

Standard Operating Procedure:
Oyster Tissue Processing & Preservation

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

Procedure Owners: _____ **Date:** _____

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1 Purpose

The purpose of this document is to provide a standard method of processing live oysters and preserving tissue samples for various diagnostic assays using sterile techniques. This protocol enables multiple diagnostics on a single oyster reducing the number of sacrificed animals.

2 Scope

This SOP applies to Northeast Fisheries Science Center, Milford Laboratory staff processing live eastern oysters collected for the Long Island Sound Study (LISS) funded oyster health and disease project and being preserved for microbial culture, histology, and molecular diagnostics.

3 Definitions/Acronyms

RFTM - Ray's Fluid Thioglycollate Medium

4 Reagents/Media

- 95% Ethanol
- 70% Ethanol
- Penicillin-Streptomycin Solution
 - Penicillin G Sodium Salt 98% (1636.6 IU/mg), 455690050, Acros Organics
 - Streptomycin sulfate (720 IU/mg), 61224-0500, Acros Organics
- Nystatin Solution
 - Nystatin 5g Amber Glass, BP29495, Fisher BioReagents
 - Deionized water
- Bleach
- Davidson's Fixative
 - Glycerin, Reagent Grade, LabChem, LC148502
 - Formaldehyde, 37% by weight (Histology), Fisher Chemical, F75P-20
 - Ethanol (95%)
 - Sodium chloride (NaCl) [7647-14-5] S-3014. Sigma Chemical Company, St. Louis, MO
 - Deionized water
- RFTM
 - Sodium chloride (NaCl) [7647-14-5] S-3014. Sigma Chemical Company, St. Louis, MO
 - Fluid Thioglycollate Medium (FTM), Powder, R45352, Remel

5 Supplies/Materials

- Gloves (Nitrile and Shucking)
- Lab coats
- Lab markers/pens/pencils

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- Scale
- Bristle Brush (for cleaning oysters)
- Paper towels
- Spray bottles for bleaching equipment
- Freshwater
- Caliper
- Sand
- Tin foil or weigh boats
- Trays
- Shucking knife
- Dissection tools - scalpel, scissors, probes, and forceps
- Pathology blades
- Glass or histology safe plastic jars
- Bunsen burner or ethanol lamp flame
- Delicate task laboratory wipes
- Histology cassettes
- 1.5 mL Microcentrifuge tubes PCR clean
- 15mL glass culture tubes and screw caps
- Pipette set (1000µL, 200µL, 50µL, 10µL, 2µL)
- Pipette tips (1000µL, 200µL, 50µL, 10µL, 2µL)

6 Equipment

- Refrigerator

7 Safety Precautions

Laboratory members will wear gloves to process the samples. Nitrile gloves will be worn when handling tissue and shucking gloves will be worn when shucking/opening live oysters. 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 in a fume hood if necessary. Steps within the process will also be conducted under a fume hood (see below). Blades and scalpels will be kept covered when not in use and disposed of in a sharps container. Bunsen burners/Ethanol lamps will not be left unattended and must be turned off. 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 (nitrile or shucking) 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 histology cassettes will be pre-labeled prior to processing tissue 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 process

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samples at the same time to ensure everyone is using the same methods and corrections can be made when necessary. Guidelines for measuring and other steps in the process will be discussed between members prior to start to ensure uniformity. For tissue sectioning for histology, there will be a gauge at the station to ensure proper thickness of the cross section and photos will also be posted in the area for easy reference.

