Proposal Subject:	Method to determine the Presence of Male Specific Coliphage in Shellfish Meats and the Microbiology Checklist for Male-specific Coliphage (MSC)
Specific NSSP Guide Reference:	ISSC Constitution, ByLaws, and Procedures Procedure XVI, Procedure for Acceptance and Approval of Analytical Methods for the NSSP and Section IV. Guidance Documents, Chapter II. Growing Areas .11 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists.
Text of Proposal/ Requested Action	The laboratory procedure is based on the methods described in Burkhardt, W., III, W.D. Watkins, and S.R. Rippey. 1992. Seasonal effects on accumulation of microbial indicator organisms by *Mercenaria mercenaria*. Appl. Environ. Microbiol. 58:826-831; DeBartolomeis, J. and Cabelli, V.J. 1991. Evaluation of an *Escherichia coli* host strain for enumeration of F male specific bacteriophages. Appl. Environ. Microbiol. 57: 1301-1305; Burkhardt, W. III *Enumeration of Male-specific Bacteriophage in water and shellfish tissue.* 2004. Gulf Coast Seafood Laboratory, Office of Seafood, U.S. Food and Drug Administration, Dauphin Island, AL. 31 pg. The laboratory procedure is to be reviewed by the Laboratory Methods Review Committee for consideration as a Type IV Method according to Procedure XVI. The Laboratory Evaluation Checklist – Pages 2, 16, 17, and 18, Microbiology of the Guidance Documents, Chapter II. Growing Areas, .11 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists is attached. It includes a section for the Male-specific Coliphage (MSC). MSC is an important microorganism for monitoring the microbial quality of waters (e.g., sewage treatment, growing area, etc.).
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). State Laboratory Managers and Laboratory Evaluation Officers need this document to correctly perform the analysis and to evaluate any laboratory performing the Coliphage (Bacteriophage) procedure.
Cost Information (if available):	None
Action by 2005 Laboratory Quality Assurance Committee	Recommended referral of Proposal 05-113 to the appropriate committee as determined by the Conference Chairman.
Action by 2005 Task Force I	Recommended adoption of the Laboratory Quality Assurance Committee recommendation on Proposal 05-113.
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 05-113. Rationale – The "no action" on Proposal 05-114 eliminated the need for checklist adoption. The submitter will submit the checklist with the data for method approval to the Executive Office for Conference approval consistent with Procedure XVI.

Action by 2007 Task Force I Recommended adoption of the Laboratory Methods Review Committee recommendation of no action on Proposal 05-113.

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.

.11 – Laboratory Evaluation Checklist – Microbiology – 2

Check the applicable analytical methods:	
	Multiple Tube Fermentation Technique for Seawater (APHA)[PART II]
	Multiple Tube Fermentation Technique for Seawater Using MA-1 [PART II]
	Multiple Tube Fermentation Technique for Shellfish Meats (APHA)[PART III]
	Standard Plate Count for Shellfish Meats [Part III]
	Elevated Temperature Coliform Plate Method for Shellfish Meats [PART III]
	Male Specific Bacteriophage for Shellfish Meats [PART III]

PART 1 – QUALITY ASSURANCE			
CODE	**REF**	**ITEM**	
K	8, 11	**Quality Assurance Plan**	
		1. Written Plan (Check √ those items which apply.)	
			a. Organization of the laboratory
			b. Staff training requirements
			c. Standard operating procedures
			d. Internal quality control measures for equipment calibration, maintenance, repair and for performance checks.
			e. Laboratory safety
			f. Internal performance assessment

