

**Standard Operating Procedure:***Ray's Fluid Thioglycollate Medium (RFTM) assay for detection of *Perkinsus marinus**

Revision History		
Version No.	Effective Date	Description
1.0	01/01/2024	Original composition

**Procedure Owners:****Date:**

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Mariah Kachmar, Field Project Lead,  
LISS Oyster Health Project

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Meghana Parikh, Veterinary Medical Officer

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Katherine McFarland, Research Biologist

**Approved By:****Date:**

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Lisa Milke, EAD Chief

## Scientific Standard Operating Procedure

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### 1 Purpose

The purpose of this SOP is to provide concise guidance and standard methodology on how to determine presence and infection intensity of *Perkinsus marinus* in oyster tissue using the Ray's Fluid Thioglycollate Medium (RFTM) assay.

### 2 Scope

This SOP applies to Northeast Fisheries Science Center, Milford Laboratory staff performing the RFTM assay on tissues from eastern oysters, *Crassostrea virginica* collected for the Long Island Sound Study (LISS) funded oyster disease project.

### 3 Definitions/Acronyms

RFTM - Ray's Fluid Thioglycollate Medium

### 4 Reagents/Media

- Fluid Thioglycollate Medium (FTM), Powder, R45352, Remel
- Lugol's Iodine (10g Potassium Iodide and 5g Iodine per 100mL DI H2O), R40029, Remel
- Penicillin G Sodium Salt 98% (1636.6 IU/mg), 455690050, Acros Organics
- Sodium chloride (NaCl) [7647-14-5] S-3014. Sigma Chemical Company, St. Louis, MO
- Streptomycin sulfate (720 IU/mg), 61224-0500, Acros Organics
- Nystatin 5g Amber Glass, BP29495, Fisher BioReagents

### 5 Supplies/Materials

- Flasks (1L and 250mL)
- Distilled water
- Deionized water (DI)
- Stirring rods
- Autoclave Mitts
- 50mL sterile falcon tubes
- Coverslips, various sizes, Corning Inc. Corning, NY.
- Culture Tubes with Screw Cap, Pyrex, 14-932A. Fisher Scientific, Pittsburgh, PA.
- Dissecting Tools- scissors, scalpels, probes, forceps
- Microscope Slides, Frosted, 2948-78X25. Corning Inc., Corning, NY.
- Pasteur Pipettes, 13-678-20A. Fisher Scientific, Pittsburgh, PA.

### 6 Equipment

- Corning Bottle Top Dispenser, 5mL, 6841, Corning Inc., Corning, NY.
- Hot plate with magnetic stirrer, PC-620D, Corning Inc., Corning, NY.
- Vortex mixer, Vortex-Genie 2 Model G-560, 12-812. Fisher Scientific, Pittsburgh, PA.
- Autoclave

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- Refrigerator
- Scale
- Compound Microscope
- Alcohol lamp

### 7 Safety Precautions

Laboratory members will wear gloves and protective eyewear when using chemicals. Members will follow standard laboratory procedures in the event of an accident (i.e. spilling chemicals, eye contamination, chemical burns) as well as for disposal of chemicals and any hazardous wastes. Chemicals will be clearly labeled and kept under a fume hood if necessary. Blades and scalpels will be kept covered when not in use and disposed of in a sharps container. Bunsen burners/EtOH lamps will not be left unattended and must be extinguished when not in use. Lab members will have knowledge of the location of the nearest first-aid kit. All Safety Data Sheets (SDS) for chemicals can be found in the binders on lab doors.

### 8 Laboratory Quality Control

Laboratory members will wear gloves and lab coats to reduce the risk of contamination. All equipment will be sterilized before and after use, as well as in between individual organisms. Tubes and slides will be pre-labeled prior to microscopy to streamline the process and prevent samples from being incorrectly identified. There will be designated stations/lab areas for each process. Multiple members of the lab will be processing samples at the same time to ensure everyone is using the same methods and corrections can be made when necessary. Additionally, multiple lab members will read slides to ensure agreement and consistency in results. Guidelines for assigning infection intensity and other steps in the process will be discussed between members prior to start to ensure uniformity. Samples will also be done in subsets at regular intervals to increase accuracy.