9 Preparation

1. At least one day prior to field sampling, 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. Check the supply list for the procedures and take inventory. Make sure there are adequate amounts of supplies and reagents needed.
3. All laboratory stations should be set up and cleared of unnecessary equipment for easy work flow.
4. Calibrate any equipment used in the procedures (i.e. calipers, scales, etc).
5. Cut and label foils for numbering oysters and keeping track.
6. Cut clean paper towel pieces for sampling individual oyster tissues.
7. Label all microcentrifuge tubes for qPCR, jars and cassettes for histology, and culture tubes for RFTM.
 - a. Cassettes for histology should be labeled using a pencil with the individual sample ID, following the scheme: MMYY SITE_##
 - i. MMYY refers to the collection month and year
 - ii. SITE is the 4 letter abbreviation for the collection site (ASHC = Ash Creek, FENC = Fence Creek, GOLD = Goldstar 2022 reef, and STAR = Goldstar 2023 reef)
 - iii. ## is the 2 digit individual oyster sample number
 - iv. The histology jar should be labeled with the sample ID set and PI last name. Leave room to add fixative details after samples are added.
 - b. Microcentrifuge tubes will be split into main and duplicate sets.
 - i. The main set will be labeled with permanent marker with the individual animal ID (MMYY SITE_##) on the side, and A/F/G/S## on the lid.
 - ii. The duplicate set should be labeled by printing full identifying details on waterproof paper (example [here](#)) and taping to the tube with packing tape (this will assure that split ethanol does not remove labels written with marker and that all information is legible).
 - iii. The main samples will go into a single freezer box and will be grouped by collection month and state (ie. CT samples from the same month in one box). The duplicate samples will go into a separate freezer box with the same grouping. Freezer boxes should be labeled on the top and side of the bottom with the following information:
 1. Sample IDs of individual oysters included in the set. This is typically 30 oysters.
(MMYY SITE_##-->##; ie. 0423ASHC_01→30)

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2. Tissue type (ie. Oyster Gill, Gut, Rectum)
3. Main or Duplicate
4. Preservation type (fresh frozen or 95% EtOH)
5. PI Name
8. Prepare any media/reagents needed for processing. Pre-fill duplicate set of microcentrifuge tubes with 95% ethanol for the qPCR samples. Prepare RFTM, (see "[RTFM](#)" SOP) and pre-fill labeled culture tubes. Prepare Davidson's fixative (see "[Davidson's fixative & Histology slide preparation](#)" SOP).
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_TissueProcessing](#).
 - a. The template data sheet should be copied and renamed as MYYYSITE_TissueProcessing. This data sheet should be saved in the Google Drive data repository (Project Planning > Data Management > Lab Data > Tissue Processing > Working_Folder).
 - b. Cells B2 and C2 should be updated to reflect the collection date and site for the sample set.

10 Procedures

1. Upon arrival at the laboratory, animals are kept refrigerated until ready for processing. Processing should occur within 48hrs of collection to maintain integrity and quality of tissue.
2. Prepare a work station by setting up the sterilization station described in step 10.7.a. below. Make sure there are adequate dissection tools, trays, and take inventory of the labeled tubes for RFTM, qPCR ethanol preserved, and histology cassettes. Make sure there are pre-cut foil and paper towels. Obtain the caliper and scale to measure and weigh the animals. Prep the oyster cleaning sink with a 6% bleach solution, brushes, and shucking knives.
3. Using cold freshwater, scrub animals with the bristle brush and rinse animals well. With a shucking knife, remove all fouling organisms such as spat, barnacles, and algae.
4. Scrub hinges with a toothbrush and 6% bleach (diluted 1 part bleach: 10 parts water) to sanitize the area. Use a paper towel to remove excess water and set aside on an individually labeled piece of foil.
5. Using digital calipers, measure the shell height from the hinge to the free edge (anterior-posterior axis) of the shell to the 0.1 millimeter (Figure 1).

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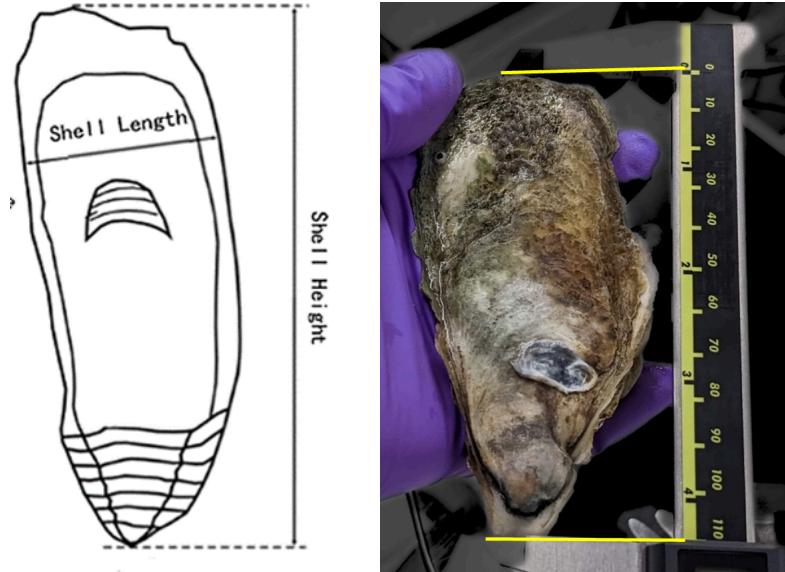


Figure 1. Demonstration of shell measurements (mm) on external anatomy of an oyster shell.