			g. External performance assessment
C	8		2. QA Plan Implemented
K	11		3. Participates in a proficiency testing program annually. Specify Program(s)_____
CODE	**REF.**	**Work Area**	
O	8, 11		1. Adequate for workload and storage.
K	11		2. Clean, well lighted.
K	11		3. Adequate temperature control.
O	11		4. All work surfaces are nonporous, easily cleaned and disinfected.
K	11		5. Microbiological quality and density of air is < 15 colonies/plate in a 15 minute exposure determined monthly and results recorded.
O	11		6. Pipet aid used, mouth pipetting not permitted.
CODE	**REF.**	**Bacteriological Examination of Shellfish by Male-specific Bacteriophage Equipment & Supplies**	
			SEE PAGE 3, 4 & 5 FOR RELEVENT EQUIPMENT ITEMS.
K	**31**		**1. Sample containers are sterile, made of glass or some other inert material (i.e., polypropylene), hold 100-125 mL, and treated with sodium thiosulfate.**
C	**27,28,29,30**		**2. The refrigerated centrifuge must have the capacity to accommodate the amount of shellfish samples required for procedure, perform at 9000 x G, and maintain a temperature of 4°C ± 1°C.**
C	**27,28,29,30**		**3. The water bath must be able to maintain 44-46°C and 50-52°C temperature ranges.**
K	**9**		**4. The level of water in the water bath covers the level of liquid and agar in the containers and culture tubes.**
K	**13**		**5. Working thermometers are tagged with identification, date of calibration, calibrated temperature and correction factor.**
K	**4**		**6. All working thermometers are appropriately immersed.**
K	**11**		**7. A standards thermometer has been calibrated by NIST or one of equivalent accuracy at the points -20°, 0°, 35°, 44.5°C, 50° and 121°C. Calibration records maintained.**
K	**9**		**8. Standards thermometer is checked annually for accuracy by ice point determination. Results recorded and maintained.** **Date of most recent determination_____.**
K	**13**		**9. Incubator, freezer, refrigerator, autoclave and water bath working thermometers are checked annually against the standards thermometer at the temperatures at which they are used. Records maintained.**
C	**32**		**10. Sterile 0.22 or 0.45µm pore size filters are used to prepare the antibiotic solutions using sterile disposable syringes. Check sterility of each lot.**
K	**27,28,29,30, 31**		**11. Pre-sterilized plastic or sterile glass syringes are used to filter sterilize the stock antibiotic solution. Check sterility of each lot.**
K	**31**		**12. Colonies are counted with the aid of magnification or light box device.**
C	**32**		**13. Balance provides a sensitivity of at least 0.01 g.**
C	**31**		**14. The temperature of the incubator is maintained at 35-37°C.**
K	**27,28,29**		**15. Reusable or disposable pipets-pipettors are used and sterility is checked with each lot.**
K	**2727,28,29**		**16. Sterile disposable 15 and 50 mL centrifuge tubes are used and sterility is checked with each lot.**
		Media Preparation and Storage	
			SEE PAGES 5 & 6 FOR RELEVENT MEDIA PREPARATION AND

			STORAGE ITEMS.
K	27,28,29		1. Media is prepared from individual components.
K	27,28,29		2. Media is prepared and sterilized according to the method procedure.
C	27,28,29		3. Streptomycin/ Ampicillin solution is added after the autoclaved bottom agar has tempered to 44 – 46 ° C.
O	27,28,29		4. Storage of MSB bottom agar under refrigeration does not exceed 1 month.
O	27,28,29		5. Unsterilized DS soft agar is stored in a – 20° C freezer for up to 1 month
K	27,28,29		6. The DS soft agar is removed from the freezer and sterilized for 15 minutes at 121° C before use.
O	27,28,29		7. Storage of Growth broth in the refrigerator in loosely capped tubes/bottles does not exceed 1 month and in screw capped tubes/bottles does not exceed 3 months.
C	27,28,29		8. Host *stock E. coli* F_{amp} is ATCC 700609.
K	27,28,29		9. The host stock used for growth broth host cells is less than 1 week old.
O	27,28,29		10. Media is warmed to room temperature before use.
			Preparation of Shellstock for Examination
K	2, 11		1. Shucking knives, scrub brushes and blender jars are (autoclave) sterilized for 15 minutes prior to use.
O	2		2. Blades of shucking knives are not corroded.
O	9		3. Prior to scrubbing and rinsing debris off shellstock, the hands of the analyst are thoroughly washed with soap and water.
O	2		4. The faucet used to provide the potable water for rinsing the shellstock does not contain an aerator.
K	9		5. Shellstock are scrubbed with a stiff, sterile brush and rinsed under water of drinking water quality.
O	9		6. Shellstock are allowed to drain in a clean container or on clean towels prior to opening.
K	9		7. Prior to opening, the hands (or gloved hands) of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol.
K	9		8. Shellstock are not shucked directly through the hinge.
C	9		9. Contents of shellstock (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.
K	9		10. At least 12 shellstock are used for analysis.
K	2, 19		11. The sample is weighed to the nearest 0.1 gram
C	9		12. Samples are blended at high speed for 60 seconds.
K	9		13. For other than shellstock, APHA *Recommended Procedures* is followed for the examination of freshly shucked and frozen shellfish meats.
			Sample Analysis
C	27,28,29		Samples are analyzed according to the approved method.
K	27,28,29		Growth Broth is tempered to 35 – 37° C and vortexed (or shaken) to aerate prior to inoculation
K	27,28,29		Several host cell colonies are transferred to a tube of growth broth to provide log phase growth host cells for sample procedure.
C	27,28,29		Growth broth with host cells is incubated 35 – 37° C for 4 to 6 hours to provide culture in log phase growth.
C	27,28,29		The host cell growth broth is not shaken.
O	27,28,29		At least 30 to 50 grams of blended shellfish meat is weighed into sterile centrifuge tubes; weight is recorded .