### 9 Preparation

1. At least one day prior to performing RFTM assay, examine the lab to make sure the area is ready for processing. There should be adequate space in the sharps containers and chemical waste bins. If there is not, properly dispose of materials to create a clear working environment. Trash bins containing non-biohazard waste should also be emptied in the facility dumpster in the parking lot. Bins should not exceed 75% full.
2. Take inventory of required supplies. Make sure there are adequate amounts of supplies and reagents needed.
3. Ensure any glassware or dissection tools have been properly cleaned and sterilized for the procedures (See Appendix A).
4. All laboratory stations should be set up and cleared of unnecessary equipment for easy work flow.

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5. If needed, calibrate any equipment used in the procedures (i.e. scales, etc).
6. Make sure all equipment is running properly such as heat plates and microscopes.
7. Make sure all culture tubes and tube racks have been labeled prior to tissue processing.
  - a. Tubes should contain the first initial of the site name (A,F,G,S) and the sample number, 1–30. The tube rack should contain a label with full sample ID details (MMYY SITE\_01->30) and tissue processing date.
8. **RFTM (steps 1-3 in procedures) and preparation of culture tubes should be done at least one day prior to sampling.**
9. New electronic data collection sheets should be generated, organized, and dated, and they should be printed or electronically available for data recording. See [TEMPLATE\\_RFTM](#).
  - a. The template data sheet should be copied and renamed as MMYY SITE\_RFTM. This data sheet should be saved in the Google Drive data repository (Project Planning > Data Management > Lab Data > RFTM > Working\_Folder).
  - b. Cells B2 and C2 should be updated to reflect the collection date and site for the sample set, and the date the slides are read should be entered in column E (and I if the slide is read by more than 1 person).

### 10 Procedures

*Advance preparation of solutions needed for tissue sampling.*

1. Antimicrobial Solution Preparation
  - a. Penicillin-Streptomycin solution (*This solution can be prepared up to 6 months in advance*)
    - i. Autoclave 500mL of deionized (DI) water for 15 minutes at 121°C (15psi) on the slow exhaust setting.
    - ii. After sterile DI water is cool to the touch, add 0.33 g of Streptomycin Sulfate and 0.159 g of Penicillin G. \*\*This amounts to a final concentration of 520 IU/mL Penicillin G and 475 IU/mL Streptomycin sulfate.\*\*
    - iii. Swirl lightly until powder is dissolved.
    - iv. The solution can be stored and maintain potency at 4°C (refrigeration) for 1-2 weeks. For larger batches aliquot desired amount (15mL), label, and

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store the remaining in the freezer (-20°C) until needed. The solution will maintain potency in the freezer for ~6 months.

- v. Record the preparation in the lab reagent log and assign a batch number.
  - b. Nystatin Solution (*This solution should be prepared on the same day that RFTM tubes are being prepared*)
    - i. In a small falcon tube mix 4mg of nystatin powder with 4mL of sterile DI water. (0.1g/100mL). This volume is needed to make 70 RFTM culture tubes. Adjust volumes as needed.
    - ii. Mix ingredients well. This solution should be immediately mixed with the Penicillin-Streptomycin solution (10.1.a) and added to the final RFTM tubes (10.2.k).
  - c. Combining for final antimicrobial solution (Penicillin-Streptomycin-Nystatin) (*This solution should be prepared on the same day that RFTM tubes are being prepared*)
    - i. Thaw Penicillin-Streptomycin to room temperature in a warm water bath. 40mL of penicillin-streptomycin and 4mL nystatin solution (see 10.1.b) is needed to make 70 RFTM culture tubes. Adjust volumes as needed.
    - ii. Combine penicillin-streptomycin and nystatin solutions in a 50mL sterile tube. Cap the tube and invert several times to mix. Keep aside until ready to combine with RFTM (See 10.2.k)
2. Ray's (1952) Thioglycollate Medium (RFTM) preparation