6. Using a sterile shucking knife, open each oyster by severing the adductor muscle at its attachment to the upper (right) shell. ***Be sure to keep the body intact (not damaging any tissue with the shucking knife) and in the cup of the left shell*** (Figure 2).

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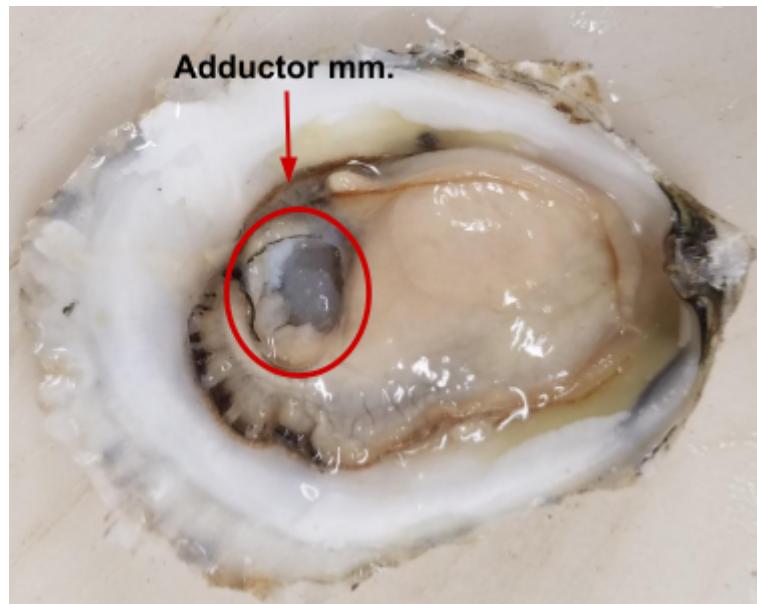


Figure 2. Shucked Oyster

- a. 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; 6%)
 - iii. Freshwater dip
 - iv. 95% ethanol dip
 - v. Ethanol is burned off using a flame (allow to cool before reusing)
7. After shucking the animal, visually inspect the body and assign a **body condition rating** (Table 1). This will serve as an indicator of the oyster's overall health status.

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Table 1. Oyster condition rating key (from Quick and Mackin 1971; Histological Techniques for Marine Bivalves Mollusks and Crustaceans. NOAA Technical Memorandum NOS NCCOS 5, 2004.)

Condition Index	Score	Oyster appearance	Photo reference
Very good	1	Animal firm and filling shell cavity; coloration creamy white and evenly textured; usually ready to spawn	
Good	2	Not quite as firm or large as above; usually ready to spawn	
Good minus	3	Coloration less opaque, often slightly yellow or gray	

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Fair plus	4	Animal distinctly not filling shell cavity; coloration often mottled, with blood vessels and muscle fibers showing through the more translucent epithelium	
Fair	5	Oyster well-developed but not opaque or tending toward white; grayish and translucent; flesh flaccid	
Fair minus	6	Translucency more pronounced	

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Poor plus	7	Oyster not well-developed, darker gray, often greenish; pericardial cavity clear; small portion of shell cavity filled	
Poor	8	Negative qualities more accentuated	
Very poor	9	Animal distinctly atrophied; coloration dark and uneven, very translucent; seldom more than third of shell cavity occupied; adductor muscle often discolored and transparent even in the normally white sector	

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***** All individuals will be preserved for each of the following processes *****

