Standard operating procedure for total RNA preparation from tissue samples

Leibniz Institute for Farm Animal Biology (FBN)

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FOR FARM ANIMAL BIOLOGY

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Prerequisites

Equipment

- Precellys 24 homogenizer (Bertin Technologies, VWR)
- Agilent 2100 Bioanalyzer (Agilent Technologies)
- NanoDrop 1000 spectrophotometer (PeqLab, VWR)
- Qubit fluorometer (Invitrogen, Thermo Fisher Scientific)
- Bench Top centrifuge (Eppendorf centrifuge 5417 R)
- Sterilized mortar and pestle, forceps, spatula
- Liquid nitrogen and appropriate cryoboxes
- Tight thermo-insulated/cryogenic gloves
- Protective glasses/goggles)
- Disposable laboratory gloves

Hazard warning:

- Liquid nitrogen is a hazardous chemical. It is nontoxic but cryogenic, asphyxiant gas that can reduce the normal oxygen concentration in breathing air. Exposure to oxygen-deficient atmosphere may cause dizziness, drowsiness, nausea, diminished mental alertness, loss of consciousness and suffocation. Contact of liquid nitrogen with skin and eyes can cause severe cryogenic burns.
- Anyone handling cryogenic liquids and asphyxiant gas should be familiar with the hazards presented by such materials and trained in how to handle them safely. Liquid nitrogen should be used in a well ventilated area. To prevent personal hazards due to cryogenic liquids users have to wear personal protective equipment.

Kits and Chemicals

- NucleoSpin RNA II kit (Macherey & Nagel, Cat # 740955.50)
- NucleoSpin RNA Cleanup kit (Macherey & Nagel Cat, # 740948.50)
- rDNase set (Macherey & Nagel, Cat # 740963)
- TRIzol reagent (Invitrogen, Cat # 15596-026, Thermo Fisher Scientific)
- Tissue lysing kit from Precellys with 2 mL Soft Tissue Homogenizing Ceramic Beads for soft tissue homogenization (Bertin Technologies, Cat # 91-PCS-CK14, VWR)
- Qubit RNA HS Assay Kit (Invitrogen Cat # Q32852, Thermo Fisher Scientific)
- Agilent RNA 6000 Nano Kit (Agilent Technologies, Cat # 5067-1511)
- Roti nucleic acid free - solution for removal of nucleic acid contaminations from surfaces, (Roth, Cat # HP69.1)
- Chloroform p.A.
- 2-Propanol p.A.
- Freshly prepared 75% Ethanol
- Freshly prepared 70% Ethanol
- 96% Ethanol
- Nuclease-free water

Samples

- Tissue samples were collected from jejunum mucosa, liver (*Lobus caudatus*), skeletal muscle *(M. longissimus dorsi*) and rumen (*Saccus ventralis*, papillary base) according to the Standard operating procedure for cryo-freezing of tissue samples of the Leibniz Institute for Farm Animal Biology (FBN), Institute Genome Biology, Genome Physiology Unit (https://www.fbn-dummerstorf.de/fileadmin/media/I3.0/FBN_GenomePhysiology_SOP_CryofreezingTissueSsamples_20160331.pdf).
- The samples were taken directly after slaughtering and dissection of the animals in the institute's own slaughterhouse located on the institute's campus. The samples were immediately shock frozen in liquid nitrogen and subsequently stored at -80°C until further use.

Total RNA preparation

- *Total RNA was prepared from frozen tissue samples using an on-column-purification procedure with the NucleoSpin RNA II kit including a DNA digestion step according to the manufactuer's instructions for liver and jejunum tissue.*
- *For more fibrous tissue, such as muscle and rumen, this procedure was modified and combined with a preceding TRIzol trreatment.*

Total RNA extraction from muscle and rumen:

1. Add 1.0 ml TRIzol reagent to 100 mg frozen tissue pieces and immediately homogenize the sample using the bead beating Precellys 24 homogenizer.
2. Homogenize the sample in 2 mL tubes containing 1.4 mm ceramic beads from the Precellys tissue lysing kit.
3. Centrifuge the sample for two times at 5,000 rpm for 15 sec with a 20 sec in between both centrifugation steps (cooling step to avoid warming of the samples).
4. Incubate the sample at room temperature for 5 min; centrifuge at 4°C and 12,000 x g for 10 min
5. Aspirate the supernatantsand transfer them into a new 2 ml tube, add 200 µl chloroform and shake vigorously for 15 sec
6. Incubate the sample at room temperature for 5 min; centrifuge at 4°C and 12,000 x g for 5 min
7. Aspirate the supernatant and pipette it into a new 2 ml tube, add 500 µl 2-propanol and mix by inverting the tube
8. Incubate the sample on ice for 10 min; centrifuge at 4°C and 12,000 x g for 10 min
9. Aspirate the supernatant carefully with a pipette tip and discard it
10. Add 500 µl 75% freshly prepared ethanol to the pellet, twirl gently, centrifuge at 4°C and 12,000 x g for 10 min
11. Repeat this washing step (steps 9 and 10)
12. Aspirate and discard the supernatant; air dry the pellet for approx. 5 min on cellulose with the tube upside down.
13. Dissolve the pellet in 50 µl nuclease-free water