- a. In a flask, add Sodium chloride (NaCl) and dehydrated Fluid Thioglycollate medium (FTM) into distilled water. Adjust quantity of ingredients based on the desired number of tubes. Suggested amounts:

Final volume	# of culture tubes	NaCl (g)	FTM (g)	Distilled water (mL)
1L	200	22	29.3	1000
650mL	130	14.3	19	650
350mL	70	7.7	10.3	350

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- b. Preheat the heat/stir plate at the highest setting (varies by heat plate), put the flask on a heat plate, and stir continuously with the stir bar until dissolved.
- c. The mixture will be pink and change to a golden-yellow color as it begins to boil. This can take 30-40 minutes depending on the heat plate. Keep a close eye on the flask. It will start from a gray/blue to pink color. Once it turns to pink, turn the heat down slightly to avoid overflow.
- d. While the solution is heating, prepare a water bath at room temperature. Obtain clamps/tongs and heat safe gloves to transport the flask.



**Figure 1:** Image showing the media color just prior to boiling. Vortex shows an indicator of the speed of the stir bar.

- e. The solution will begin to change to a golden-yellow color (Figure 1). When this color is achieved immediately take the mixture off of the heat plate. **Do not let it overflow from boiling.**
- f. Using tongs or heat gloves, carefully place the flask into a tub of room temperature water to cool down. Note that as the solution cools it will turn back pink.
- g. After the flask is cooled to the touch, dispense 5mL of solution into each 15mL culture tube using the "Corning Bottle Top Dispenser".

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- i. If needed, obtain the instruction Manual.
- ii. Attach the telescopic tube (adjustable) to the dispenser. Be sure that the tube touches the bottom of the flask. This may take a few length adjustments of the telescopic tube.
- iii. Make sure the reflux tube (smaller tube) is attached to the dispense.
- iv. Screw the dispenser onto the desired bottle.
- v. To purge air from the dispenser, turn the discharge tube (outer spout) to 90 degrees. Set a small volume (1-2 mL). Raise and press the dispenser a few times until liquid exits the reflux tube.
- vi. Return the discharge tube to 0 degrees or the position to allow liquid to dispense.
- vii. To dispense, adjust to the desired volume (5mL). Raise the housing until it stops and press the housing to the lowest point. The movement should be slow and consistent to avoid air bubbles and achieve the exact amount desired.
- h. Screw the caps loosely onto the tubes.
- i. The tubes with medium are then autoclaved for 15 minutes at 121°C (15psi) on the slow exhaust setting. When autoclaving is finished, allow tubes to cool to the touch.
- j. Pipette 550µL of antimicrobial solution (prepared in 10.1.c) to each RFTM culture tube. As the solution is added to each tube, cap tightly and vortex quickly for 1-2 seconds. Continue until solution is added to all tubes.
- k. Label the culture tubes and tube racks according to the instructions in 9.7.
- l. Record the preparation in the lab reagent log and assign a batch number. Log reagents used to prepare original RFTM media, nystatin solution, and the penicillin-streptomycin solution batch number.
- m. Place the culture tubes in the refrigerator and in the dark until ready for use.  
 \*\*The RFTM may change color from yellow to pink when vortexed. They should return to the yellow once cooled in the refrigerator\*\*
  - i. Unused autoclaved tubes of RFTM can be stored for many months in the dark without deterioration.
  - ii. Discard them if they become cloudy or the RFTM congeals.

