RNA ISOLATION FROM LASER CAPTURE MICRODISSECTION BIOLOGICAL SAMPLES USING PICOPURE RNA ISOLATION KIT

(Updated November 2006, AB)

References:

1. *PicoPure RNA Isolation Kit (cat# KIT0204, Molecular Devices).* ***Please read the original protocol****.*
2. *Kerk et al (2003) recommended using the PicoPure RNA Isolation kit for Laser Capture Microdissection materials.*
3. *According to a personal communication between Julie Pelletier (Harada lab at U.C. Davis) and a tech support at Arcturus in early 2006, RNA purification columns contain nucleic acid carrier poly(dI) or poly(dI:dC), which is also mentioned on page 9 in the manual of RiboAmp HS RNA Amplification Kit. This carrier contributes to the concentration of isolated RNA regardless whether RNA concentration is determined using a UV spectrophotometer or a fluorescent dye Ribogreen and fluorescent dye reader or fluorescent spectrophotometer (such as Nanodrop Fluorospectrometer); moreover, we still experience some degreen of nucleic acid carrier contamination in RNA samples after DNase I treatment and purification via RNeasy Mini kit (Qiagen). Therefore, the actual concentration and amount of isolated RNA are unknown.*
4. *This protocol is written based on the protocols of PicoPure RNA Isolation (Molecular Devices), DNA-free (Ambion), RNeasy Mini RNA Isolation kit (Qiagen), and RiboGreen Assay for RNA (NanoDrop Technologies, Inc.).*

PROCEDURE

NOTE:

(1) CARRIED OUT AT ROOM TEMPERATURE

(2) USE AROSOL-BARRIER (or PCR) PIPET TIPS TO PREVENT CONTAMINATION

A. CLEANSING THE PURIFICATION COLUMNS

(Purpose: to remove most of poly(dI or poly(dI:dC) carrier in the purification columns so that more accurate RNA concentration can be determined).

1. Precondition the purification column as followings:
   a. Pipet **250 µL** of **Conditioning Buffer (CB)** onto the membrane of the purification column.
   b. Incubate the column for **5 minutes**.
   c. Spin the column at **16,000 *xg*** for **1 minute**.
2. In a 1.5-mL RNase-free microcentrifuge tube, mix **20 µL** of **Extraction Buffer (XB)** with **20 µL** of **70% Ethanol** solution by pipetting up and down 10 times.
   a. Pipet the mixture onto the **membrane** of the **purification column**.
   b. Spin at **16,000 xg** for **1 minute**.
3. Pipet **100 µL** of **Wash Buffer 1 (W1)** to the column. Spin at **8,000 *xg*** for **1 minute**.
4. Pipet **100 µL** of **Wash Buffer 2 (W2)** to the column. Spin at **8,000 *xg*** for **1 minute**.
5. Pipet another **100 µL** of **Wash Buffer 2 (W2)** to the column. Spin at **16,000 *xg*** for **2 minutes**. *Inspect the column to ensure the membrane is dry (i.e., free of alcohol solution in the W2 solution. If the membrane is not dry, spin at 16,000 xg for another 1 minute*.
6. Label a 1.5-mL RNase-free microcentrifuge tube as "**BLANK**" for the eluted water.
7. Transfer the purification column from its collection tube to the newly labeled tube in step 6. Discard the solution in the collection tube, but **save** the collection tube for RNA isolation of your sample.

8. Pipet **30 μL** of **DEPC-treated water** or **Elution Buffer (EB)** to the membrane of the column. <u>*Note:*</u> *while pipetting,the solution, touch the pipet tip to the membrane to enhance the absorption of water solution to the membrane.*
9. Incubate the column for **1 minute**.
10. Spin the column at **16,000 *xg*** for **1 minute**.
11. Transfer the column back into its collection tube. ***The column is ready for RNA isolation***.
12. **Save** the **eluted solution** passed through the column as **BLANK** for blanking the Nanodrop spectrophotometer.

