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Sample fixation and RNA extraction of brackish and marine samples with high sediment loads for metatranscriptomics sequencing

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This protocol has been used for a time-series experiment and confirmed as working.

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Abstract

Water samples from estuaries are brackish to marine and in cases of many subtropical rivers, such as the Brisbane River in Queensland, Australia, have high sediment loads that pose a challenge for RNA fixation and extraction.

Here we provide a protocol that has been tested on water samples of the Brisbane River Estuary, in Queensland, Australia. The estuary has a high sediment load and pH ranging from 6 - 6.5 in the brackish zone. We optimized sample collection by using 3.6L of estuary water field fixed with 400ml of 5% phenol in ethanol solution. This protocol yielded 0.5 - 2ug of RNA with RIN value 7-9.

Sampling equipment, reagents and kits required

1 **Reagents:**
Phenol - Sigma P4557 pH6.9
Ethanol
10% Hydrochloric Acid
10% SDS
Liquid N_2
RNase*Zap*™ RNase Decontamination Solution - Thermofisher AM9780

Sampling equipment:
Acid-washed carboys/containers, pre-marked to 3.6L and 4L
Sampling bucket
Merck filtration towers
Merck 8um pre-filter 147mm
Merck 0.22um Sterivex filter - SVGP01050
Parafilm
Whirlpak Bags
Ice bricks

Kits :
PurelinkTM RNA Mini Kit - Thermofisher 12183018A
TURBO DNA-*free*™ Kit - AM1907
2ml/1.5ml Eppendorf/RNase-free tubes

Prepare aqueous 5% phenol in 100% ethanol solution for sample fixation in fume hood

2 Buffer Sigma Phenol from pH 4 to pH 6.9 using a pH meter and kit-supplied pH 10 concentrate.Around ~500ul of this concentrate is usually added to 60ml of 0.1M Citrate buffered phenol to reach pH 6.9 using a pH meter.

Note : If this phenol is unavailable, an alternate can be prepared from 0.1M Citrate buffered phenol (Merck, USA) which includes a pH 10 concentrated solution.

3 Prepare 400ml of 5% aqueous phenol in ethanol solution: Pour 380ml of 100% ethanol into a dark glass bottle (or bottle wrapped in aluminium foil), and pipette 20ml pH 6.9 phenol slowly along the sides of the bottle. Care should be taken to pipette in phenol from below the buffer layer. This is suitable for one sample collection (ie 3.6 L) of estuary water.

Sample fixation in field

4 Pre-wash sample collection containers with 10% Hydrochloric acid followed by distilled water 3x times

5 Using a sample collection bucket, pour upto pre-marked 3.6L line on the sample collection containers.

6 Add 400ml of 5% aqueous phenol in ethanol solution carefully without spilling.
Place bottle(s) into a large esky in the dark, filled with ice bricks to keep the solution cool during sampling

Sample processing in the laboratory

7 Use equipment such as filters, filtration towers and lines in the fume hood, for RNA purposes only. Wipe down all surfaces with 70% Ethanol, and use Alfoil (Aluminium foil) to cover working area (taped down). Use RNase*Zap*™ RNase Decontamination Solution to wipe down all surfaces.

8 Place vortex mixer, centrifuge, and tube racks wiped down with 70% ethanol and RNase*Zap*™ RNase Decontamination Solution onto the Alfoil area.

9 In the fume hood, set up a peristaltic pump (Masterflex, USA) with 18 gauge tubing connected to 24 inch tubing inserted into the phenol-fixed sample. The other end of the tubing is connected to 142 mm Merck Filtration Towers fitted with 8um pre-filters, and subsequently connected to 0.22um Sterivex filter. (SVGP1050H). Insert a tubing to the other end of the Sterivex filter and connect it to a drain sink.

10 Run the peristaltic pump (Masterflex, USA) at 100rpm watching carefully for leaks. This process should take around 20-30 mins. Ensure that all the water is dispensed from sterive without allowing it to dry, and keep the pressure under 30 psi.

11 Plug the Sterivex filter using parafilm or sterivex caps, place them in Whirl-Pak Bags and snap-freeze in liquid N_2.

Extract RNA using PureLink RNAqueous Mini-kit with modifications

12 Pre-heat the Elution buffer provided with the kit in heat blocks set to 72^{o}C.

13 Defrost Sterviex within 1-2 minutes in the fume hood, and remove the parafilm/caps from one end. Keep the other end tightly sealed.

14 Add 900ul of lysis buffer provided with the kit and slowly add 100ul of 10% SDS along the sides of the sterivex filter.

15 Place on a vortex shaker at maximum speed and shake for 15 minutes. After 15 minutes, rotate the sterivex filter 180 degrees and shake for another 15 minutes.

16 Extract the buffer + SDS mixture containing cells using a 3ml syringe. First, draw air into the syringe, attach to a luer-lock on the Sterivex filter, and slowly push air into the filter with minimal bubbling. Release the syringe, and in this process the buffer mixture flows into the syringe.

17 Add the buffer + SDS Mixture into sterile 2ml tubes.

18 Add equal volume of 100% Ethanol and mix well.

19 Load 300ul of the buffer + SDS mixture into a spin column from the kit provided and spin at 16,000g for 15 seconds. Do not load more than this volume. You can load upto 2 times before dispensing the flow-through from the collection tube. Repeat this step until all of the buffer + SDS mixture is finished.

20 Load 180ul of Wash Solution I onto the spin column and spin 15,000g for 15 seconds.

21 Load 180ul of Wash Solution II/III onto the spin column and repeat spin.

22 Load 180ul of Wash Solution II/III onto the spin column and repeat spin.

23 Dry spin the spin column at 16,100 g for 1 minute, and replace the collection tube with 1.5ml eppendorf tube.

24 Quickly add 11ul of pre-warmed Elution buffer to the centre of the spin column. Allow to stand for 1 minute at room temperature and spin at 15,000g for 30 seconds to elute RNA

25 Repeat with a second addition of 11ul of pre-warmed Elution buffer and allow to stand for 1 minute at room temperature and spin at 15,000g for 30 seconds to elute RNA

26 Discard the spin column and immediately place the RNA samples on ice rack.

DNAse treatment of eluted RNA samples

27 Add 2ul of 10X Turbo DNAse I buffer and 1ul of Turbo DNAse I to the sample along the sides of the tube. Mix gently using a vortex shaker and spin down.

28 Incubate in a shaking incubator for 40 minutes at 37^{o}C.

29 Add 2.3 ul of DNase Inactivation Reagent and mix thoroughly by vortexing. Incubate at room temperature for 2 minutes.

30 Centrifuge the reaction mixture at 16,100g for 1 minute to pellet the DNase Inactivation Reagent. Repeat spin if needed.

31 Transfer the supernatent (approx. 20ul of RNA sample) into a fresh RNAse-free tube taking care to avoid the pellet.

32 Quantify using RNA tapestation or Qubit. The typical RNA yield is around 0.5 - 2ug, and RIN value is 7 - 9