Proposal Subject:	Method to Determine the Presence of Male Specific Coliphage in Shellfish Meats and the Microbiology
Specific NSSP Guide Reference:	Section IV. Guidance Documents Chapter II. Growing Areas .10 Approved Laboratory Tests
Text of Proposal/ Requested Action	The MSC method must be reviewed and adopted prior to use in determining the acceptability of shellfish growing waters for reopening.
Public Health Significance:	FDA is submitting a proposal to ISSC to allow MSC to be used as a re-opening criterion in cases where unexpected, unusual sewage contamination occurs that may have impacted shellfish harvest areas (not for conditional re-openings). The MSC method must be reviewed and adopted prior to use in determining the acceptability of shellfish growing waters for reopening.
Cost Information (if available):	Not available.
Action by 2005 Laboratory Methods Review Committee	Recommended referral of Proposal 05-114 to the appropriate committee as determined by the Conference Chairman.
Action by 2005 Task Force I	Recommended adoption of the Laboratory Methods Review Committee recommendation on Proposal 05-114.
Action by 2005 General Assembly	Adopted recommendation of 2005 Task Force I.
Action by USFDA	Concurred with Conference action.
Action by 2007 Laboratory Methods Review Committee	Recommended no action on Proposal 05-114. Rationale – The data necessary to approve the method is not available. The submitter will send data to the Executive Office for Conference approval consistent with Procedure XVI.
Action by 2007 Task Force I	Recommended referral of Proposal 05-114 to an appropriate committee as determined by the Conference Chairman.
Action by 2007 General Assembly	Adopted recommendation of 2007 Task Force I.
Action by USFDA	December 20, 2007 Concurred with Conference action with the following comments and recommendations for ISSC consideration. The Conference has made considerable progress in its efforts to recognize new and developing analytical methods for the detection of indicators, pathogens, and marine toxins. Much credit goes to the Laboratory Methods Review Committee and its leadership for ensuring a scientifically defensible process for adopting analytical methods under the NSSP.

At the 2007 meeting numerous analytical methods were proposed for ISSC adoption. However, many of these methods were lacking the validation and associated data needed by the Laboratory Methods Review Committee to make a final determination regarding their efficacy for use in the NSSP. As a result the General Assembly voted "No Action" on analytical method Proposals 05-107, 05-108, 05-109, 05-111, 05-113, and 05-114. It is FDA's understanding that the intent of the "No Action" vote was not to remove these Proposals from ISSC deliberation as "No Action" normally suggests, but rather to maintain them before the Conference pending submission of additional data for further consideration. The Voting Delegates, by requesting the Proposal submitters provide additional data to the Executive Office for methods approval consistent with Procedure XVI, clearly recognized the importance and utility of these methods and intended to maintain them before the Conference for possible adoption following additional data submission. FDA requests that the ISSC Executive Board confirm FDA's understanding of this outcome. FDA fully supports such a Conference action and encourages the Executive Office to pursue submission of additional data as necessary to move forward with acceptance of these methods.

Enumeration of Male- specific bacteriophage in water and shellfish tissue

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What are male- specific (f-specific) bacteriophage?

•Lytic viruses of bacteria- (killing of host bacteria)

E. coli and S. typhimurium

(production of E. coli pili*)*

•Requires a piliated host cell for adsorption, they do not attach to cell surface (somatic).

•Requires host cell in log- phase of growth- cells do not produce pili at < 30°C

•Optimal growth temperature: 35- 37°C.

•Plaque size is generally self- limiting

Two Predominant Host Strains

•*E. coli* HS(pFamp)RR

Section IV. Resistant to Streptomycin and Ampicillin

•*Salmonella typhimurium* WG49

Result of mating: *E. coli* WG27 (piliated)

x

S. typhimurium WG45

-Resistant to Naladixic acid and Kanamycin

--Pili production in each strain is plasmid mediated

Media Composition
E. coli Famp

Bottom Agar

•Tryptone 10.0 g
•Dextrose 1.0 g
•NaCl 5.0 g
•Agar 15.0 g
••Water 1000 ml

•Autoclave 121℃ 15 min
- temper to 50°C.
•Add 0.05 g Streptomycin sulfate
0.05 g Ampicillin (aseptically)

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
••Water 500 ml
••Boil- Dispense in 2.5 ml aliquots (16 x 100 ml tubes) and freeze (-20°C)
••Autoclave prior to use;
temper to 50- 52° C

Growth broth- same formulation as Bottom Agar w/o agar or antibiotics

Media Composition
S. typhimurium WG49
Bottom Agar

•Trypticase Peptone 10.0 g
•Yeast Extract 1.0 g
•Dextrose 1.0 g
•NaCl 8.0 g
•Agar 15.0 g
••Water 1000 ml

•Autoclave 121℃ 15 min
- temper to 50°C.
•Add 0.10 g Naladixic Acid
0.02 g Kanamycin sulfate (aseptically)

