ISOLATION OF TOTAL RNA USING GUANIDINE THIOCYANATE

"Crude Susan"

This procedure will allow you to isolate total RNA (i.e., cytoplasmic plus nuclear) from virtually any animal cell or tissue. Guanidine thiocyanate is an extremely potent chaotropic agent; thus, it quickly denatures macromolecules and inactivates nucleases. Phenol extractions are then done at 60°C, thereby preferentially extracting RNA into the aqueous phase and leaving the DNA behind.

This procedure is particularly useful for the following situations:

(1) Tissues rich in RNase (e.g., pancreas)
(2) Frozen tissue or other tissue in which the nuclei and cytoplasm cannot be easily separated
(3) Red blood cells (in other procedures, heme sticks to the RNA and you will purify brown RNA)
(4) Cells transiently transfected (a lot of plasmid DNA remains in the cytoplasm or stuck to the surface of cells)

Materials:

1. Guanidine Thiocyanate Lysis Buffer (a.k.a. "Crude Susan"):
   Dissolve 100 g of guanidine thiocyanate in:
   90 ml dH_2O
   10.6 ml 1 M Tris-Cl (pH 7.6)
   4.24 ml 0.5 M EDTA.
   Allow to stir until completely dissolved (1 hour to overnight). You may wish to warm the solution to assist dissolving.

   Add: 21.1 ml of 20% Sarkosyl (N-lauroyl sarcosine)
   2.12 ml of ß-mercaptoethanol.
   Bring to a final volume of 212 ml. Store at 4°C.

   The final concentration of the components will be:
   4 M guanidine thiocyanate
   2% Sarkosyl
   50 mM Tris, pH 7.6
   10 mM EDTA
   1% ß-mercaptoethanol
2. Phenol containing 0.1% 8-hydroxyquinoline (used as an antioxidant) and saturated with 0.1 M Tris pH 7.4.
3. Chloroform:Isoamyl alcohol (24:1).
4. 0.1 M and 3 M sodium acetate, pH 5-6 (with H0Ac).
5. A 60°C bath.

Procedure:

1. Pre-warm Crude Susan, Phenol, Chloroform and 0.1 M Na0Ac to 60°C.

2. Add a "suitable" volume of Crude Susan to the material of interest (cell pellet, lump of tissue, liquid nitrogen powder, etc.). A "suitable" volume usually means about five or more volumes of Crude Susan relative to the tissue. Here are some suggestions:
   i. One plate (100 mm, or T-75 flask) of cells can be scraped into PBS and pelleted into an Eppendorf tube. 200 ul of Crude Susan is added to the fresh or frozen cell pellet; subsequent manipulations can then all be performed on an Eppendorf tube-sized scale.
   ii. To a pellet of 2-5 X 10^9 red cells (pellet volume is several hundred microliters) use 2 ml of Crude Susan. The final Et0H ppt can then be performed in one 15 ml Corex tube.
   iii. If you want your final Et0H precipitate to be kept to one tube per sample, I suggest the following volumes of Crude Susan:
      a) for Eppendorf tube, use 200 µl Crude Susan.
      b) for 15 ml Corex tube, use 2 ml Crude Susan.
      c) for 30 ml Corex tube, use 5 ml Crude Susan.

   These volumes can be increased by 50%, if you precipitate with isopropanol instead of ethanol.

   When in doubt, it is better to use too much than too little Crude Susan. Even if you use 2 ml of Crude Susan per 100 mm plate of cells, the RNA will not be too dilute to precipitate with ethanol.

   It is not absolutely necessary for the Crude Susan to be pre-warmed to 60°C, but the cells will lyse faster if it is hot.

3. Homogenize the sample to disrupt and solubilize the material and to shear the DNA. (Initially, the mixture will be very "goopy" due to DNA liberated from the nucleus.) The object here is two-fold: first, to rapidly homogenize the sample to inactivate nucleases (especially for tissues containing RNase; however, this is not a big concern for most cultured cell lines); second, to shear the DNA to small enough pieces such that the viscosity is reduced for ease of pipetting.

