MOLECULAR BIOLOGY PROTOCOLS

I. Tissue Homogenization & RNA Isolation for cDNA synthesis

1. Pre-spin 2ml Phase lock Gel-heavy tubes briefly (ie- 3,000rpm for 2 min) @ RT.

2. **HOMOGENIZATION**: Obtain two rat eyes from –80ºC freezer and place on ice in a clean uncontaminated 1.5 ml eppendorf tube. To homogenize add 1ml TRIZOL reagent and 10 µl of a 20 mg/ml concentration of glycogen* and homogenize.

Necessary Reagents
2ml phase lock tubes
1.5 ml eppendorf tubes
1.5 ml teflon tissue homogenizer
TRIzol RNA extraction reagent
20mg/ml glycogen *optional
Choroform/isoamyl alcohol (24:1)
Isopropyl Alcohol
75% EtOH
Rnase free water

SUPERSCRIPT II
5X First-Strand Buffer

3. Add cell lysate to the tubes containing the pre-spun Phase lock tubes and incubate for 5-10 minutes @ 15 – 30ºC to allow complete dissociation of nucleoprotein complexes.

4. **PHASE SEPARATION**: Add 0.2ml of chloroform-IAA per 1 ml of TRIzol Reagent. Shake vigorously by hand for 15 seconds and incubate them @15-30ºC for 2-3 minutes.

5. Centrifuge the samples at no more than 12,000 x g for 15 minutes @ 4ºC. After centrifugation the mixture should separate into a lower red , phenol chloroform phase, an intermediate phase, and a colorless upper aqueous phase (RNA will be in the upper aqueous phase). The volume is typically about 60% of the aqueous phase.

6. **RNA PRECIPITATION**: Transfer the aqueous phase to a fresh tube, and save the organic phase if isolation of protein or DNA is desired. Precipitate the aqueous phase by mixing with isopropyl alcohol. Use 0.5 ml of isopropyl per 1 ml of TRIzol reagent. Mix samples by repeated inversion. Allow samples to incubate at –20º C for 10 minutes, then spin at 12,000 x g for 10 minutes @ 4º C.

7. Decant the supernatant by dumping the liquid into a wastebasket (don't worry the RNA will remain attached to the inside of the tube).

8. **RNA WASH**: Add 1 ml of 75% EtOH per 1 ml TRIzol Reagent used. Mix samples to dislodge the pellet and wash salts.

9. Centrifuge samples at 7,500 x g for 5 minutes @ 4º C.

10. Carefully decant the alcohol and remove all the residual alcohol. Dissolve the pellet in 100λ RNAse free ddH20. Incubation @ 55-60º C for 10 minutes may be helpful in dissolving this solution. RNA should be stored at –80ºC after this step.

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Note* typical yields that I have observed are ~ 1µg/µl or 100
µg total for two whole rat eyes.
Sample calculation:
Concentration = OD260 x dilution factor x 40µg/ul
Concentration = .226 x 300/3 x 40 = 904µg/ml or 0.9µg/µl
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11. Obtain the OD $^{260/280}$ readings by spectrophotometry to assess the purity and concentration of this extract.

12. **DNaseI Treatment for cDNA synthesis**: Mix together

- 1µg RNA sample
- 1 µl 10x Dnase Rxn Buffer
- 1µl DnaseI, amp grade, 1U/µl
- DEPC-treated H_2O to 10µl

12. Incubate for 15 minutes @ RT

13. Inactivate DNaseI with 1µl 25mM EDTA solution @ 65ºC for 10 min. Store RNA at –80ºC until needed.