B. RNA ISOLATION

<u>Note:</u> *The PicoPure RNA Isolation kit protocol suggests to perform an on-column DNase I treatment after step 7 below. I have not tried it because I had a bad experience with on column RNase-free DNase I using Qiagen kit in early 2002. The problem was that DNase I did not digest DNA completely. After I found Ambion's RNase-free DNase I digested DNA completely, I have used it since (see Section C below).*

1. Thaw tubes containing Laser Microdissected samples in Extraction Buffer (XB, Arcturus) on a microcentrifuge tube rack at room temperature for a few minutes or until the frozen solution is completely thawed.
2. Spin tubes in a microcentrifuge to bring all liquid and samples from the caps to the bottom of the tubes for 30 seconds. <u>*Note:*</u> *use 1.5-mL microcentrifuge tube as an adapter for* ***0.5-mL PCR tube*** *or 0.5-mL PCR tube in a 1.5-mL microcentrifuge tube as the adapter for* ***0.2-mL PCR tube***.
3. Inspect the content in the tubes for the complete thawing of solutions.
4. Incubate the samples at **42°C** for at least **30 minutes**. <u>*Note:*</u> *I used an AIR Incubator to ensure that the WHOLE tube is exposed to the same temperature, NOT just the bottom of the tube when use the heating block.*

5. (Optional) If there are more than 2 tubes of the SAME samples, e.g. Embryo proper, **pool** all solutions from individual tubes into a single tube of 1.5-mL microcentrifuge tube. Determine the **total volume** of the sample solution.
6. Pipet an **equal volume** of **70% Ethanol** solution to the tube containing **LMD'd Sample** in **Extraction Buffer** by pipetting up and down **10 times**. For example, mix **20 µL** of **70% Ethanol** solution to **20 µL** of **RNA solution in Extraction Buffer (XB)**.
    a. Pipet the mixture onto the membrane of the pre-cleansed purification column.
    b. Spin at **100 *xg*** for **2 minutes** and immediately followed by **16,000 *xg*** for **1 minute**.
7. Pipet **100 µL** of **Wash Buffer 1 (W1)** to the column. Spin at **8,000 *xg*** for **1 minute**.
8. Pipet **100 µL** of **Wash Buffer 2 (W2)** to the column. Spin at **8,000 *xg*** for **1 minute**.
9. Pipet another **100 µL** of **Wash Buffer 2 (W2)** to the column. Spin at **16,000 *xg*** for **2 minutes**. Inspect the column to ensure the membrane is dry (i.e., free of alcohol solution in the W2 solution. If the membrane is not dry, spin at 16,000 xg for another 1 minute.
10. Label a 1.5-mL RNase-free microcentrifuge tube according to your **RNA Sample.**
11. Transfer the purification column from its collection tube to the newly labeled tube in step 10.
12. Pipet **10-30 µL** of **DEPC-treated water** or **Elution Buffer (EB)** to the membrane of the column. *Note: (1) while pipetting,the solution, touch the pipet tip to the membrane to enhance the absorption of water solution to the membrane; (2) the volume depends on an estimated amount of RNA being isolated. I recommend using at least 2**0 µL** to ensure that the membrane is well covered with water. I used 30 µL.*
13. Incubate the column for **1 minute**.
14. Spin the column at **1,000 *xg*** for **1 minute** and **immediately followed** by **16,000 *xg*** for **1 minute**.
15. Transfer the column back into its collection tube.
16. Put the tube containing the **eluted RNA solution on ice**.
17. Determine the recovery volume of the nucleic acid solution using a pipetman.

C. REMOVING GENOMIC DNA FROM TOTAL RNA USING RNase-FREE DNase I

Reference: Modify from the Ambion protocol accompanying the DNase-treatment components (Cat # 1906).

1. Add **0.1 volume** of **10X DNase I buffer** and **0.5-1 µL** of **2 Units/µL DNase I** (Ambion) to the RNA solution. One unit of DNase I degrades 1 µg of DNA at 37ºC for 10 minutes (Ambion).

	Example			
DEPC'd Water	3.0 µL			
Vol. of RNA	28.0 µL			
Vol. of 10X DNase I buffer	3.5 µL			
Vol. of DNase I (2 U/µL)	0.5 µL			
Total volume	**35.0 µL**			

2. Mix the content gently by flicking the tube several times. Spin the tubes briefly (5 seconds).
3. Incubate at **37ºC** for **20-30 minutes**. Spin the tubes briefly.
4. To inactivate DNase I, add **0.1 volume** of the **DNase inactivation reagent** (**slurry)** to the sample. Mix well by flicking the tube several times.
   *Note: Make sure the slurry is **WHITE**. If the solution is **CLEAR**, revortex the mixture.*
5. Incubate at room temperature for **2 minutes**. Flick the tube once more during the incubation to redisperse the DNase inactivation reagent.
6. Label an RNase-free 1.5-mL microcentrifuge tube according to your RNA sample.
7. Spin the tubes at **10,000 x g** for **1 minute** to pellet the DNase inactivation reagent.

