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Total RNA Isolation from Bulk Tissue or Isolated Cells

Abstract: The purpose of total RNA preparation from bulk tissue is several fold for the KPMP. It will be used: a) as a benchmark to validate single-nucleus RNA sequencing data for genes missed or artefacts such as stress response genes; b) to compare the quality of RNA made across KPMP Tissue Interrogation Sites (TISs) receiving adjacent kidney cortex sections from the same source; and c) determine if transportation conditions affect RNA quality. The sites will use the RNAlater protected kidney tissue shipped on dry-ice and extract total RNA locally. The method involves a combination of RNA extraction-precipitation using TRIzol reagent and RNA clean up using Qiagen RNAeasy kit. An aliquot will be quality checked locally on Bioanalyzer 2100 and an aliquot sent to a lab serving as a central core to perform the QC on all samples if needed. Since this protocol involves double extraction and purification, it generally will cause some loss of sample due to these extra steps but will still provide high quality RNA. gDNA elimination steps are not included. For bulk nephrectomy tissues RIN > 6.5 will be considered as acceptable for gene level expression data. For single cell or small segments of tissue, RIN cutoffs are not used. As part of the KPMP Pilot 1, this protocol was used to determine if transportation of frozen tissue affects RNA quality and RNA-seq results. It was found that tissue processed in RNAlater and transported frozen on dry ice does not have an adverse effect on RNA quality despite different methods of RNA extraction used at the various TISs.

Materials and Reagents

1. TRIzol: Thermo Fisher Scientific, #15596-026
2. Phase Lock Gel tubes: 5PRIME, #2302830
3. Qiagen RNeasy Plus Micro kit: Qiagen #74034 (for less than 5mg tissue)
4. Qiagen RNeasy minikit: Qiagen #74104 (for 5-30mg tissue)

Procedure

1. Measure and Weigh the frozen sample

   Note: up to 100mg can be used to homogenize in 1ml TRIzol

2. Collect samples in **960 µl** of TRIzol. Do homogenize or other mechanical treatment to get the samples completely dissolved if required. For tissue chunks, we use powergen 125 homogenizer at setting 6 (Fisher Scientific), usually up to 3 x 10-15 sec bursts with 30 sec of cooling on ice in between works well for kidney tissue

   Tip: Keep the probe immersed in the solution to avoid frothing

   QA/QC: Inspect visually for presence of large chunks after each homogenization burst to judge if additional step is needed

3. Add **40 µl** of glycogen solution to each tube (**5 mg/ml**, final 200 µg/ml of glycogen) and mix well. Glycogen can be omitted if sample being used is not small

4. Transfer TRIzol mixture to Phase Lock Gel (5PRIME, #2302830) after pre-centrifugation to get gel down to the bottom of the column (12,000 xg for 5 min)
5. Incubate for 5min at room temperature
6. Add 250 µl of chloroform and mix vigorously by shaking for 15 sec
7. Centrifuge at 12,000 xg for 10 min at 4°C
8. Collect supernatant to a new 1.5 ml tube
9. Add same amount of isopropanol **(~600 µl)**
10. Mix well by vortex and incubate the tube at -20°C for ~ 30min
11. Centrifuge at 20,000 xg for ~ 30 min at 4°C
12. Discard supernatant by pipette and add **600 µl** of 80% EtOH (DEPC-treated or RNAase free water). (There may be a visible precipitate or pellet at this stage)
13. Mix well by vortex and centrifuge at 12,000 xg for 10 min at 4°C
14. Discard supernatant by pipette completely and air dry for a couple of minutes. Not over dry
15. Add **280 µl** of RLT plus buffer (*Qiagen RNeasy Plus Micro kit, #74034*) containing 2-ME (10 µl of 2-ME for 1ml of RLT plus buffer, 1% 2-ME) and mix well with a pipette

    Note 1: For bigger pieces of tissue (expect more than 5µg of RNA or more than 5-10 mg tissue use Qiagen RNeasy mini kit (Qiagen, #74104) and follow directions. If TRIzol step is already done, you can use these same volumes for up to 30mg of starting tissue

    Note 2: The Plus kit has columns to get rid of gDNA which you may not need as the TRIzol RNA step usually takes care of that, so you can save money by skipping gDNA step or use a Qiagen RNeasy Micro kit. If this RNA is needed for downstream NGS analysis gDNA elimination steps can be followed that are in the kit

16. Add **420 µl** of 100% EtOH (1.5 volumes) and mix well. May see precipitation
17. Transfer **700 µl** of the sample to RNeasy MinElute spin column (Qiagen RNeasy Plus Micro kit) and centrifuge for 30 sec at 12,000 xg at 24°C
18. Transfer/reload the collected flow through to the same column and centrifuge for 30 sec at 12,000 xg at 24°C (**This double column loading is especially useful for very small samples**). This time discard the flow through
19. Add 700 µl of RW1 solution to the column and centrifuge for 30 sec at 12000 xg

    NOTE: This step essentially washes away contaminants such as protein, fat, etc. but may also wash fragmented RNA or micro RNAs. If you need these you can skip RW1 wash, however, we have included this in this protocol

20. Add 500 µl of RPE buffer to the column and centrifuge at 12,000 xg for 30 sec at 24°C
21. Discard flow through and add 500 µl of RPE buffer to the column. Centrifuge at 12,000 xg for 2 min at 24°C
22. Transfer the column to a new collection tube. Discard the old collection tube with flow through
23. Open the lid of the column and centrifuge at 12,000 xg for 5 min at 24°C to dry up the column membrane completely
24. Place the column in a new 1.5 ml tube and add 8 µl (if using micro kit) of RNase free water on the center of the column membrane. Incubate for 2 min at RT

    NOTE: For minicolumn, 30µl

25. Centrifuge at 12,000 xg for 3 min at 24°C

26. Add **8 µl** (if using micro kit) of RNase free water again on the center of the column membrane and incubate for 2 min at RT

    NOTE: For minicolumn, 30 µl

27. Centrifuge at 12,000 xg for 3 min at 24°C. (**Double Elution**)
28. Perform **additional 60 µl elution for >10 mg starting tissue** (**3rd elution**) if using minicolumn, this will significantly increase yield
29. Total elution volume would be **~ 15 µl (**if using micro kit)* and 120 µl if using minikit
30. Analyze obtained RNA using a Bioanalyzer

    * For **bigger tissue (up to 30mg)**, use *Qiagen RNeasy minikit (Qiagen, #74104)*
    * Elution volume will be increased depending on the initial tissue size

RNA quantification and analysis

1. Can use nanodrop or similar for a rough idea of quantity and purity, we find overestimation by up to 20% by nanodrop
2. Agilent Bioanalyzer model # G2939A
   a) for **very low** RNA - Agilent RNA 6000 Pico Kit', cat # 5067-1513 (2100 expert Eukaryotic Total RNA pico setting)
   b) for **RNA conc >25ng/ml** - Agilent RNA 6000 **Nano** Kit, cat # 5067-1511 (2100 expert Eukaryotic Total RNA nano setting)