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n. *IMPORTANT: RFTM maintains anaerobic conditions in the culture tube as well as providing nutrients and an appropriate osmotic environment. Therefore, tubes are kept sealed tightly and only opened briefly to add antibiotics and tissue as described below. After tissue is added, seal the tubes and return them immediately to the dark for the tissue to incubate.*

- i. There is a color change indicator that is yellow when anaerobic and will change to pink when conditions become aerobic (due to the presence of resazurin). If the tubes demonstrate >30% oxidation (color change to pink), they can be reheated 1 time. Heat tubes with caps loosened for approximately 10 minutes in a boiling water bath (100°C) until the pink disappears. Cool to <45°C before inoculating with tissue.

### 3. Lugol's Iodine Solution for staining

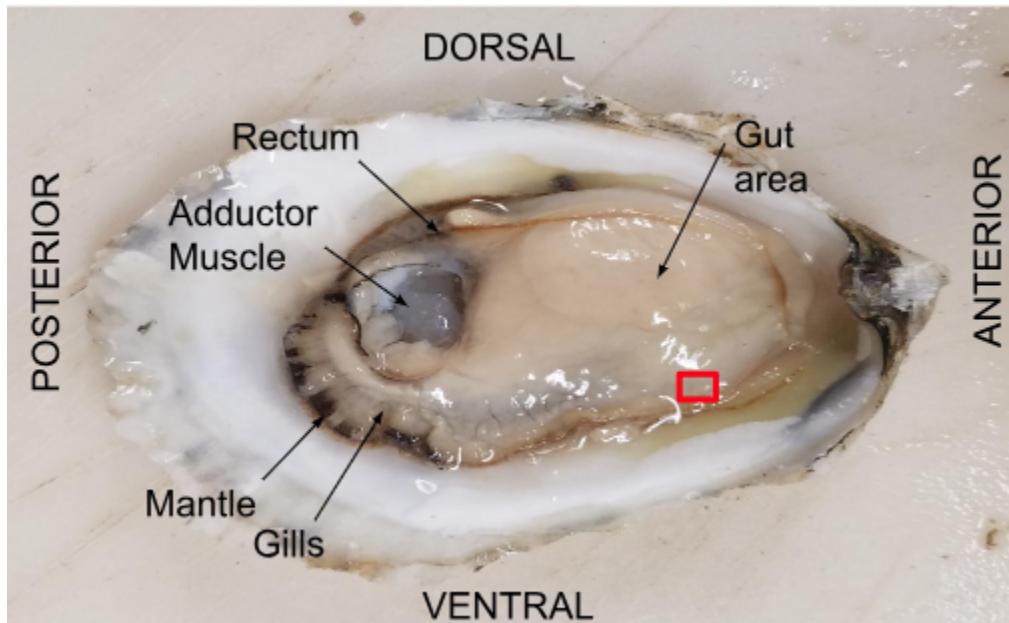
- a. Stock product being used is Lugol's Iodine containing 10g Potassium Iodide and 5g Iodine per 10mL of DI water.
- b. Calculations for working solution:
  - i. 10mL stock Lugol's + 20mL DI water = 3.33g Potassium iodine and 1.66g Iodine per 100mL

WOAH recommends: 2g Potassium Iodide and 1.3g Iodine per 100mL

### 4. Inoculation and Incubation of oyster tissues in culture tubes

- a. Using sterile dissecting scissors and forceps, a 5x5mm piece of mantle-edge tissue is excised from just over the palps. (Figure 2) \*\*Full protocol for tissue excision can be found in the Oyster Tissue Processing SOP\*\*
- b. Place the tissue in a culture tube containing 5 mL (500µL) RFTM, 0.5mL Penicillin-Streptomycin and 50µL nystatin solutions (See 10.1.k). Use a sterile probe to fully submerge the tissue in the bottom third of the RFTM.
- c. Screw cap back on tightly.
- d. Incubate culture tubes with tissues in the dark at room temperature and for at least 5 days.
- e. If not analyzed within 7 days of inoculation, the tube should be placed in a refrigerator in the dark. Tissues should be analyzed within 2 weeks after being placed in the refrigerator.