C	27,28,29		The blended shellfish meat is centrifuged for 15 minutes at 9000 x g at 4° C.
K	27,28,29		Only supernatant is pipetted off and weight recorded.
K	27,28,29		Supernatant is allowed to warm to room temperature – 20 to 30 minutes.
K	27,28,29		The autoclaved DS soft agar is tempered and held at 50 – 52° C throughout sample procedure.
K	27,28,29		The supernatant is shaken or vortexed before adding to DS soft agar.
K	27,28,29		At least, a total of 7.5 ml of shellfish meat supernatant are plated.
C	27,28,29		2.5 ml of sample are added to 2.5 ml of DS soft agar and 0.2 ml of log phase host cell in growth broth while in the tempering waterbath.
C	27,28,29		DS soft agar/sample/host cell mixture is gently rolled between palms to mix.
C	27,28,29		The soft agar mixture is overlaid bottom agar and swirled gently to distribute.
K	27,28,29		Negative and positive control plates accompany samples.
K	27,28,29		Growth broth is used for negative (blank) control plates.
K	27,28,29		MS2 male specific bacteriophage is used as the positive control.
K	27,28,29		A negative control plate is the first plate and the last plate.
K	27,28,29		The positive control plate is set up after all samples and just before the final negative plate.
C	27,28,29		All plates are incubated at 35 – 37° C for 16 to 20 hours.
		Computation of Results	
C	31		1. Circular zones of clearing (of any diameter) in lawn of host bacteria are plaques.
C	32		2. The desired range of 30 to 300 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).
K	27		3. The equation used is: $\text{PFU/100grams} = \frac{\text{Avg of plate counts}}{\text{ml analyzed/plate}} \times \frac{\text{grams of homogenate}}{\text{grams of supernate}} \times 100$
O	9		2. Round off at the end of your computation using the information in ***Recommended Procedures for the Examination For Sea Water and Shellfish.***
K	27		4. Results are reported as PFU/ 100 g for shellfish samples.

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1.	*Compendium of Methods for the Microbiological Examination of Foods*, 2nd Edition, APHA. 1984.
2.	Good Laboratory Practice.
3.	"Interim Guides for the Depuration of the Northern Quahog, *Mercenaria mercenaria*, Northeast Marine Health Sciences Laboratory, North Kingstown, RI. 1968.
4.	NBS *Monograph 150*, U.S. Department of Commerce, Washington, D.C. 1976.
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	APHA/WEF/AWWA. 1992.
12.	Title 21, Code of Federal Regulations, Part 58, "Good Laboratory Practice for Nonclinical Laboratory Study", Washington, D.C.
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17.	*Handbook for Evaluating Water Bacteriological Laboratories*. 1975. US EPA, 670/9-75-006.
18.	Adams, W.N., 1974. NETSU. Personal communication to Dr. Wallace Andrews, FDA.
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20.	*NSSP Guide to the Control of Molluscan Shellfish*. 1997. FDA/ISSC.
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22.	Furfari, Santo. March 21, 1972. Personal Communication to Dan Hunt, FDA.
27.	Burkhardt, W. III *Enumeration of Male-specific Bacteriophage in water and shellfish tissue*, 2004. Gulf Coast Seafood Laboratory, Office of Seafood, U.S. Food and Drug Administration (or just FDA), Dauphin Island, AL. 31 pg.
28.	Burkhardt, W., III, W.D. Watkins, and S.R. Rippey. 1992. Seasonal effects on accumulation of microbial indicator organisms by Mercenaria mercenaria. Appl. Environ. Microbiol. 58:826-831.
29.	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.
30.	DeBartolomeis, J. and Cabelli, V.J. 1991. Evaluation of an *Escherichia coli* host strain for enumeration of F male-specific bacteriophages. Appl. Environ. Microbiol. 57:1301-1305.
28.	United States Environmental Protection Agency, *Method 1601: Male-specific (F+) and Somatic Coliphage in Water by Two-step Enrichment Procedure,* EPA 821-R-01-030, EPA, Washington, DC, April 2001.
29	United States Environmental Protection Agency, *USEPA Manual of Methods for Virology*, Chapter 16, EPA 600/4-84/013 (N16), Washington DC, June 2001.

