Tumor mRNA Isolation Protocol

Part 1: Total RNA Isolation

1. **Tumors are surgically removed from patients and rapidly transported to pathology where a pathologist removes a piece(s) for microarray analysis. These tumor samples are rapidly frozen in Liquid Nitrogen and stored at –80°C until use.**

2. **The frozen tumor specimen is removed from the freezer and, using a scalpel, small pieces (approximately 50-100 milligrams each) are cut off of the larger tumor specimen while it is still frozen. As the small pieces are cut off, they are immediately placed into 10-12mls of TRIzol Reagent (GibcoBRL Life Technologies) (http://www2.lifetech.com:80/catalog/techline/molecular_biology/Manuals_PPS/15596.pdf) that is contained within a 50ml Screw Cap Tube at RT. For a given tumor sample, up to 1 gram can be placed into this volume of TRIzol, and typically 0.5-1 gram of tissue is used per isolation if possible. Note, that as little as 0.2 gram has been successfully used in this volume but it may be better to use 5-8mls TRIzol for specimens of this size (the TRIzol Reagent Protocol calls for 1ml TRIzol per 50-100mg tissue processed).**

3. **The tumor sample in TRIzol is homogenized using a PowerGen 125 Tissue Homogenizer (Fisher Scientific) starting at 5000 RPM and gradually going up to approximately 20,000 RPM over a period of 30-60 seconds at RT. The homogenization is performed until a homogeneous solution is obtained and very few visible tumor pieces can be seen.**

4. **Incubate at RT for 5-10 minutes after homogenization.**

5. **The TRIzol/tumor homogenate is transferred to a 50ml Oak Ridge Centrifuge tube and centrifuged at 12,000g (10,000RPM on SS34) for 5-10 minutes at 4°C.**

6. **Remove the upper fat layer by using a Pasteur pipette hooked up to a vacuum flask (the upper fat layer, if present, has a yellowish appearance while the TRIzol homogenate remains red).**

7. **NOTE: From here to Step 12, the protocol is exactly as written in the TRIzol Reagent Protocol (see above for URL).**

8. **Add 0.2ml Chloroform per 1ml TRIzol Reagent used in Step 2. Shake tube vigorously for 15-30 seconds by hand and incubate at RT for 5 minutes.**

9. **Spin at 12,000g for 15 minutes at 4°C. Carefully remove the upper aqueous phase, which contains the total RNA, and place this in a new 50ml Oak Ridge Centrifuge tube.**

10. **Precipitate the RNA by adding 0.5ml Isopropyl alcohol per 1ml TRIzol Reagent used in Step 2. Incubate at RT for 10 minutes, then spin at 12,000g for 10 minutes at 4°C.**

11. **Carefully decant the supernatant and wash the pellet once using 75% ETOH by adding 1ml 75% ETOH per 1ml TRIzol used in Step 2. Spin at 7,500g (7500 RPM in SS34) for 5 minutes at 4°C.**

12. **Carefully decant the supernatant and air dry the pellet for 10-20 minutes at RT.**

13. **Resuspend the RNA pellet in 200-300µl DEPC-DH20 or DEPC-TE, and take A260 if necessary.**

Part 2: mRNA Isolation

14. **NOTE: From here to the last step I followed the INVITROGEN FastTrack 2.0 Kit Protocol (http://www.invitrogen.com/pdf_manuals/ft_man.pdf) as described, except that I started the mRNA isolation protocol as described for "mRNA Isolation from Total RNA" except that I use 15ml Lysis Buffer.**

15. **Add the 200-300µl of total RNA from Step 13 to 15ml FastTrack Lysis Buffer (which should contain the added RNAse inhibitors, etc) and mix gently.**

16. **Heat to 65°C for 5 minutes and then place on ice for 1 minute.**

17. **Add 950µl of 0.5M NaCl to the 15ml Lysis Buffer plus RNA and mix by swirling.**

18. **Add the Oligo dT aliquot, let sit 2 minutes at RT, and mix by swirling until the Oligo dT is completely dispersed.**

19. **Incubate at RT with gentle shaking for 15-20 minutes.**

20. **Spin at 3000g (2000 RPM in tabletop) for 5 minutes at RT.**

21. **Very Carefully remove the supernatant, then resuspend the pellet in 20ml Binding Buffer and spin as in Step 20.**

22. **Remove supernatant and resuspend in 10ml Binding Buffer and spin again.**

23. **Remove supernatant and wash two more times with 10ml Low Salt Buffer each time.**

24. **Follow FastTrack Protocol and transfer the Oligo dT into the provided 2ml Eppendorf tube as described.**

25. **Wash Oligo dT once using 0.5ml Low Salt Buffer as described.**

26. **Elute the mRNA into a fresh 2ml Eppendorf tube as described using 250µl of FastTrack Elution Buffer that has been preheated to 65°C.**

27. **Repeat Step 26 and pool the eluates so that the total elution volume equals 500µl.**

28. **Precipitate the mRNA by adding 75µl of 2M Sodium Acetate (provided) and 1.25mls of 200Proof ETOH (not provided) to the 500 µl mRNA eluate from Step 27, and then place the sample at –80°C.**

29. **The sample should remain at –80°C at least until it freezes solid, but can also remain in this state for days to weeks.**

30. **Precipitate the mRNA by spinning it at top speed in an Eppendorf Centrifuge at 4°C for 20-30 minutes.**

31. **Decant the supernatant and air dry the pellet for 15-25 minutes at RT.**

32. **Resuspend the mRNA pellet in 10-20 microliters of DEPC-DH20 or DEPC-TE and use 1-2µl to measure the A260 to determine the mRNA.**

33. **Store the mRNA samples at –80°C until use, or bring up the volume to 100µl with DEPC-DH20 and reprecipitate using ETOH and the appropriate salts for long term storage and/or mailing.**