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**Figure 2:** Anatomy of *Crassostrea virginica* for aid in dissection. The **Red square** is the location at which tissue should be extracted for RFTM preservation.

## 5. Tissue Analysis/Diagnostics

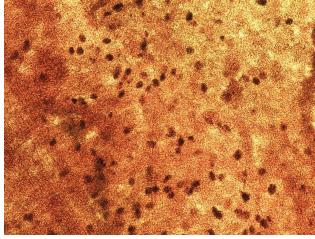
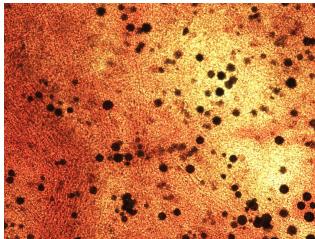
- a. After the tissue incubation period, remove the oyster tissue from the RFTM using a sterile probe and place it on a microscope slide.
- b. Tease apart the tissue sample using a sterile probe to ensure even staining with Lugol's iodine solution. **\*\* Instruments are ethanol and flame sterilized between samples. See Appendix A. \*\***
- c. Add 1-2 drops of Lugol's Iodine solution to the tissue with a Pasteur pipette and then cover with a cover slip.
- d. Examine microscopically on a compound microscope. Entire tissue is examined under the microscope at 10x, 40x, or 100x as required to visualize *P. marinus* hypnospores. RFTM will enlarge parasite hypnospores of all sizes and Lugol's Iodine will stain hypnospores black. The size range for hypnospores is 5-300µm. Hypnospores are spherical in shape and to be carefully differentiated between parasite and debris.
- e. Presence is recorded and infection intensity is assigned according to the Mackin Scale (Table 1).

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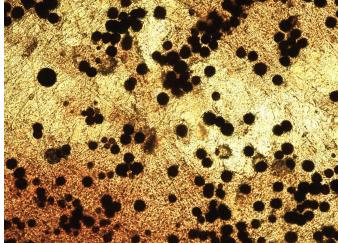
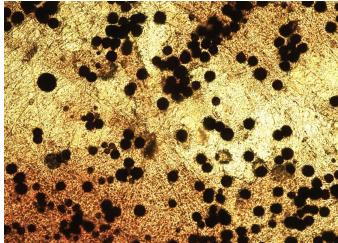
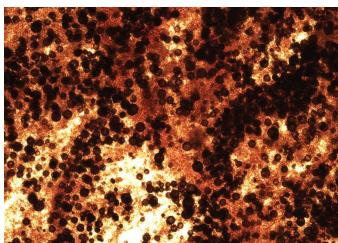
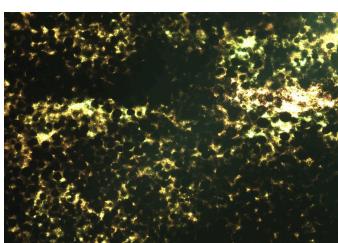
- i. Each month, 10% of all samples should be independently read by two people for quality control.

**Table 1:** Mackin Scale (Ray 1954a, 1954b). Ray's Fluid Thioglycollate Method images from Ray, S.M.

Infection intensity	Numerical Value	Description	
Negative (N)	0	No hypnospores	
Very light (VL)	0.5	1-10 hypnospores	 (note single dermo cell among many brown cells)
Light (L)	1	11-125 hypnospores; less than 25% of the tissue is hypnospores	

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Light/Moderate (LM)	2	25% of tissue is hypnospores	
Moderate (M)	3	50% of tissue is hypnospores	
Moderately heavy (MH)	4	75% of tissue is hypnospores	
Heavy (H)	5	>75% of tissue is hypnospores to 100%	

- f. Calculations of prevalence and intensity will be reported using the equations below:

$$\text{Prevalence \%} = \frac{\text{\# of infected individuals per site}}{\text{total \# of individuals collected per site}}$$

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$$\text{Weighted Prevalence \%} = \frac{\text{Sum of intensity scores}}{\text{total \# of individuals collected per site}}$$

$$\text{Intensity (population)} = \frac{\text{Sum of intensity scores}}{\text{\# of individuals infected per site}}$$

- g. See [Triplex qPCR protocol](#) for molecular approach to quantification of *P. marinus* DNA in oyster tissue.

