Author: Pat Pramoonjago

Author of latest version: Pat Pramoonjago

Authorizer: Christopher Moskaluk, LCBRN Principal Investigation

Last review date: 4/12/2013

Purpose

To obtain DNA, RNA and protein from tissue specimens (denaturing guanidine-based sample lysis and silica-membrane purification). This procedure does not retain low molecular weight RNA species well.

Responsibility

BTRF personnel are responsible for carrying out the sample collection, processing and freezing procedures competently and safely. Record results obtained in lab notebook or in the BTRF/CHTN folder in the CHTN server under investigator’s name. All personnel handling human biospecimens must have training in, and adhere to, universal biohazard precautions and human subject research ethics/confidentiality principles.

Equipment/Reagents

1. AllPrep DNA/RNA/Protein mini kit (Cat#80004,Qiagen)
2. Tissuelyser II and 5mm stainless steel bead( Qiagen)
3. NanoDrop 1000 Spectrophotometer (Thermo Scientific)
4. Protective gear (biosafety cabinet, eye/face-shield, disposable gloves, appropriate lab attire).
5. Clinical centrifuge capable of delivering 1300 x g centrifugal force, with appropriate rotors and adaptors to fit the tubes.
6. Sterile disposable pipets, tips, 1.5 and 2 ml microfuge tubes(RNase and DNase free)
7. ß –mercaptoethanol
8. 100% ethanol
9. RNase-free DNase set (Cat#79254, Qiagen)

Procedure

1. This protocol is used for simultaneous isolation of DNA, RNA and protein from whole tissue fragments (up to 50 mg). All tissue samples should be stored in 2.0 ml microfuge tubes in a –80o C freezer. When working with the samples, the frozen tissue samples MUST remain frozen on dry ice before being placed directly into RLT buffer, and must be disrupted IMMEDIATELY upon being transferred to the RLT buffer and proceed WITHOUT DELAY until the end of the procedure.
2. Prepare all reagents before starting by adding 10 μl of ß–Mercaptoethanol per 1 ml buffer RLT. Buffer RPE, Buffer AW1, and Buffer AW2 are each supplied as a concentrate. Before using for the first time, add the appropriate volume of ethanol (96–100%), as indicated on the bottle.
3. Add 350 μl of Buffer RLT containing ß–Mercaptoethanol into the 2.0 ml microfuge tube containing the frozen tissue sample. Immediately place a 5 mm stainless steel bead in the tube. Place the tubes in the TissueLyser and operate the TissueLyser at 20 Hz for 2 minutes, 2 times. The time depends on the tissue being processed and can be extended until the tissue is completely homogenized.
4. Spin down the tubes briefly, then transfer the lysate to a fresh 1.5 ml microfuge tube and spin the tube for 3 minutes at full speed. Carefully remove the supernatant by pipetting, and transfer it to the AllPrep DNA spin column placed in a 2 ml collection tube. Close the lid gently, and centrifuge for 30 s at ≥8000 x g.
5. Place the AllPrep DNA spin column in a new 2 ml collection tube and store at room temperature (15–25ºC) for later DNA purification (steps 21). Use the flow-through for RNA purification (step 6).

**RNA purification**

6. Add an equal volume (350 ul) of 70% ethanol, vortex briefly and transfer the sample including any precipitate to an RNeasy mini spin column sitting in a 2 ml collection tube, close the lid gently and centrifuge for 15 seconds at ≥8000 x g. Keep the flow-through for protein preparation (step 17).

7. Add 350 μl buffer RW1 to the column, close led gently and centrifuge for 15 second at ≥8000 x g to wash the spin column membrane. Discard the flow through.

8. Prepare DNase1 mixture by adding 10 μl DNase stock solution into 70 μl buffer RDD, mix well by pipette up and down 4-5 times.

9. Add the DNase mixture directly to the RNeasy spin column membrane and place on the bench top for 15 minutes.

10. Add 350 μl buffer RW1 to the spin column and centrifuge for 15 seconds at ≥8000, discard flow through.

11. Add 500 μl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 second at ≥8000 x g to wash the membrane. Discard the flow-through.

12. Add 500 μl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x g to wash the membrane. The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.

13. Place the RNeasy spin column in a new 2 ml collection tube, and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min. Perform this step to eliminate any possible carryover of Buffer RPE.

14. Place the RNeasy spin column in a new 1.5 ml collection tube. Add 30 μl RNase-free water directly to the spin column membrane. Close the lid gently and centrifuge for 1 min at ≥8000 x g to elute RNA,

15. Repeat step14 using the eluate from step 14.

16.Measure RNA concentration by A260 spectrophoscopy (Nanodrop 1000, Thermo Scientific), then adjust concentration to 100-150 ng/ul with RNase free water, and keep the RNA at -80 °C.

**Protein precipitation procedure**

17. Divide the flow-through from step 6 into 2 microfuge tubes. Add 300 µl of Buffer APP into each tube. Mix vigorously and incubate at room temperature for 10 min to precipitate protein.

18. Centrifuge at full speed for 10 min, and carefully decant the supernatant.

19. Add 500 µl of 70% ethanol to the protein pellet. Centrifuge at full speed for 1min, and remove the supernatant by pipetting or by decanting as much liquid as possible.

20. Dry the protein pellet for 5–10 min at room temperature, then keep the protein pellet at -80 °C.

**Genomic DNA purification**

21. Add 500 µl Buffer AW1 to the AllPrep DNA spin column from step 5. Close the lid gently, and centrifuge for 15 s at ≥8000 x g. Discard the flow through.

22. Add 500 µl Buffer AW2 to the AllPrep DNA spin column. Close the lid gently, and centrifuge for 2 min at full speed to wash the spin column membrane. The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during DNA elution. Residual ethanol may interfere with downstream reactions.

23. Place the AllPrep DNA spin column in a new 1.5 ml collection tube. Add 100 µl Buffer EB (preheated to 70°C) directly to the spin column membrane and close the lid. Incubate at room temperature for 2 min, and then centrifuge for 1 min at ≥8000 x g to elute the DNA.

24. Repeat step 23 by using the eluate from step 23 to elute further DNA.

25. Measure DNA concentration by A260 spectrophoscopy (Nanodrop 1000, Thermo Scientific), then adjust concentration to 100-150 ng/ul with Buffer EB and keep the DNA at -80 °C.

DATA ANALYSIS/CALCULATIONS

RNA and DNA concentrations and estimation of purity are determined by using Nanodrop spectrophotometer readings of A260, A260:A280 and A260:A230 ratios.