**Poly (I:C) Pacific Oyster RNA Extraction Protocol (Madeline Baird)**

**Samples**:

Multiple oyster lifestages in tubes with DNA/RNA Shield in the -80 freezer next to the cold room in FTR

**Materials**:

Zymo Quick DNA/RNA Mini Prep Plus Kit and Protocol (Catalog Number D7003) (Lot number: 253939)

Eppendorf AG 22331 Hamburg Centrifuge

Fisher Scientific Vortex Mixer

Next Advance Bullet Blender 5E Gold+ (Serial Number: BB5EAUPD-55917)

Brady M510 Label Printer

Qubit 3.0 Fluorometer (with Flashdrive)

Eppendorf Pippettes (10,20,200 and 1000 microliter) (many 1000 microliter needed)

USA Scientific Pipette tips (10,20,200,1000 microliter)(many 1000 microliter needed)

Plastics

* DNA LoBind Tube 0.5 mL SafeLock Tubes (for bead beating)
* Olympus Plastics 1.7 mL Microtubes (Cat. No. 22-281S)(Lot No. 13822016) (Labeled and used for RNA only)
* Fisher brand Disposable Centrifuge tubes (Cat. No. 05-539-4)
* Thermofisher Scientific Qubit Assay Tubes

100% Ethanol

Invirtogen Qubit RNA HS Assay Kit, 100 assays (REF Q32852)

Brady M5-120-492 Label Printer Labels (with circle and rectangle areas)

Next Advance ZROB05 0.15 and 0.5 mm Zirconium Oxide Beads

Next Advance MSP01-RNA 0.10 microliter measuring spoon

Next Advance SLV1E5A 1.5mL Tube Sleeves (for bead beating)

Plastic tube holders

Sample freezer boxes

Ice bucket

Standard consumables (gloves, KimWipes, pipette tips) \*note that many 1000 µL tips are needed

**Protocol:**

Sample Preparation

* Obtain ice from Support room 216 to thaw DNase I (DNase in the -20 C Freezer 24). Use an ice bucket to hold the ice and DNase I as it thaws.
* Remove Quibit RNA Assay kit from Fridge 23 (Red Bag)
* Remove Samples from the -80 C Freezer

Bead Beating

* Obtain Dry Ice from Health Sciences
* Have Husky Card to get into building
* Bring ice bucket that is by the bead beater machine in FTR 213
* Down the stairs and to the left
* Sign the clipboard with Lab Information
* Place ice into the right side of the bead beater machine
* Bead beat the samples to break down debris
* Beads and scoop are in 213
* Scoop 0.10 G of 0.15mm beads into Safe Lock Eppendorf tubes (located on the shelf closest to the door in 209) (or used 0.5 beads when I ran out of 0.15 and scooped about half of the 0.10 G scoop)
* Pipette the liquid up and down a couple times to suspend the oysters in the liquid rather than having them sit on the bottom
* Pipette to move the mixture from the original sample tube to the Safe Lock Eppendorf tube with the beads.
* Place Safe Lock Eppendorf tubes into the white casings for them (in 213 next to Bead Beater) to sit in the Bead Beater machine
* Run machine at speed 12 for 5 minutes
* Let the foam dissipate

**NOTE**: Some samples used different sized beads due to what we had in inventory.

Samples 107-114 used 0.5 mm beads (½ 0.10 scoop)

Sample 127 used 0.15 and 0.5 half and half beads

Samples 115-126 0.15 mm beads (½ 0.10 scoop)

Samples 128-160 0.5 mm beads (½ 0.10 scoop)

Potential Storage OR RNA Isolation; Can stop here if needed between days and store samples at -80°C

RNA Extraction

* Use Zymo Research DNA/RNA MiniPrep Plus Kit (Tubes for this come in the box) (I opened all of the kits, mostly by need of columns and tubes)
* If using a new kit, add Ethanol to the Wash Buffer bottles (pg 5 of protocol) (don’t need the last two steps/checkmarks)
  + Add 96 ml 100% ethanol ( 104 ml of 95%) to the 24 ml DNA/RNA Wash Buffer concentrate(D7003T)
* Add 275 microliters of DNase free water to DNase if new tube
  + Reconstitute lyophilized DNase 1 with DNase/RNase-Free Water and mix with gentle inversion, use immediately or store frozen aliquots
  + #E1009-A 250 U add 275 ml of water

Sample Preparation; Cells and Tissue

Samples were already stored in DNA shield , so add an equal volume of DNA/RNA Lysis Buffer (1:1), mix well (vertex for 30 seconds and spin for 30 seconds) and proceed to purification.

