**Isolating RNA for Microarrays**

**Important:  RNase is everywhere!  Wear gloves and change them if you touch anything that may have RNase (such as your skin, hair, etc).  Use only baked glassware or virgin plastic.  Make sure solutions are RNase free: DEPC-treat solutions noted below.  Be very careful to avoid introducing RNase to isolation procedure**.

For this procedure, you will need the following;

1)  **\***Chaos Buffer

2) **\***2M NaAc, pH 4.0 – bring to correct pH with glacial acetic acid (GAA)

**Note: leave a lot of room because you will have to use quite a bit of GAA to correctly pH the reagent.  For example, you will probably have to add 150ml of GAA to make a final volume of 250ml 2M NaAc pH 4.0.**

3)  3M NaAc – to add to 100% EtOH for storage of RNA after isolation

4)  Phenol – acidic (pH 4.3) – make sure it is warmed to room temp

5)  Chloroform Iso-Amyl Alcohol (C.I.A. 23:1)

6)  Isopropyl Alcohol

7)  **\***70 % Ethanol – use DEPC – treated water and Rnase-free EtOH which is stored @ - 20oC

8)  **\***dH2O

9)  Qiagen RNA Isolation Kit

10) b-Mercaptoethanol (for Qiagen Kit)

11)  100% EtOH (RNase-free) – a fresh bottle of EtOH that is only used for RNase-free work.  Store at –20oC.

Chaos Buffer for RNA isolation

\***note: below are final concentrations.  You should start from a working stock of 0.5M EDTA and 1M Tris-HCl**

For 100 mls 200 mls

4.5M Guanidinium thiocyanate 53.2 g 106.4 g

2% N-lauroylsarcosine (sarcosyl 2.0g 4.0g

**\***50mM EDTA, pH 8.0 10 mls of 0.5M 20 mls

25mM Tris-HCl, pH 7.5 – use DEPC-H2O 2.5 mls of 1M 5 mls

0.1M b-Mercaptoethanol 700ul 1.4mls

0.2% Antifoam A (Sigma) 200 ul 400 ul

**\* = Must be RNase Free**

To make Rnase Free: make reagent 0.2% DEPC (diethyl pyrocarbonate – Sigma D-5758 – located at 4oC).  Mix well for one hour.  Autoclave 15 min per liter.

Aliquot reagents in to smaller tubes: 1.5 ml, 15 ml, or 50 ml.  You can use a nitro-cellulose 0.2 micron syringe filter to remove any additional proteins.  RNase-free water can be heated to 95oC and then cooled to room temp before using.

**Protocol**

**\*Note: if you are doing many dissections, you can store the tissues in Ambion RNA later per its protocol and isolate the RNA from the tissues at a later date.**

**\*Note: place acidic phenol at room temperature now.**

1.  Weigh fish.  Record species, location, sample number, sex and weight.  Remove organ.

**\* Note - Mix thoroughly after next 5 steps.**

2.  Immediately place organ in 400 ul of Chaos buffer and quickly homogenize (use electric tissue homogenizer).  Clean tissue homogenizer in 2% SDS and rinse twice in nanopure water.

3.  Add 40 ul 2M NaAc pH 4.0.  Mix by vortexing.

4.  Add 400 ul of **acidic** phenol.  Mix well.

\*Acidic phenol pulls off DNA.  Remember that there is a H2O layer on top of phenol, so draw from bottom of bottle.  Warm phenol to room temp.  If phenol is colored at all, use a new bottle.

5.  Add 200ul of C.I.A.  Mix again.

6.  Let sit on ice for 10 minutes.  Transfer to a 1.5ml tube if not already in one.

7.  Centrifuge on max speed for 20 minutes at 4oC.

**\*While samples are centrifuging, clean work area, racks, and pipette ends with Rocal (diluted 1 part per 1000 part water).**

8.   Remove 400 ul of supernatant, leaving some behind.  To avoid contamination by DNA trapped at the interface, do not take the lowest part of the aqueous phase.

9.  Add 1 volume of isopropanol to supernatant  (should be 400ul).  Vortex, and mix by inverting.

10.  Store for 30 minutes at –20°C.  If you need to take a break or lunch, this is the time to do it.  You can store for longer than 30 minutes.  \*Note: I usually leave my samples in the freezer overnight and spin in the morning.  This has seemed to improve my RNA yield.

11.  Spin at max speed for 30 minutes at 4oC.

12. There should be a small pellet (may be clear, so be careful).  Draw up and remove the liquid, leaving pellet at bottom of tube (be careful not to draw up the pellet)

13.  Rinse pellet carefully with 400 ul of 70% EtOH.  Pull off EtOH.  The pellet should be clear so be careful.  Air dry or speed-vac until all of EtOH is evaporated.

**14. Follow Qiagen RNeasy Mini Protocol for RNA Cleanup:**

**\*use the following notes as guidelines:**

For Qiagen Columns:

\*Use MiniPrep for approx 100ug (e.g. for hearts).

\*Use MidiPrep for up to 1000 ug (e.g. for livers).

\*Use MaxPrep for up to 6000 ug.

\*There is approximately 1.0 ug of RNA/gram body weight in Fundulus hearts.  \*There is approximately 93 ug of RNA/gram body weight in Fundulus LIVERS

\*At beginning of Qiagen RNA Isolation procedure:

**\*After adding 100ul RNase-free water to pellet, make sure RNA goes into solution.  This takes time!  Heat at 70oC for 5 minutes and vortex for 5 minutes or until pellet is no longer visible.**

\*At beginning of procedure,  place RNA reagents for Bioanalyzer at room temp**.**

\*For elution step:

\*For heart: add 30 ul RNase-free water to column.  Let sit at room temp for 5 minutes and then spin according to protocol.  Repeat with another 30ul for a total volume of 60 ul.

15.  Determine RNA concentration using Nanodrop.

16.  Determine RNA quality using Bioanalyzer.  Follow Bioanayzer protocol.

17.  After spec-ing, the RNA needs to be stored in 1/10 volume **3M NaAc** and 2.5 volume EtOH.  Mix and store in –20oC or lower.

18.  When you want to use RNA, take out of freezer.  Vortex well.  Take 2 x the amount needed.  Centrifuge for 20 min.  Wash pellet in EtOH and bring up in H2O or 0.1% SDS (for microarray).