    - The RNA samples were then subjected to purification and DNase treatment using the NucleoSpin RNA II kit (Macherey & Nagel, Düren, Germany)

14. Add 175 µl buffer RA1 and 1.75 µl ß-mercaptoethanol) to the 50 µl RNA solution and mix by vortexing

15. Add 225 µl 96% ethanol to adjust for binding conditions, mix by pipetting up and down (5 times)
16. Transfer the mixture into a Nucleospin RNA column (light blue ring) placed in a 2 ml collection tube
17. Centrifuge at room temperature for 30 sec 13,000 rpm (11,000 x g), discard the flowthrough and place the column in a new collection tube
18. Pipette 175 µl membrane desalting buffer (MDB) in the column, centrifuge at room temperature for 30 sec at 13,000 rpm, discard the flowthrough
19. Mix 20 µl rDNase solution with 80 µl DNase reaction buffer (both included in the Nucleospin kit) and add 95 µl rDNase reaction mixture directly on the center of the membrane of the column without touching it
20. Incubate for 20 min at room temperature
21. First wash: Add 200 µl RA2 buffer to the column, centrifuge for 30 sec at 13,000 rpm (room temperature), discard the flowthrough and place column in a new collection tube
22. Second wash: Add 600 µl RA3 buffer to the column, centrifuge for 30 sec at 13,000 rpm (room temperature), discard the flowthrough and place column in a new collection tube
23. Third wash: Add 250 µl RA3 buffer to the column, centrifuge for 1 min at 13,000 rpm (room temperature), discard the flowthrough and place column in a 1.5 ml collection tube
24. Add 30 µl nuclease-free water centrically on the column without touching the membrane, incubate at room temperate for 10 min, centrifuge for 1 min at 13,000 rpm to elute the purified RNA
25. Store the RNA at -80°C until further use

Total RNA extraction from liver and jejunum:

1. Grind frozen tissue samples were ground in liquid nitrogen with pestle and mortar and use 30 mg of the pulverized tissue samples for total RNA isolation using the NucleoSpin RNA II kit according to the instructions in the manufacturers' instructions.
2. Prepare lysis buffer by mixing 3.5 µl ß-mercaptoethanol with 350 µl buffer RA1,
3. Add the lysis buffer to the pulverized tissue sample and vortex vigourously
4. Filtrate the lysate through a NucleoSpin filter by centrifuging at room temperature for 1 min at 13,000 rpm (11,000 x g)
5. Discard the NucleoSpin filter and add 350 µl freshly prepared 70% ethanol to the flowthrough to adjust for binding conditions, mix by pipetting up and down (5 times)
6. To bind the RNA, transfer the mixture to a NucleoSpin RNA column placed in a 2 ml collection tube
7. Centrifuge at room temperature for 30 sec 13,000 rpm (11,000 x g), discard the flowthrough and place the column in a new collection tube
8. For desalting, pipette 350 µl membrane desalting buffer (MDB) in the column, centrifuge at room temperature for 30 sec at 13,000 rpm, discard the flowthrough
9. For DNA digestion, mix 20 µl rDNase solution with 80 µl DNase reaction buffer and add 95 µl rDNase reaction mixture directly on the center of the membrane of the column without touching
10. Incubate for 20 min at room temperature
11. First wash: Add 200 µl RA2 buffer to the column, centrifuge for 30 sec at 13,000 rpm (room temperature), discard the flowthrough and place column in a new collection tube
12. Second wash: Repeat washing step 11 with 600 µl RA3 buffer on the column
13. Third wash: Repeat washing step 12 with 250 µl RA3 buffer on the column and place the column after centrifugation in a 1.5 ml collection tube
14. Add 30 µl nuclease-free water centrically on the column without touching the membrane, centrifuge for 1 min at 13,000 rpm to elute the purified RNA
15. Repeat step 14
16. Store the collected RNA (60 µl) at -80°C until further use

Quantification and Quality check of the total RNA samples

- Finally, the RNA samples were tested for remaining traces of genomic DNA via PCR with primers amplifying genomic DNA (according to Weikard et al. 2009, doi: 10.1186/1471-2164-10-186) and, in case of remaining DNA residues, the RNA sample was further cleansed by additional DNase treatment using the NucleoSpin RNA Cleanup kit and rDNase set.
- RNA concentration and purity were quantified on a NanoDrop 2000 spectrophotometer and on a Qubit 2.0 fluorometer using 1 µl purified RNA and the Quant-it Qubit RNA HS Assay kit according to the manufactuer's instructions.
- RNA integrity was evaluated on the Agilent Bioanalyzer 2100 using 1 µl purified RNA and the RNA 6000 Nano kit according to the manufactuer's instructions.