DS Soft Agar

•Trypticase Peptone 10.0 g
•Yeast Extract 1.0 g
•Dextrose 1.0 g
•NaCl 5.0 g
•1M $CaCl_2$ 0.5 ml
•Agar 7.0 g
••Water 500 ml
••Boil- Dispense in 2.5 ml aliquots (16 x 100 ml tubes) and freeze (-20°C)
••Autoclave prior to use;

temper to 50- 52° C

Growth broth- same formulation as Bottom Agar w/o agar or antibiotics

Differentiation of RNA and DNA Bacteriophage

•RNAse Type I-A Sigma # R4875
••Final conc= 100ug/ ml of media
•Stock concentration= 10 mg/ml (100X)
••Dissolve at a conc. Of 10 mg/ml in 0.01 M Sodium Acetate (pH 5.2); Boil for 15 min and allow to cool to RT; PH by adding 0.1 vol of 1M Tris HCl (pH 7.4)
••Store @ -20C

Propagation of *E. coli* Famp

Bottom Agar Streak plate-
Transfer preferable < 1 week old.
•Broth Growth medium tempered to 35- 37°C- vortex to aerate.
•Using 10ul loop collect material from of several colonies and transfer to broth medium.
•Shake briefly to mix, then incubate at 35- 37°C for 4-6 hours
(turbidity ≈ 10^7cells/ ml; O.D @540 nm= 0.4)

% RECOVERY OF BACTERIOPHAGE F-2W/ VARIOUS AGES OF FAMP CULTURE

Age of a 10 ml host cell culture(h)	% Recovery (Mean ± SD)3.0
	105.4 ± 2.5
3.5	97.4 ± 2.2
4.0	96.0 ± 2.8
4.5	95.0 ± 3.3
5.0	92.5 ± 1.2
6.0	90.9 ± 1.8

Adapted from DeBartolomeis, 1999

For MSB density determinations in shellfish tissue

1. Homogenize by blending 12 shellfish for 1 min at high speed.
2. Aliquot 30- 50 g from each sample into centrifuge bottle.
3. Centrifuged for 15 min. @ 9,000 x g; 4°C.
4. Collect and weigh supernatant in a sterile container.
5. Allow supernatant to warm to RT (20- 30 min)
6. Combine 2.5 ml aliquot of supernatant, 2.5 ml DS Soft agar (tempered to 52°C) and 0.2 ml of *E. coli* HS(pFamp)RR
7. Overlay onto a tryptone agar plate containing streptomycin/ ampicillin (50 µg/ml final).
8. Plates are inverted and incubated for 18- 24 h @ 35- 37°C

Information needed for

Bacteriophage density determinations:

Plate counts of plaques

g Shellfish homogenate centrifuged
g Shellfish supernatant recovered

Calculations

MSB/ 100 grams=

1) Ave PFU/ plate ÷ number of ml added/ plate= Average PFU/ml

2) Average PFU/ml x grams of supernatant x 100 g . = PFU/ 100 g

g homogentate

Example: Plate counts- 75, 73,80; 2.5 ml/ plate

50 g homogenate; 33 g supernatant

76 ÷ 2.5 x 33 g supernatant x 100 grams = 2006 PFU/ 100 grams

50 g homogenate

To determine level of sensitivity

3 plates containing 0, 0, 0; 2.5 ml/ plate
50 g Homogenate; 33 g Supernatant
Assume 1 plaque on 1 plate then calculate
1÷ 3 plates ÷ 2.5 ml x 33 x (100 ÷ 50) =
Reported as < 9 pfu/ 100 grams

For MSB density determinations in low contaminated water- Concentration technique

1. Weigh 100 ml of water in a sterile container centrifuge bottle.
2. Allow water to warm to RT (20- 30 min).
3. Add 1g tryptone and 1 g beef extract to water aliquot, shake to dissolve.
4. Add 10 ml of *E. coli* Famp culture- Do not shake
5. Incubate at 35- 37°C for 50 min – rotate at 100 rpm.
6. Centrifuged for 15 min. @ 9,000 x g; 4°C.

For MSB density determinations in highly contaminated water (> 100 pfu/ 100 ml)

1. Allow an aliquot of water to warm to RT (20- 30 min)
2. Combine 2.5 ml aliquot of supernatant, 2.5 ml DS Soft agar (tempered to 52^{o} C), and 0.2 ml of *E. coli* HS(pFamp)RR
3. Overlay onto a tryptone agar plate containing streptomycin/ ampicillin (50µg/ml final).
4. Plates are inverted and incubated for 18- 24 h @ 35- 37°C

Problems that may arise

Multiple layers are formed after centrifugation

Reason- glycogen- lipids associated w/ shellfish physiological state

Sliding pellet- not solid

Reason- waited too long to remove supernatant

Clumping Agar

Reason- sample was too cool

Runny plaques

Reason- wet plates; too much condensation

No plaques/ individual bacterial colonies on agar plates

Reason- no phage present or inadequate amount host cell

Ways of Enhancing Plaque Visibility

Addition of 2,3,5- triphenyl tetrazolium chloride (TTC), 1% solution in ethanol
65 ul / tube of tempered DS soft agar
Assuming: 2.5 ml of DS agar and
2.5 ml sample
or
Grams Safrin 1:100 in water- differentiates lawn from plaque

Storage of *E. coli* Famp

Selective pressure- Streptomycin and Ampicillin

Bottom Agar Streak Plate
Storage: Refrigerator (2-3 weeks)
•Tryptic Soy Agar Deep w/ Mineral oil overlay
Storage: Room temperature in Dark (2-5 years +)
•Addition of glycerol (10% final) into broth culture. Storage: Freeze at – 80°C (Indefinite?)