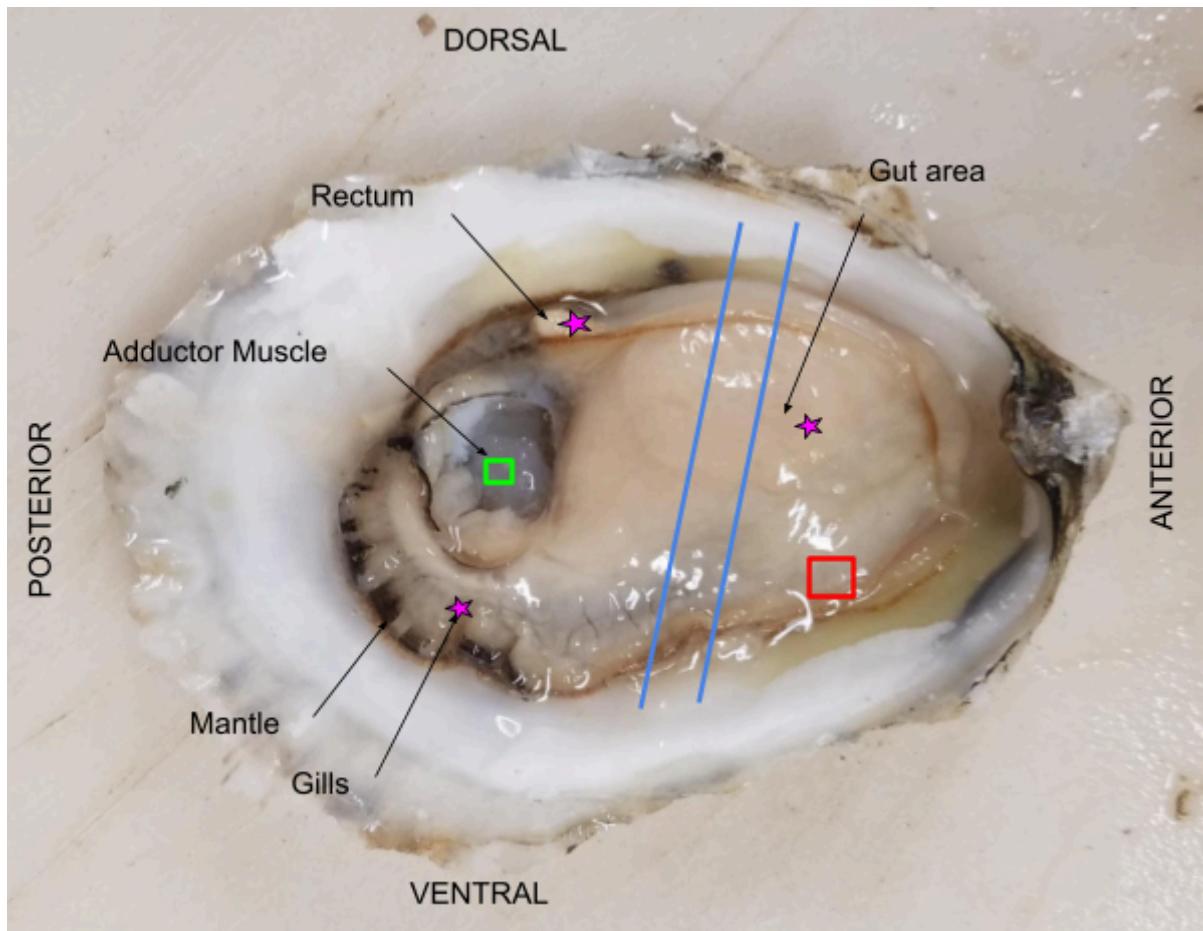


Figure 3: Anatomy of *Crassostrea virginica* for aid in dissection. Blue parallel lines are the location at which histological sectioning should occur. The Red square is the location at which tissue should be extracted for RFTM preservation. Pink stars are the locations at which tissue should be taken for qPCR samples. Green square is the location at which adductor muscle tissue should be taken for archiving.

8. For RFTM:

- Using sterile dissecting scissors and forceps, a 5x5mm piece of mantle-edge tissue is excised from just over the palps. Use a ruler as a guide (Figure 3).
- Place the tissue in a culture tube containing 5 mL RFTM. Use a sterile probe to fully submerge the tissue in the bottom third of the RFTM.

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- c. Screw cap back on tightly.
 - d. Culture tubes with tissues are placed in the dark at room temperature and incubated for at least 5-7 days.
 - e. If not analyzed within 7 days of processing, the tube should be placed in a refrigerator in the dark (samples should be analyzed within 2 weeks of being put in the refrigerator).
 - f. For tissue analysis, see "[RFTM](#)" SOP.
9. For DNA Extractions for triplex qPCR diagnostic assay and tissue archiving:
- a. Using sterile instruments, duplicate samples of gill, gut and rectal tissues are collected. One set of each tissue is stored together in empty 1.5mL microcentrifuge tubes and held on ice (or cold blocks) until processing is complete. The duplicate set is stored together in 1.5mL microcentrifuge tubes in 95% ethanol. To enable additional samples to be collected from the same individual, tissues must be precisely excised from specific locations noted in Figure 3, and no more than 0.5mg of tissue is removed (Figure 4). *****A duplicate sample from each oyster will be collected and stored for backup *****
 - b. Instruments used to retrieve tissues are sterilized in between oyster samples by the same methods described in section 6a.