NSSP Form LAB-100 rev. 2005-02-18

Action by 2009 Laboratory Quality Assurance Committee

Recommended a substitute checklist for the Male-Specific Coliphage in Proposal 05-113 with the Male-Specific Coliphage Laboratory Method recommendation for acceptance by the Laboratory Methods Review Committee with the changes recommended by the Laboratory Quality Assurance Committee (Changes are denoted in bold).

(1) Request that the ISSC Executive Board appoint a workgroup to review the current format of the checklists on the ISSC Website and report their findings back to the Laboratory Quality Assurance Committee via email and conference call set by the ISSC Executive Office. Laboratory Quality Assurance Committee will report to the Executive Board with revisions to the checklists posted on the website.

(2) Request that the ISSC Executive Board charge the Laboratory Quality Assurance Committee to review the SLV Protocol for Acceptance of a New Method for compliance with quality assurance requirements and specifically when a developer of a newly accepted method by the ISSC is required to submit a checklist for the method to the Laboratory Quality Assurance Committee for review.

(3) Request the ISSC Executive Office make available on the ISSC website the step-by-step procedures for newly accepted lab methods for use in the NSSP.

(4) Request the ISSC Executive Board to change the structure of the Laboratory Quality Assurance Committee to a subcommittee of the Laboratory Methods Review Committee for better use of the member's expertise.

Action by 2009 Task Force I Recommended adoption of Laboratory Quality Assurance Committee recommendation on Proposal 05-113 with additional recommendations.

Actionby 2009 General Assembly Adopted recommendation of 2009 Task Force I on Proposal 05-113.

Action by USFDA 02/16/2010 Concurred with Conference on Proposal 05-113.

Check the applicable analytical methods:			
	Multiple Tube Fermentation Technique for Seawater (APHA) [Part II]		
	Multiple Tube Fermentation Technique for Seawater using MA-1 [Part II]		
	Membrane Filtration Technique for Seawater using mTEC [Part II]		
	Multiple Tube Fermentation Technique for Shellfish Meats (APHA) [Part III]		
	Standard Plate Count for Shellfish Meats [Part III]		
	Elevated Temperature Coliform Plate Method for Shellfish Meats [Part III]		
	Male Specific Coliphage for Soft-shelled Clams and American Oysters		
CODE	**REF**		**Bacteriological Examination of Soft-shelled Clams and American Oysters for Male Specific Coliphage (MSC)**
			Equipment and Supplies
K	30		1. Sample containers used for the shucked sample are sterile, made of glass or some other inert material (i.e. polypropylene) and hold 100 – 125 ml.
C	27, 28		2. The refrigerated centrifuge used must have the capacity to accommodate the amount of shellfish sample required for the procedure, perform at 9000 x g and maintain a temperature of 4°C.
C	27, 28		3. The tempering bath(s) must be able to maintain the temperature within 2°C of the set temperature.
K	9		4. The level of water in the tempering bath covers the level of liquid and agar in the container or culture tubes.
C	27, 28		5. Sterile 0.22 µm pore size syringe filters and pre-sterilized plastic or sterile glass syringes are used to sterilize the antibiotic solutions.
K	1		6. The sterility of each lot of pre-sterilized syringes and syringe filters is determined.
K	1		7. The sterility of each batch of reusable glass syringes is determined.
C	27, 28		8. The balance used provides a sensitivity of at least 10 mg.
C	27, 28		9. The temperature of the incubator used is maintained between 35 – 37°C.
C	**28**		**10. Sterile disposable 50 ml centrifuge tubes are used and their sterility is determined with each lot.**
			Media Preparation
K	28		1. Media preparation and sterilization is according to thevalidated method.
K	27, 28		2. Bottom agar, double strength soft agar and growth broth are prepared from their individual components.
K	27, 28		3. Soft agar is prepared double strength in volumes of 2.5 ml.
C	**27, 28**		**4. The streptomycin and ampicillin solutions are added to tempered bottom agar.**