Source of Bacterial Host Strains

•*E. coli* HS(pFamp)R; ATCC #700891
•*Salmonella choleraesuis* subsp. *Choleraesuis* (Smith) Weldin serotype Typhimurium aka WG49; ATCC #700730

Types and Sources of Positive MSB Controls

Bacteriophage MS2; ATCC# 15597-B1

Bacteriophage Fd; ATCC# 15669 –B2

Municipal Wastewater

Bacteriophage Stability in Shellfish Homogenate

		Time (h)		
Temperature	**Addition**	**0**	**4**	**10**
1- 3 °C	**-**	**2.57**	**2.58**	**2.42**
25°C	**-**	**3.81**	**3.64**	
25°C	**Log Famp[a]**	**3.81**	**3.86**	**3.89 (24h)**
35°C		**3.81**	**3.45[b]**	

[a]Famp added at a density of 270 cells/ g
[b]Significant decrease at 95% Confidence limit

Bacteriophage Stability in Shellfish Supernatant

		Time (h)		
Temperature	**Addition**	**0**	**4**	**24**
25°C	**-**	**3.81**	**3.74**	**2.60[b]**
25°C	**Log Famp[a]**	**3.81**	**3.13[b]**	**2.90[b]**
35°C	**-**	**3.81**	**3.73**	**2.90[b]**
35°C	**Log Famp[a]**	**3.81**	**3.56[b]**	**5.51[c]**

[a]Famp added at a density of 270 cells/ g
[b]Significant decrease at 95% Confidence limit
[c]Significant increase at 95% Conifidence limit

Action by 2009 Laboratory Methods Review Committee Recommended adoption of the substitute MSC method as a Type IV method for analysis of soft shell clam and oyster tissue to determine impacts of wastewater treatment plant effluent spills.

Action by 2009 Task Force I Recommended adoption of Laboratory Methods Review Committee recommendation on Proposal 05-114.

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force I on Proposal 05-114.

Action by USFDA 02/16/2010

Concurred with Conference on Proposal 05-114 with the following comments and recommendations for ISSC consideration.

FDA concurs with Conference action to adopt the male specific coliphage (MSC) method as a NSSP Type IV lab method for determining the presence of MSC in soft shell clams and oysters. However, the Summary of Actions states that its use is only for determining impacts associated with wastewater treatment plant effluent *spills*. It was not intended that its application be limited in this regard. Action by the Laboratory Methods Review Committee (LMRC) intended the use of this method for assessing the impact of wastewater effluents "*in general"* and not just the impact of wastewater effluent "*spills"*. FDA has confirmed this with the Chairperson of the LMRC and therefore recommends that the Summary of Actions be amended to reflect its use in the broader context of assessing the impact of wastewater effluents in general.

Action by ISSC Executive Board March 2010

The Executive Board rejected FDA's recommendation in Response to Summary of Actions. The ISSC Executive Director will add section for clarification in the 2009 Summary of Actions on Proposal 05-114 Microbiology Method to Determine the Presence of Male Specific Coliphage in Shellfish Meats and the Microbiology and develop guidance in the NSSP Guide for the Control of Molluscan Shellfish.

Modified Double Agar Overlay Method
for Determining Male-specific Coliphage
In Soft Shelled Clams and American Oysters
March 2009 Revision

This method for determining levels of male-specific □upernata in soft shelled clam and American oyster meat is based on the method described by DeBartolomeis and Cabelli[1,2]. FDA has 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.

Modification of the FDA (2004) Method

Spinney Creek Shellfish, Inc. (SCS) further refined these procedures for soft-shelled clam and oyster meat in work funded by the Maine Technology Institute 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, 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.

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
600 ml beaker
500 ml jars, autoclavable with caps
Inoculating loops (3 mm in diameter or 10 □L 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- 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 $F_{amp}^{\sim}$ *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.
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 3 months.

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 $1/5^{th}$ 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 @ 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 and American Oyster 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 and American Oysters. For each soft shelled clam or American oyster 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 or American oysters 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.

2. Record all pertinent information on the collection form.
3. During transportation store samples in a cooler at 0 to 10°C.
4. At laboratory, store samples in a refrigerator at 0 to 4 °C.
5. 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 upernata in soft-shelled clams and American oysters 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 set-up 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 $10,000. There are no special skill sets required beyond those required to operate a state-approved shellfish laboratory under the NSSP.