Figure 4: Size comparison of 0.5mg of gut, mantle, and gill tissue samples from eastern oysters for DNA extraction. Size of each tissue type is no larger than the tip of a fine-point forceps.

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10. For Histology:

- a. Following sample collection for qPCR and RFTM analyses, the remaining oyster shell and body will be transferred to a fume hood to be processed for histology.
All processing steps involving Davidson's fixative should be completed in a fume hood.
- b. Use a shucking knife to carefully remove the oyster body from the shell and place on a flat surface. The shell can be discarded in the regular waste stream according to Milford Laboratory protocol.
- c. Using a pathology blade, make a single clean diagonal cut across the body of the animal (Figure 3). Ensure the cross-section includes gill tissue and avoids the palps. Larger or more elongated oysters may call for a steeply angled diagonal cut to ensure that the proper tissues are being sectioned.
- d. Make a second diagonal cut on the bottom half (without the adductor muscle) of the oyster (Figure 3). *The cross-section should be 4mm thick and include portions of the mantle, stomach, digestive tubules, gonads, kidney, and gills.*
- e. Take the cross section and place it into the cassette (Figure 5), which was prelabeled using pencil.
- f. Each pathology blade should only be used on 1-2 oysters, as needed to ensure sharp,clean cuts.



Figure 5. Cross section placed into histology cassette.

- g. Place the cassettes with tissue into Davidson's fixative (see "[Davidson's Fixative and Histology](#)" SOP) as samples are being processed. Cassettes should always

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be fully immersed in solution. Approximately 500mL of Davidson's fixative should be used per sample set of 30 oysters. Clearly label the jar with the date and time samples were fixed as well as sample site information and "in Davidson's fixative". Store in the refrigerator for a minimum of 24 hours (maximum 48 hours).

- i. After the fixation period is complete and while under a fume hood, drain the fluid into a clearly labeled chemical waste container and fill the jar with 70% ethanol to cover all of the cassettes. Add the date of ethanol transfer to the bottle label and initial. *This is a wash step and tissue should be soaked for at least 24hrs.*
- ii. Cassettes should be soaked in ethanol for at least 24 hours before being embedded in paraffin, but they can be stored in a cool secure location for several weeks while preserved in ethanol.
- h. For processing and slide preparation, see "[Davidson's Fixative and Histology SOP](#)".

11 Clean up

1. All oyster tissue should be thrown in the trash and removed from the laboratory at the end of the day.
2. Oyster shells should be moved to the basement walk-in fridge and stored in a labeled, closed container for donation to shell recycling.
3. All tools, trays, cutting boards, tripours, etc. should be washed with Alconox detergent, rinsed, dried, and put away.
4. All lab surfaces should be sprayed with the appropriate disinfectant and wiped down.
5. Any hazardous chemicals should be returned to the fire safety cabinet.
6. Tissues collected in microcentrifuge tubes for qPCR analysis are stored in the -80°C freezer in Building 1, Room 17. Fresh frozen tissues are placed on racks labeled for short-term processing, while tissues in ethanol are added to storage racks for archived tissue.
 - a. Document addition and location of new samples in the [Molecular Lab Inventory Sheet](#)
7. Histology samples preserved in ethanol should be placed in the fire safety cabinet following fixation in Davidson's.
8. All collected samples should be documented in the [LISS Oyster Health Project Sample Inventory sheet](#).
9. Inventory of supplies should be taken to prepare for the next sampling period.
10. All tissue processing activities should be recorded in the laboratory notebook and data sheets should be updated and checked for quality assurance and control.

12 Data Quality Control

Laboratory data will be recorded directly into digital data sheets or recorded and transcribed to digital data records on the secure NOAA server within 3 business days of the date of



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collection and processing. 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 and notebooks 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.

13 References

Quick, J. A., and J. G. Mackin (1971) Oyster parasitism by *Labyrinthomyxa marina* in Florida. Fl. Dept. Nat. Resour. Mar. Res. Lab. Prof. Pap. Ser., 13:1-55.

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