Sampling and extraction of RNA from salmonid embryos

Authors:

Diego.Perojil@ed.ac.uk (PhD researcher), queries regarding protocol

Daniel.Macqueen@roslin.ed.ac.uk (PI), all queries

This document describes different steps of RNA sampling and extraction using salmonid embryos. This protocol is divided into “Sampling of RNA” and “RNA extraction”.

1. Sampling of RNA

Overview: This section describes the method used to sample the embryos and stabilize the RNA for later extraction.

Consumables:

- 1.5ml Eppendorf tubes with safety lock
- TRIzol™ (or equivalent)
- Sterile homogenization beads (e.g, tungsten, zirconium or glass beads)
- Tissuelyser or equivalent homogenization device.
- Water from egg trays.

1.1. Label the required number of 1.5ml Eppendorf tubes depending on the required number of biological replicates. No more than 3 eggs per tube should be used in salmonids.

1.2. Fill each tube with the required amount of homogenization beads.

The amount of beads is dependent on the type of beads used. We initially used two 3mm tungsten beads, later ~500ul of 1mm Zirconium beads, and finally around the same volume of 1mm glass beads. It may be necessary to monitor

the homogenate after homogenization and use more beads/homogenization time if necessary.

1.3. In a flow hood, add 1000ul of TRIzol™ to each tube. Store tubes in fridge while collecting embryos.

The number of tubes used will depend on the sampling strategy for each species.

1.4. Place the required number of eggs for all replicates in water from egg trays within a Petri dish on ice.

Using water from egg trays to temporarily keep the eggs while sampling allows for easy identification of dead/dying eggs, which will turn white and opaque and should not be sampled. If eggs are kept in PBS they will not turn white when they die, instead the chorion will turn more transparent and the yolk/embryo mass will clump into a ball. This makes it more difficult to tell between dead and alive eggs.

1.5. Using watchmaker forceps, transfer each egg to an empty petri dish, and puncture it with a pair of closed watchmaker forceps (pair 1), while holding the egg with a second pair of watchmaker forceps (pair 2). Do not remove forceps from puncture, instead, continue puncturing until pair 1 comes out through the other side. Place egg over an open tube while still held by both pairs of forceps, and tear open the puncture by opening pair 1, dechorionating and depositing the egg in the tube at the same time. Add no more than three punctured eggs per tube*. **Make sure tubes close tightly.**

**Salmonid specific, this is to avoid overfilling of tubes due to egg size. Egg puncturing is not necessary if using heavy 3mm tungsten beads.*

1.6. Homogenize using a Tissuelyser at a setting of 30Hz for 6min.

1.7. Store tubes at -80ºC until RNA extraction.

2. RNA extraction

Overview: This section describes the method used to extract and purify total RNA from previously sampled TRIzol™ homogenates.

Consumables:

- 1.5ml Eppendorf tubes
- Isopropanol, anhydrous
- 1-Bromo-3-Chloropropane
- NF-H_2O
- Ethanol, 70%
- Sodium chloride
- Sodium citrate sesquihydrate
- RNase AWAY™
- Milli-Q® water.

2.1. Preparation of precipitation solution: It consists of 1.2M NaCl and 0.8M Sodium citrate sesquihydrate. It can be stored in ready-made aliquots at -20°C.
   2.1.1. Apply RNase AWAY™ to pre-autoclaved glass bottle (100ml volume required). Rinse with Milli-Q® water several times.
   2.1.2. Dissolve 7g of NaCl in 50ml of NF-H_2O.
   2.1.3. Add 21.05g of sodium citrate sesquihydrate. Use a sterile, magnetic stirrer and slight heat to dissolve.
   2.1.4. Make up to 100ml of NF-H_2O in a measuring cylinder (decontaminated as in 2.1.1.).
   2.1.5. Sterile filter solution into new bottle (decontaminated as in 2.1.1.) using a 0.2-micron syringe filter. Store at 4°C.
   2.1.6. Alternatively, the solution can be aliquoted for later use and stored at -20°C.
2.2. Thaw TRIzol™ tubes at room temperature. While the tubes thaw, cool microcentrifuge to 4°C and label 1.5ml Eppendorf tubes as required (3 tubes for each sample tube will be needed).
2.3. Transfer homogenate to new tube, leaving the beads behind.
2.4. Centrifuge at 12000RCF, for 10min, at 4°C.
2.5. Transfer supernatant to a new tube.

A pellet will form at the bottom of the tube, while a layer of oil will form at the top. Extract only the middle layer between the pellet and the oil. If the oil is not visible, avoid aspirating trace amounts by keeping the pipette tip well below the surface.

2.6. Add 100ul of 1-Bromo-3-Chloropropane.
2.7. Vortex vigorously for 15s. Incubate for 30min at room temperature.
2.8. While tubes are incubating, prepare precipitation solution:
   2.8.1. Thaw required number of aliquots of precipitation solution.
   2.8.2. Prepare one tube for each sample.
   2.8.3. Add 100ul of precipitation solution and 100ul of anhydrous Isopropanol to each tube.

   The precipitation solution and the isopropanol do not mix, hence one tube per sample is used to avoid adding solution in incorrect proportions.

2.9. Centrifuge tubes at 20000RCF, for 15min at 4°C.
2.10. Carefully place tubes on ice without disturbing newly formed layers, and transfer 2x 200ul of aqueous phase, slowly and carefully, into pre-labelled 1.5ml Eppendorf tubes. Use pipette tip with blunt end and keep tip in the middle, as far away from the interphase, the meniscus and the sides of the tube as possible. If there is debris stuck to the sides of the tube, stop pipetting before the meniscus level reaches the debris.
2.11. Add 200ul of precipitation solution to each of the tubes where the aqueous layer was placed. Gently invert 6 times.
2.12. Incubate at room temperature for 10min – or incubate overnight at -20°C for increased RNA recovery.
2.13. Centrifuge at 20000RCF, for 10min at 4°C.

2.14. Remove supernatant and resuspend in 1000ul of 70% Ethanol.
2.15. Incubate on rotation 30min.
2.16. Repeat steps 2.13.-2.15. Then, repeat step 2.13. again.
2.17. Remove supernatant, including remaining ethanol with a p-10 pipette.
2.18. Air dry pellets for 10min at room temperature.
2.19. Resuspend in 20-100ul NF-H_2O (depending on size of pellet).
2.20. Incubate at room temperature for 60min, flicking the tubes every 10min.
2.21. Assess RNA concentration using Qubit.
2.22. Dilute samples as necessary with NF-H_2O, aiming for ~400ng/µl.
2.23. Assess the presence of contaminants using Nanodrop™

Samples with 260/230 ratios well below recommended RNA values of ~1.8 can generally still be sequenced with no ill effect, up to a value of ~1.0. 260/280 values should not be <1.8. QC requirements may vary depending on sequencing provider.

2.24. Assess RNA integrity using Tapestation. RIN values above 7 are preferred, values below 5 should be avoided.
2.25. Store samples at -80°C.