O	27, 28		5. Storage of the bottom agar under refrigeration does not exceed 1 month.
K	27, 28		6. Unsterilized soft agar is stored at -20°C for up to 3 months.
K	27, 28		7. The soft agar is removed from the freezer and sterilized for 15 minutes at 121°C before use.
K	27, 28		8. Storage of growth broth in the refrigerator in loosely capped tubes/bottles does not exceed 1 month and in screw capped tubes/bottles does not exceed 3 months.
K	27, 28		9. Bottom agar plates are allowed to reach room temperature before use.
		PREPARATION OF THE SOFT-SHELLED CLAMS AND AMERICAN OYSTERS FOR ANALYSIS	
K	2, 11		1. Shucking knives, scrub brushes and blender jars areautoclave sterilized for 15 minutes prior to use.
O	2		2. The blades of the shucking knives used are not corroded.
O	9		3. The hands of the analyst are thoroughly washed with soap and water prior to scrubbing and rinsing of debris off the shellfish.
O	2		4. The faucet used to provide the potable water for rinsing the shellfish does not contain an aerator.
K	9		5. The shellfish are scrubbed with a stiff, sterile brush and rinsed under water of drinking water quality.
O	9		6. The shellfish are allowed to drain in a clean container or on clean towels unlayered prior to shucking.
K	9		7. Prior to shucking, the hands (or gloved hands) of the analyst are thoroughly washed with soap and water and rinsed with 70% alcohol.
K	9		8. The shellfish are not directly shucked through the hinge.
C	9		**9. The contents of the shellfish (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	9		10. At least 12 shellfish are used for the analysis.
C	**2, 19**		**11. The sample is weighed to the nearest 0.1 gram.**
CODE	REF	**SAMPLE ANALYSIS**	
C	**28**		**1. *E.coli* F_{amp} ATCC 700891 is the bacterial host strain used in this procedure.**
K	27, 28		2. Host cell growth broth is tempered at 35 – 37°C and vortexed (or shaken) to aerate prior to inoculation with host cells.
K	27, 28		3. Several host cell colonies are transferred to a tube of tempered, aerated growth broth and incubated at 35 – 37°C to provide host cells in log phase growth for sample analysis.
C	27, 28		**4. Inoculated growth broth is incubated at 35 – 37°C for 4 to 6 hours to provide a host cell culture in log phase growth.**
C	27, 28		**5. After inoculation, the host cell growth broth culture is not shaken.**
C	28		**6. A 2:1 mixture of growth broth to shellfish tissue is used for eluting the MSC.**
C	**28**		**7. The elution mixture is prepared w/v by weighing the**

			sample and adding two equal portions of growth broth by volume to the shellfish tissue.
C	**28**		**8. The elution mixture is homogenized at high speed for 180 seconds.**
C	**28**		**10. Immediately after blending , 33 grams of the homogenized elution mixture are weighed into centrifuge tubes.**
C	**28**		**11. The homogenized elution mixture is centrifuged for 15 minutes at 9000 x g at 4°C.**
C	**27, 28**		**12. The supernatant is pipetted off, weighed and the weight recorded.**
C	**27, 28**		**13. The supernatant is allowed to warm to room temperature about 20 to 30 minutes.**
K	27, 28		14. The autoclaved soft agar is tempered and held at 50 – 52°C throughout the period of sample analysis.
K	27, 28		15. Two hundred microliters (0.2 ml) of log phase host strain *E coli* is added to the tempering soft agar immediately prior to adding the sample supernatant.
K	27, 28		16. The sample supernatant is shaken or vortexed before being added to the tempering soft agar.
C	**27, 28**		**17. 2.5 ml of sample supernatant is added to each tube of tempering soft agar.**
C	**27, 28**		**18. The soft agar/sample supernatant/host cell mixture is gently rolled between the palms of the hands to mix.**
C	**27, 28**		**19. The soft agar/sample supernatant/host cell mixture is overlaid onto bottom agar plates and swirled gently to distribute the mixture evenly over the plate.**
C	**28**		**20. 10 plates are used, 2.5 ml per plate for a total of 25 ml of supernatant analyzed per sample.**
K	27, 28		21. Negative and positive control plates are prepared and accompany each set of samples analyzed.
K	27, 28		22. Growth broth is used as the negative control or blank.
K	27, 28		23. Type strain MS2 (ATCC 15597) male specific Bacteriophage is used as the positive control.
K			24. A negative control plate is plated at the beginning and end of each set of samples analyzed.
K	27, 28		25. The positive control is plated after all the samples are analyzed and immediately prior to the final negative control.
C	**27, 28**		**26. All plates are incubated at 35 – 37°C for 16 to 20 hours.**
			COMPUTATION OF RESULTS
C	**27**		**1. Circular zones of clearing or plaques of any diameter in the lawn of host bacteria are counted.**
C	**28**		**2. The working range of the method is 1 to 100 PFU per plate. When there are no plaques on all ten plates, the count is <6 PFU/100 gm for soft-shelled clams and <7 PFU/ 100 gm for American oysters. If the density exceeds 100 PFU per plate on all plates, the count is given as > 10,000 PFU/100 gm.**

K	28		The formula used for determining the density of MSC in PFU/100 gm is: $(0.364)(N)(W_s)$, where N = total number of plaques counted on all 10 plates and W_s = weight of the supernatant used.
O	9		3. The MSC count is rounded off conventionally to give a whole number.

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