Experimental Protocol – RNA-seq of frozen sheep tissue samples

Summary

This experimental protocol describes the generation of RNA-seq libraries from frozen sheep tissues at the Roslin Institute. The protocol was developed to investigate gene expression and regulation in a number of tissues from Texel x Scottish Blackface sheep at different developmental time points in relation to growth traits. The experiment was split into three steps that are described below.

STEP 1

Collection of frozen tissue samples for RNA-Seq from large animals

(NB: Work quickly using reagents maintained at appropriate temperatures.)

1 Purpose / Introduction

To collect tissues for RNA-Seq from large animals during post mortem for subsequent extraction of RNA.

2 Equipment/Reagents/Materials

15ml Falcon tubes containing 5ml Sucrose Buffer with protease inhibitor tablet.

Cryotubes for snap freezing tissues.

Liquid Nitrogen dewar filled with liquid nitrogen.

No.11 and No. 22 Swan Morten Scalpels

Sterile Plastic Forceps

Sterile Scissors

Dry Ice

Wet Ice

Ziplock bags

Petri Dishes

3 Principle

Animals are euthanized according to University of Edinburgh protocol and a post mortem performed. Tissues are dissected out and cut into small pieces (~0.5cm diameter/200mg). For preservation of RNA, and for downstream isolation of nuclei from frozen tissue, the pieces of tissue are snap frozen separately in liquid nitrogen in 3-5 aliquots. A larger number of tissue pieces can be stored in one tube to use as a 'back-up' if the individual aliquots are insufficient, and/or for DNA isolation.

4 Procedure

4.1 At the farm all surfaces should be cleaned with disinfectant then RNAlater and all participants should wear gloves and ensure they regularly change these gloves throughout the harvest.

4.2 Tissues should arrive from the post mortem team on a plastic petri dish. The tissue should be cleaned of any excess blood or gut contents (for GI tract tissues only) by dipping in sterile PBS using plastic forceps. A No. 22 Swan Morton disposable scalpel should then be used to chop the tissue into small pieces that are <0.5cm diameter in any direction. To secure the tissue for chopping either a No. 11 Swan Mortem Scalpel or sterile plastic forceps can be used. A set of new scalpels should be used for each sample.

4.3 Tissue pieces should be spread evenly and singly across cryovials for immediate preservation in liquid nitrogen. One aliquot can contain multiple pieces of tissue to be used as a 'back-up' should the single aliquots be insufficient. For transfer back to the lab decant the cryovials into dry ice and then then move to a -80°C freezer.

STEP 2

Isolation of total RNA from frozen tissue samples

Equipment and reagents

Liquid Nitrogen in LN2 storage flask

Dry ice

Wet ice

RNase Zap

Trizol 15596026 100mls Life Technologies

1-bromo-3-chloropropane Sigma # B9673-200ml

Ethanol E0650DF17EB Fisher Scientific

RNase free water

Durx 670 clean room wipes SLS CLE4012

Precellys tissue homogeniser and Precellys lysing matrix CKM tubes for RNA

Pipettes and filter tips

Petri dishes

No.11 Swann Morton Scalpel Blades

RNeasy Mini Kit C-74104EB

Agilent Tapestation, RNA Screentape and RNA Sample Buffer

Procedure

- Care must be taken during all steps to prevent degradation or contamination of RNA.
- Reagent bottles should be RNase free and kept specifically for RNA prep
- Bench surfaces, racks, ice boxes, pipettes and equipment should be scrupulously clean and treated with RNase Zap
- Sample tubes and reagent bottles should be open only for the minimum time required for each step

1. Collect ~50-100mg tissue aliquots into pre-labelled 1.5ml screw-topped tubes
2. Snap freeze in Liquid nitrogen. Store tubes at -70°C until required for RNA prep.
3. For each RNA prep, IN FUME HOOD, add 1ml Trizol to a Precellys lysing matrix tube and chill on wet ice.
4. Collect samples from -70°C freezer on dry ice.
5. Set lysing speed and time on Precellys homogeniser:
   speed 2000
   time 20 seconds
   Speed and time may change for tissues that are difficult to lyse, but the above settings are suitable for most tissues
6. Remove tissue from cryovial into petri dish on dry ice and cut to between 60-100mg carefully using a scalpel.

7. Add a maximum of 100mg of frozen tissue to 1ml of chilled Trizol in lysing matrix tube. Snap frozen tissues should be kept frozen and lysed immediately after adding to Trizol, so homogenise no more than 2 – 4 at a time.
8. Homogenise samples on the Precellys. A small amount of non-lysed tissue may remain after lysis. Although this will reduce yield, increasing the lysis time and speed can lead to shearing and poor quality RNA.
9. If samples feel warm after homogenising, cool briefly on ice before equilibrating to room temperature.
10. Incubate at room temperature for 5 minutes.
11. IN FUME HOOD add 200ul BCP, shake tube vigorously by hand for 15 seconds.
12. Incubate at room temperature for 3 minutes.
13. Centrifuge samples at 12000 x g for 15 minutes at 4°C.
14. Taking great care not to disturb interface, transfer 450ul of upper aqueous phase containing the RNA to a new 1.5ml tube.

<u>RNeasy clean-up (refer to manufacturer's protocol)</u>

15. Add 1 volume of 70% ethanol, and vortex. Do not centrifuge. Proceed at once to step 15.
16. Transfer up to 700 µl of the sample to an RNeasy Mini spin column in 2 ml collection tube (supplied with kit). Centrifuge at room temperature for15 s at 10,000 x g, and discard flow-through.
17. Using the same collection tube, repeat step 15 using the remainder of the sample. Discard the flow-through
18. Add 700 µl Buffer RW1 to RNeasy column. Close lid, centrifuge for 15 s at 16,000 x g, and discard flow-through.
19. Add 500 µl Buffer RPE to RNeasy column. Close lid, centrifuge for 2min at 16,000 x g, and discard flow-through.
20. Add 500 µl Buffer RPE to RNeasy column. Close lid, centrifuge for 15 s at 16,000 x g, and discard flow-through
21. To further dry membrane, place RNeasy column in new 2 ml tube, close lid, and centrifuge at full speed for 1 min.
22. Place RNeasy column in a new 1.5 ml tube. Add 30–50 µl RNase-free water, close lid, leave for 1 min then centrifuge for 1 min at 10,000 x g. Elution can be repeated with an additional 30 – 50ul RNase-free water to increase yield, or the first elution can be passed though the column for a second time.

23. Place RNA on ice and check RNA concentration and 260/280 ratio on the Nanodrop. Remove a 1ul aliquot for RIN QC. Store remaining stock RNA at -80°C
24. Dilute the 1ul QC RNA if required using RNase free water. Run samples on the Agilent Tapestation using the RNA Screentape manufacturer's protocol. RIN values of $\geq$ 7 are recommended for RNA seq.

STEP 3

Library Preparation

Total RNA samples that passed QC were sent to Novogene, Cambridge UK, who prepared NEB Next® Ultra™ RNA libraries for sequencing on the Illumina NovaSeq 6000 according to the standard work flow:

https://international.neb.com/-/media/nebus/files/manuals/manuale7530.pdf?rev=071d46347b5c4d1aa116f27abd8f1d22&hash=0E7556C4415D444456C45C27A31DA9F5