### 6. Results reporting

- a. Results will be recorded in the lab notebook and will be transcribed into the digital data sheet (see 9.9).

### 7. Clean up/Disposal of Tissue

- a. Used slides with tissue and coverslips will be disposed of in the sharps container.
- b. The spent tubes with inoculated medium are then autoclaved with the caps loosely fastened for 30 minutes at 121°C (15psi) on the slow exhaust setting. Once the liquid is cool to the touch, it can be flushed down a sink that discharges to the sewage treatment line.
- c. The Corning Bottle Top Dispenser:
  - i. Turn the discharge tube to 180 degrees to allow the remaining to flow back into the bottle.
  - ii. Screw off the dispenser from the bottle and drain the telescopic tube by purging as described above.
  - iii. Fill a bottle with distilled water or alcohol and turn the discharge tube to 0 degrees or dispense mode.
  - iv. Dispense multiple times until the dispenser is clean and cleared of any reagent.
  - v. If necessary, disassemble the dispenser and clean the components.
  - vi. The dispenser is autoclavable to sterilize. Remove the reflux tube and telescopic tube . Steam- sterilize dispenser at 121°C for 15 minutes.
- d. Dissection tools used for processing tissue should be sterilized using an ethanol flame followed by washing with soap and water. Additional supplies such as trays, beakers, etc. should be washed with soap and water.

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## 11 Data Quality Control

Laboratory data will be recorded on data sheets and transcribed to digital data records on the secure NOAA server within 3 business days of the date of collection and processing (See 9.9 and 10.9 for specific instructions). This will ensure all notes are correctly updated while the events are fresh in the team's memory. Digital data files will be QC'd by a second individual alongside the data sheets to ensure all data was correctly entered within one week of data entry. Discrepancies in the data will be resolved by comparing data sheets and digital logs alongside lab notebooks that contain site and date specific information that could explain outliers in the data. A record of the lab recorder, person entering the data, and QC completion will be logged to ensure all tasks are completed prior to final data collation. Each month after QC, data will be collated with the previous data and exploratory plots updated. This will serve as an added check for potential error in the data collection and archiving process.

All physical laboratory data sheets will be archived and available at the NOAA Milford Laboratory. Purely digital files (eg. sonde data, photos, digital data sheets, etc.) will be uploaded directly to the NOAA server which is backed up regularly.

## 12 References

Ray, S.M., 1954a. Biological studies of Dermocystidium marinum, a fungus parasite of oysters. Rice University, The Rice Institute Pamphlet Special Issue.

Ray, S.M., 1954b. Studies of the occurrence of Dermocystidium marinum in young oysters.,in: Proc. Natl. Shellfish. Assn. pp. 80–92.

Ashton-Alcox, K.A., Kim, Y., and Powell, E.N. *Perkinsus marinus* Assay protocol. Haskin Shellfish Research Laboratory.

## 13 Appendix

### A. Standard methods for sterilization of dissection tools

1. All tools are sterilized between individuals following the steps listed below:
  - i. Freshwater dip
  - ii. Bleach and sand dip to remove tissue (1:10 dilution)
  - iii. Freshwater dip
  - iv. 95% ethanol dip
  - v. Ethanol is burned off using a flame (allow to cool before reusing)



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- b. [RFTM datasheet template](#)
- c. [Oyster Tissue Processing SOP](#)
- d. [Triplex qPCR protocol](#)

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