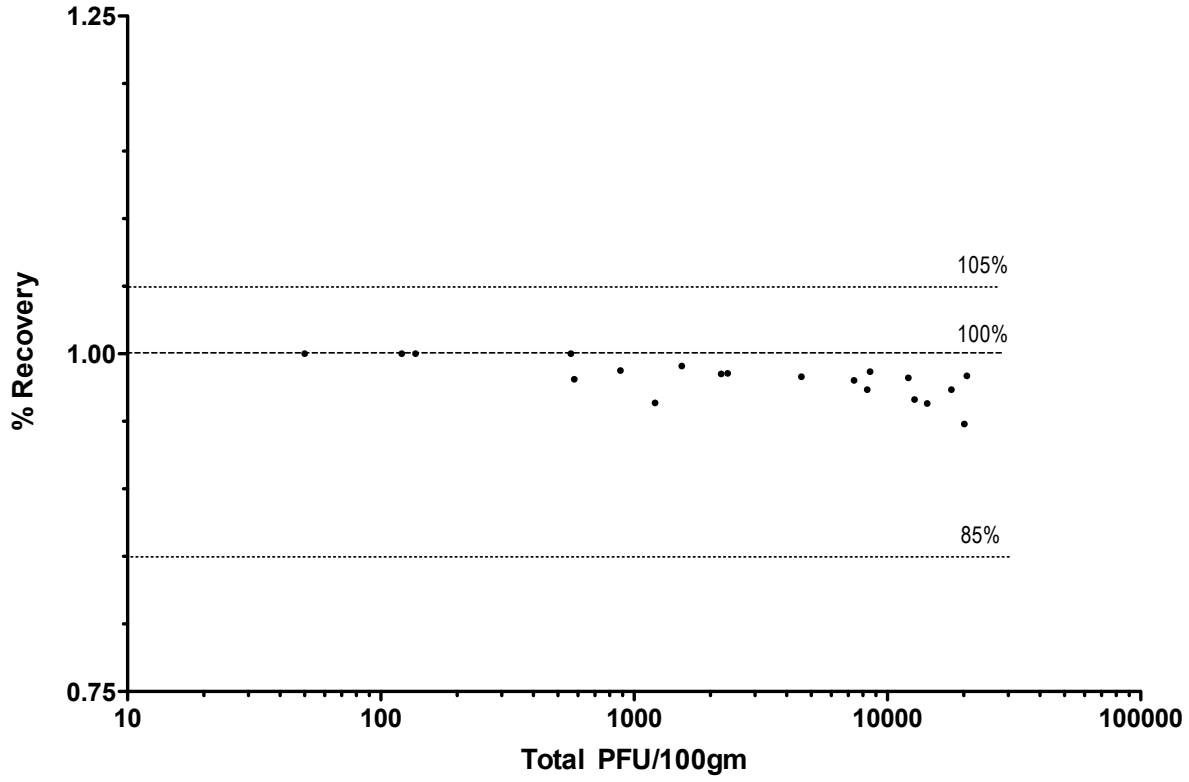
To determine the recovery of the method, a routine of re-washing the pellet into growth broth, then re-processing and re-plating twice (until depletion) was employed to directly determine the recovery. Supplemental samples 11 through 21 were spiked at lower levels to assure that recovery was consistent at low to high range concentrations along the working range. Table 6 show this recovery data for quahogs. The viral extraction demonstrated by this routine for this method varies from 94.8% to 100%.

Table 6 - Direct Recovery to Depletion for Quahogs.

	MSC Recovered			
Trial #	1 ml of Master Spike 33 gm homogenate (PFU/100gm)	Rewash Pellet and process (PFU/100gm)	Rewash 2nd Pellet and process (PFU/100gm)	Total PFU's
1	13834 **96.32%**	495 **3.45%**	33 **0.23%**	14362
2	19093 **94.80%**	1026 **5.09%**	22 **0.11%**	20141
3	20289 **98.37%**	336 **1.63%**	0 **0.00%**	20625
4	17433 **97.35%**	463 **2.59%**	11 **0.06%**	17907
5	8424 **98.68%**	113 **1.32%**	0 **0.00%**	8537
7	8117 **97.35%**	221 **2.65%**	0 **0.00%**	8338
8	12357 **96.61%**	434 **3.39%**	0 **0.00%**	12791
9	7232 **98.03%**	145 **1.97%**	0 **0.00%**	7377
10	11889 **98.22%**	216 **1.78%**	0 **0.00%**	12105
11 supplemental	4497 **98.30%**	78 **1.70%**	0 **0.00%**	4575
12 supplemental	2176 **98.51%**	22 **1.00%**	11 **0.50%**	2209
13 supplemental	2306 **98.55%**	34 **1.45%**	0 **0.00%**	2340
14 supplemental	1528 **99.09%**	0 **0.00%**	14 **0.91%**	1542
15 supplemental	1167 **96.37%**	33 **2.73%**	11 **0.91%**	1211
16 supplemental	570 **98.11%**	11 **1.89%**	0 **0.00%**	581
17 supplemental	563 **100.00%**	0 **0.00%**	0 **0.00%**	563
18 supplemental	872 **98.75%**	11 **1.25%**	0 **0.00%**	883
19 supplemental	50 **100.00%**	0 **0.00%**	0 **0.00%**	50
20 supplemental	121 **100.00%**	0 **0.00%**	0 **0.00%**	121
21 supplemental	137 **100.00%**	0 **0.00%**	0 **0.00%**	137

