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Purpose

To obtain DNA, RNA (including miRNA) and protein from tissue specimens using phenol/ guanidine-based sample lysis and silica-membrane purification.

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. RNeasy Plus Universal kit (cat#73403, Qiagen)
2. Tissuelyser II and 5mm stainless steel bead(Qiagen)
3. MixMate agitator (Eppendrof)
4. NanoDrop 1000 Spectrophotometer (Thermo Scientific)
5. Protective gear (biosafety cabinet, eye/face-shield, disposable gloves, appropriate lab attire).
6. Clinical centrifuge capable of delivering 14,000x g centrifugal force, with appropriate rotors and adaptors to fit the tubes.
7. Sterile disposable pipets, tips, 1.5 and 2 ml microfuge tubes (RNase and DNase free)
8. Chloroform, ethanol, water (Molecular grade)
9. 0.3 M guanidine HCL in 95% ethanol
10. 3M sodium acetate (Ambion)
11. Heat blocks set at 37 °C

Procedure

1. This protocol is used for simultaneous isolation of DNA, RNA (including low molecular weight miRNA) 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. The frozen tissue samples MUST remain frozen on dry ice before being placed directly into Qiazol reagent, and must be disrupted IMMEDIATELY upon being transferred to the Qiazol reagent and proceed WITHOUT DELAY at least until the end of RNA extraction procedure (step 19).
2. Buffer RPE, Buffer RWT, 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 900 μl QIAzol Lysis reagent into the 2.0 ml microfuge tube containing the frozen tissue sample. Immediately 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 tube briefly, then carefully transfer the lysate to a fresh 1.5 ml microfuge tubes.
5. Place the tube containing the homogenate on bench top at room temperature for 5 minutes to permit complete dissociation of nucleoprotein complexes.
6. Add 100 μl gDNA Eliminator Solution. Securely cap the tube containing the homogenate, and vortex for 15 seconds
7. Add 180 μl chloroform. Securely cap the tube containing the homogenate, and vortex for 15 seconds. Place the tube containing the homogenate on the bench top at room temperature for 2–3 min.
8. Centrifuge at 12,000 x g for 15 min at 4°C. After centrifugation, heat the centrifuge to room temperature if the same centrifuge will be used in the later steps of this procedure. After centrifugation, the sample separates into 3 phases: an upper, colorless, aqueous phase containing RNA; a white interphase; and a lower, red, organic phase.
9. Transfer the upper, aqueous phase (usually 600 μl) to a new 1.5 ml microfuge tube and keep the interphase and the organic phase at 4°C for DNA and protein isolation (step 20).

RNA isolation procedure

10) Add 1.5 volumes (usually 900 μl) of 100% ethanol to the aqueous phase solution, and mix thoroughly by pipetting up and down. Do not centrifuge. Proceed immediately to step 11. Note: Precipitates may be visible after addition of ethanol. Resuspend precipitates completely by vigorous shaking, and proceed immediately to step 11.

11) Transfer up to 700 μl of the sample to an RNeasy Mini spin column placed in a 2 ml collection tube. Close the lid gently, and centrifuge for 15 seconds at ≥8000 x g at room temperature. Discard the flow-through.

12) Repeat step 11 using the remainder of the sample. Discard the flow through.

13) Add 700 μl Buffer RWT to the RNeasy spin column. Close the lid gently, and centrifuge for 15 seconds at ≥8000 x g to wash the membrane. Discard the flow-through.

14) 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.

15) 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.

16) 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.

17) Place the RNeasy spin column in a new 1.5 ml collection tube. Add 50 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,

18) Repeat step19 using the eluate from step 17.

19) 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.

DNA isolation procedure

1. DNA is isolated from the interphase and phenol-chloroform layer saved previously (step 9). Remove any remaining aqueous phase overlying the interphase. This is critical for the DNA isolation.
2. Add 0.3 ml of 100% ethanol, cap the tube and invert the sample several times to mix.
3. Incubate samples for 3 minutes at room temperature.
4. Centrifuge at 5,000xg for 5 minutes at 4 oC to pellet the DNA.
5. Remove the phenol-ethanol supernatant and save it in the new tube for protein isolation. The supernatant can be stored at -70 oC for several months.
6. Wash the DNA pellet with 1 ml of sodium citrate/ethanol solution (0.1 M sodium citrate in 10 % ethanol, pH 8.5)
7. Incubate for 30 minutes at room temperature on Mixmate mixture apparatus set at 1,250 rpm.
8. Centrifuge at 5,000xg for 5 minutes at 4 oC. Remove and discard supernatant.
9. Repeat wash (step 25-27), one more time.
10. Add 1.5 ml 75% ethanol, incubate for 20 minutes at room temperature. Mix the tube occasionally through gentle conversion. DNA in 75% ethanol can be kept at 4 oC for several months.
11. Centrifuge at 8,000xg for 10 minutes at 4 oC. Remove and discard supernatant.
12. Air dry the DNA pellet for 5-10 minutes.
13. Resuspend the DNA pellet in LoTE buffer 400 ul by passing the solution up and down 20 times through a 3 ml syringe with needle gauze #23.
14. Incubate the DNA suspension in a heat block set at 37 oC for 20 minutes.
15. Centrifuge at 10,000xg for 10 minutes, then transfer the supernatant containing DNA into a new microfuge tube and measure DNA concentrations by Nanodrop, then adjust concentration to 100-150 ng/ul with LoTE buffer and keep the DNA at -80 oC

Protein isolation procedure

1. Proteins are isolated from the phenol-ethanol supernatant layer save previously (step 24). The supernatant was first divided into two 2.0 ml microfuge tubes equally.
2. Add 0.75 ml isopropanol into each tube.
3. Incubate samples for 10 minutes at room temperature.
4. Centrifuge at 12,000xg for 10 minutes at 4 oC to pellet the protein. Remove and discard supernatant.
5. Prepare a wash solution consisting of 0.3 M guanidine HCl in 95 % ethanol.
6. Was the protein pellet with 2 ml of the wash solution, incubate for 20 minutes at room temperature.
7. Centrifuge at 7,500xg for 5 minutes at 4 oC. Remove and discard the wash solution.
8. Repeat step 40-41, twice.
9. Add 2 ml of 100% ethanol to protein pellet after the third wash and vortex.
10. Incubate at room temperature for 20 minutes.
11. Centrifuge at 7,500xg for 5 minutes at 4 oC. Remove and discard ethanol wash.
12. Air dry the protein pellets for 5-10 minutes.
13. Keep the protein pellets at -80 oC.

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.