   Suggestions:
   i. For a small cell pellet (lysed with 200 µl of Crude Susan), pass the material several times through a 1 cc syringe and 21 g needle, then switch to a 23 g needle, and then a 25 g or 27 g needle.
   ii. For a large cell pellet (e.g., with red cells), use a 5 or 10 cc syringe with sequential passes through an 18 g, 21 g and 23 g needle. The sizes of the needles recommended here are not carved in stone; the main point is to shear the DNA

down to a pipettable size. The larger the volume you work with, the harder it will be to shear.

iii. For tissues (especially those that have a lot of connective tissue) a mechanical homogenizer such as a Polytron tissue homogenizer, Sorvall Omni-Mixer, Waring Blender, Ronco Veg-O-Matic, etc., is best. If you are really serious about doing this with crude tissues, I highly recommend obtaining the Polytron. In some cases, it may be necessary to spin out any insoluble material before proceeding to the next step.

4. Heat the homogenate to 60°C. Add an equal volume of phenol heated to 60°C. Mix well at 60°C. Note that the phases are completely miscible; no interphase should be observed. If you are using a syringe, pass the mixture through one more time to get the last of the homogenate out of the syringe.

   Add one volume (original starting volume) of chloroform, mix at 60°C, and one volume of 0.1 M Na0Ac. Continue to mix well at 60°C for 5-10 minutes. after you add the chloroform, you should get separation of organic and aqueous phases. Also, pressure may build up after mixing the chloroform in, so carefully release the pressure after the first few times you mix. Extractions are most safely performed in Eppendorf tubes (small volumes) or 15 ml or 50 ml capped Corning tubes. Use polypropylene; lesser plastics will dissolve in chloroform.

5. Chill on ice for a couple of minutes to bring the temperature down to room temperature. Spin to separate the phases. You will get a cleaner aqueous phase if you spin pretty hard (e.g., Eppendorf microfuge for 5 minutes for small samples; or in Corex tubes at 6,000 g for 10-15 minutes in a Sorvall for larger samples).

6. Remove the aqueous (upper) phase and transfer to a new tube. Add hot phenol (original volume added; should be half the volume of your aqueous phase), mix and bring to 60°C. Add hot chloroform (original volume added) and extract at 60°C as before. Chill and spin to separate the phases as above.

7. At this point you will have to decide how clean you want your RNA, and in what manner you wish to make it clean. You may want to continue repeated phenol extractions until the protein/aqueous interphase is clean. Alternatively, if you will use protease to clean up the sample, skip to step 9.

8. Transfer to aqueous phase to a fresh tube. Add two original volumes (i.e., same as combined aqueous volumes) of chloroform/isoamyl alcohol. Extract at room temperature for a few minutes, and spin to separate the phases.

9. Transfer the aqueous phase to fresh tube. Add 1/20 volume of 3 M NaOAc and precipitate the RNA by adding 2 to 2.5 volumes of cold absolute ethanol or one volume of cold iso-propanol. Hold at -20°C for 2 hours (or overnight) or at -80°C for 30 minutes.

10. Centrifuge the samples to collect the RNA (e.g., 10-15 minutes in a microfuge or 20-30 minutes in a Sorvall HB-4 rotor at 9000 rpm). Rinse the pellet with 70% Et0H and air dry.

11. If you wish to clean up the samples further by protease treatment, resuspend the samples in TE containing 0.5% SDS, or NETS (TE containing 0.1 M NaCl and 0.5% SDS) or Proteinase K Buffer. Treat with Proteinase K as described in the method for cytoplasmic RNA purification.

References: This protocol is an adaptation of a procedure written by Paul Thomas based on a method developed by Chirgwin et al.

1. Feramisco, J.R., J.C. Smari, K. Burridge, D.M. Helfman and G.P. Thomas. 1982. J. Biol. Chem. **257**:11024-11031.
2. Chirgwin, J., A.E. Przybyla, R.J. MacDonald and W.J. Rutter. 1979. Biochem. **18**:5294-5299.