8. Transfer the RNA solution to a newly labeled tube. Keep tube of RNA solution on ice. Record the **RNA solution volume**.
   *Note: **AVOID PIPETTING THE PELLET!***
9. Determine **RNA concentration** using either a Nanodrop spectrophotometer or an available spectrophotometer in your lab.

D. PURIFYING RNA SAMPLEAS USING RNEASY SPIN COLUMN AND REAGENTS (QIAGEN)

*Note: the RNA solution **may** contain some DNase I inactivating reagent, which can interfere with the subsequent steps. To ensure that the RNA solution is free of the inactivation reagent, I recommend carrying out RNA purification with RNeasy column.*

1. Adjust the reaction **volume to 100 µL** with RNase-free water

Volume of RNA solution				
Volume of RNase-free water or DEPC'd water				
Total Volume	**100 µL**	**100 µL**	**100 µL**	**100 µL**

2. Prepare **RLT buffer** (Qiagen) just before use in the fume hood.
   For each **100 µL of cRNA solution** in step 1, add **350 µL of RLT buffer**.
   Therefore, prepare enough volume for a number of samples.
   For example, prepare 800 µL of RLT buffer for 2 samples as follows:

RLT buffer (Qiagen)	800 µL	
Beta-mercaptoethanol	8 µL	

   Vortex briefly to mix the content well. Spin briefly.
3. Label **RNeasy column(s) (Pink)** according to your RNA sample(s).
4. Add **350 µL of RLT buffer** in step 2 to each RNA sample. Mix the content by pipetting the mixture **5 times**.

5. Add **250 µL** of **100% ethanol solution** (room temperature) to the RNA mixture. Mix the content by pipetting the mixture **5-10 times**.
6. Immediately, transfer **700 µL** of the RNA mixture to the labeled RNeasy mini spin-column sitting in a collection tube. *Note: Make sure that RNA mixture was transferred into the corresponding labeled column.* ***Be careful NOT to mix up!***
7. Spin the tubes at **10,000 rpm** for **15 seconds**.
8. Transfer the column to a **new collection tube**. Do **NOT** discard the flowthrough solution.
9. Add the **flow-through** in the first collection tube to the **same column**.
10. Spin the tube at **10,000 rpm** for **15 seconds**.
11. Transfer the column to a **new collection tube**.
12. Add **500 µL** of **RPE buffer** (Qiagen) to the column.
13. Spin the tube at **10,000 rpm** for **15 seconds**. Discard the flowthrough solution
14. Repeat steps 12-13. After discarding the flow-through, spin the column at **10,000 rpm** for **2 minutes** to get rid of ethanol.
15. Label RNase-free **1.5-mL microfuge tube**(s) according to RNA samples.
16. Transfer the column to an RNase-free 1.5-mL microfuge tube.
17. Elute RNA by pipetting **20 µL of RNase-free water** (Qiagen) or **DEPC'd** (autoclaved) water to the membrane of the column.
18. Wait for **1 min**.
19. Spin at **10,000 rpm** for **1 min**.
20. Determine the **recovery volume** of RNA solution.
21. Determine **concentration of RNA** samples using a Nanodrop Fluorospectrometer. *It is recommended to use Ribogreen dye and fluorescent reader or Nanodrop Fluorospectrometer to determine the actual concentration of RNA.*

Determination of RNA Concentration and RNA Yield Using Ribogreen and Nanodrop Fluorometer

Sample	[RNA] (pg/uL)	Dilution Factor	Adjusted [RNA] (ng/uL)	Vol. of RNA Solution (uL)	RNA Yield (ng)

22. (Optional) the volume of RNA solution can be reduced by concentrating RNA solution in a SpeedVac, which was decontaminated with a freshly prepared DEPC-treated (NOT autoclaved) water, for 5-10 minutes. **Determine the new volume using a pipetman**.