RNA Purification (at room temperature and spin refers to 30 sec at 16,000 x g )

Transfer Supernatant (the liquid) to the Spin-Away Filter (yellow)in a collection tube and centrifuge. Save the flow through for RNA purification. Spin and then follow the RNA purification steps (the 2B path)

Add one volume of ethanol (95%-100%) to the follow through and mix well by pipetting up and down.

Transfer the mixture into a Zymo-Spin IIICG Column (green) in a collection tube and centrifuge. Discard the flow through.

DNase I Treatment (in-column)

Add 400 microliters of DNA/RNA Wash Buffer to the column and discard the flowthrough.

Make a DNase I Reaction Mix in a nuclease-free tube (not included in kit) of 5 microliters DNase 1 and 75 microliters of DNA Reaction Buffer for each reaction. (10% more than you need, so If you have 10 samples, plan to make enough for 12. So 5 x 12 is 60 microliters of DNase 1 and 75 x 12 is 900 microliters of DNA Reaction Buffer. Your total volume would then be 960 microliters, then you would then distribute 80 microliters to each of the 10, with two extra samples worth if needed)

Add 80 microliters of the DNase I Reaction mix to each sample’s column matrix and then incubate at room temperature (20-30 C) for 15 minutes.

Add 400 microliters DNA/RNA Prep Buffer to the column and centrifuge. Discard the flow through.

Add 700 microliters of DNA/RNA Wash Buffer to the column and centrifuge. Discard the flow through.

Add 400 microliters of DNA/RNA Wash Buffer and centrifuge for 2 minutes to ensure complete removal of the wash buffer. Then carefully transfer the green column into a different nuclease-free collection tube.

In the column that is now sitting in a new collection tube, add 50-150 microliters of RNA DNA free water and then use a pipette to transfer the RNA in the collection tube to the RNA ONLY snap tubes (found on shelf next to Sam’s Computer)

* Use 50 microliters for smaller lifestages, 100 and 150 microliters for spat/seed
* This is the final elution volume

The RNA ONLY tubes are the final tubes that the RNA can be stored in or used immediately. RNA tubes were then stored in the -80 freezer until they were needed for the Qubit RNA High Sensitivity Assay or until they are sent for sequencing.

Qubit RNA High Sensitivity Assay

RNA Quantification assay

* Create a 200 microliter total volume of Buffer with 1 microliter of dye. There are two Standards that will run before the samples.

SAMPLE VOLUME:

200 microliters final volume

199 microliters of Qubit Assay Buffer

1 microliter of the sample

STANDARD VOLUME:

200 microliters final volume

190 microliters of Qubit Assay Buffer

10 microliter of either S1 or S2 (red or yellow)

Calculation for how much to make based on number of sample and standards:

(# reactions) x (200 microliters) = total volume of buffer dye mix

A x B = C

C - A = How much Buffer is needed

A; includes the standards in the number of reactions, also a good idea to make enough for 2-5 extra reactions

Qubit

* Plug in Qubit machine
* Tap the screen to wake it up
* Select RNA
* Select High Sensitivity
* Select Read Standards; Place S1 and then S2 into the dock at the top to be read
* Next read Samples
* Once all samples are read, select the Home button in the top left corner
* Select Data on the bottom left
* Check the check boxes for your samples and export them into the silver flashdrive (sticks out on the right of the machine)

Data

* Connect the flash drive to computer
* There will be a folder with the date it was created (.csv)
* Open the file, then sort by test date ascending as there is no ID column
* Insert column and add sample IDs
* Save As to your computer

Prep for sequencing

* Ship in box with dry ice to supplier