The average percent recovery of the method as implemented by this laboratory is calculated by averaging the above results and is reported at 98.2% with the sequential rewashing routine. Graph 3 shows the % Recovery verses Total PFU's and shows consistently high recovery over the working range.

Graph 3 - % Recovery verses Total PFU's over the Working Range



Data Summary: Quahogs

- Is the precision of the method under study consistent through the working range? **No, it varies as expected as the method approaches the LOD**
- The coefficient of variation of the test method as implemented is **6.8%** .
- Is the recovery of the method under study consistent through the working range? **Yes, it is consistently high over the working range**
- What is the overall percent recovery of the method under study? **98.2%**

Ruggedness was determined using the NSSP SOP for the Single Laboratory Validation of Marine Biotoxin and Non-MPN Based Microbiological Methods.

Different lots of agar, tryptone, and host E-coli culture and were prepared well in advance of the trials. Ten different harvest lots of quahogs were used for these analyses. Table 7 shows the data, data analysis, and the results of the paired t-test for quahogs.

Table 7 - Determination of the Method Ruggedness for Quahogs.

Media A PFU/100gm	Media B PFU/100gm	Log Media A	Log Media B
3309	3451	3.5197	3.5379
5224	5660	3.7180	3.7528
664	617	2.8222	2.7903
123	157	2.0899	2.1959
1985	2600	3.2978	3.4150
346	592	2.5391	2.7723
110	143	2.0414	2.1553
3485	3056	3.5422	3.4852
4316	3959	3.6351	3.5976
1902	1792	3.2792	3.2533
	Skew	-0.7036	-0.7246
	Variance	0.4019	0.3388
	Ratio of Larger Var to Lower Var	1.1862	

skew between -2 and 2 indicates symmetry
Ratio of Varieances < 2 indicates homogeneity of variance

Paired t-test (Media A verses Media B)	
P value	0.1442
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.600 df=9
Number of pairs	10

Data Summary: Quahogs
Value for the test of symmetry of the distribution of Media A data -.7036
Value for the test of symmetry of the distribution of Media B data -.7246
Variance of Media A data .4019
Variance of Media B data .3388
Ratio of the larger to the smaller of the variances of Media A and Media B 1.1862
Is there a significant difference between Media A and Media No

Acknowledgement

This Spinney Creek Shellfish, Inc. Single Laboratory Validation study was the culmination of previous work conducted by the FDA, Captain William Burkhardt, Dauphin Island Laboratory, AL and presented at the 2005 Conference in proposals 05-105, 05-114, and 05-113. Many thanks go to Ms. Mercuria Cumbo, Microbiologist, Maine Department of Marine Resources for her initial and patient instruction of the FDA method to Spinney Creek Shellfish, Inc. personnel. Ms. Cumbo was the first to observe viral extraction problems with soft-shelled clams and was instrumental in modifying this method to improve extraction efficiencies. Her constant technical assistance and direction throughout the SLV study was instrumental in the success of this project. Many thanks as well to Ms. Linda Chandler, FDA, College Park, MD who advised us in the modification of the method and well as constant oversight with the SLV study. Ms. Chandler's helpful insight into the SOP's, technical expertise, and review of the SLV results and document was pivotal in the completion to the project. Partial support was received from the New Hampshire Sea Grant College Program under Grant No. NA10OAR4170082 (CFDA No. 11.417) from the National Oceanic and Atmospheric Administration. Many thanks to Dr. Stephen H. Jones of the University of New Hampshire and the University staff for providing guidance and assisting with this opportunity. The findings, opinions and recommendations expressed in this report are those of the author and not necessarily those of University or of the Federal Awarding Agency. Finally, special thanks are due to Laura Stadig, Spinney Creek Shellfish, Inc. She worked tirelessly and precisely over many months, to execute the tedious task of performing the SLV laboratory work. Thanks to all, this was at all levels a group effort.