RNA AMPLIFICATION USING RIBOAMP OA KIT (ARCTURUS) AND BIOTINYLATED cRNA SYNTHESIS USING BIOARRAY HIGH YIELD RNA TRANSCRIPT LABELING KIT (ENZO) – SHORT VERSION (Updated December 08, 2006)

Experiment Name:

Objective(s):

Background Information:

Preparation of RNA Samples

1. Record **RNA samples' concentration** and **volume of amount** (**2 ng** or **4 ng**) to be used in RNA amplification in the table below. Calculate the **volume of DEPC'd water** needed to bring the **RNA volume** to **10 μL** for RNA amplification.

<u>Note:</u> For validation of genechip data using qRT-PCR, it is best to save the first-strand cDNA solution generated from the round-one amplified RNA. Therefore, if there is more than 5 ng of total RNA available from each tissue, use 4 ng of RNA solution as the starting material for RNA amplification. Then, save 10-20 ng of equivalent amplified RNA in the form of first-strand cDNA solution.

RNA Samples	[RNA] (pg/μL)	Volume of __ ng (μL)	Volume of DEPC'd Water (μL)	Total Volume (μL)
				10.0
				10.0
				10.0
				10.0
				10.0
				10.0
				10.0
				10.0
				10.0
				10.0
				10.0
				10.0
				10.0

2. Label 0.2-mL RNase-free PCR tubes with color sharpied pens and write Initial of tissues on the lid and side of tubes.
3. Set labeled tubes in a rack on ice.
4. Pipet the appropriate volume of DEPC'd water and RNA samples into each tube. Mix the content by gentle flicking. Spin tubes briefly (if necessary).
5. Proceed with Round One 1st Strand Synthesis.

Round One: 1st Strand Synthesis

Program the thermocycler Round1 and Round2 cDNA Synthesis with the following profiles (see below). *Note: it takes about 1.5 min for the MyCycler/MyiQ thermocycler to cool to 4°C from higher temperature.*

Round1 1st Strand Ribo
65°C, 5 min/ 4°C, 5 min/
42°C, 45 min/ 4°C, 5 min/
37°C, 20 min/ 95°C, 5 min/ 4°C, 5 min/

Round1 2nd strand Ribo
95°C, 2 min/ 4°C, 5 min/
25°C, 5 min/ 37°C, 10 min/ 70°C, 5 min/ 4°C, for up to 30 min

1. Prepare RNA sample in a total volume of **10 µL** (see RNA Input Recommendations) in a **0.2-mL RNase-free PCR tube**.
2. Thaw **Primer A (Beige-labeled Vial A, (Lot #)**, thoroughly mix, and spin down. Keep on ice.
3. Add **1.0 µL of Primer A**. Mix well and spin down.
4. Incubate at **65°C for 5 minutes**, then chill the samples to **4°C for at least one minute**. **Hold the sample at 4°C until ready to proceed**. Spin down the contents before proceeding to the next step.
5. Place 1st Strand Master Mix (**Red-labeled Vial 1**) on ice. 1st Strand Master Mix must be thawed, thoroughly mixed with all solids dissolved, and **maintained at 4°C until used**. 1st Strand Enzyme Mix does not require thawing and can be placed directly on ice. Mix enzyme throughly by inverting several times. Spin briefly.
6. Add 1st Strand Synthesis components in the order listed in the following table. (**BEST way**) **If you are performing several amplifications, you may wish to prepare a Complete 1st Strand Synthesis Mix based on the following table, and add 9.0 µL Complete 1st Strand Synthesis Mix** to each sample. Mix thoroughly by flicking the tube and spin down. ***DO NOT VORTEX.***

Complete 1st Strand Synthesis Mix

Component	Volume of One Reaction	Master Mix (x)	Red Vial #
1st Strand Master Mix (Lot #)	7 µL		**1**
1st Strand Enzyme Mix (Lot #)	2 µL		**2**
Complete 1st Strand Synthesis Mix	**9 µL**		

7. Incubate at **42°C for 45 minutes**, then chill the sample to **4°C for at least one minute**. Spin down briefly before proceeding to the next step.

Read all Detailed Protocol notes on the previous two pages prior to beginning.
Place components back onto ice or refreeze immediately after dispensing the reagent. Do not leave reagents at room temperature.

8. (Optional) You may remove a **2.0 µL** sample at this point in the protocol to assess the integrity of the starting mRNA by qRT-PCR. ***I do not do this step***.
9. Thoroughly mix and spin down **1st Strand Nuclease**. Mix. Place on ice.
10. Add **2.0 µL** of **1st Strand Nuclease Mix** (**Gold-labeled** Vial, **Lot #** **)** to the sample, mix thoroughly and spin down.
11. Incubate the sample at **37°C for 20 minutes** followed by **95°C for 5 minutes**.
12. Chill the sample to **4°C for at least one minute** and hold at that temperature until ready to proceed. Spin down briefly.

OK ***It is okay to stop at this point in the protocol. Sample may be stored at –20°C overnight.***

13. Thaw **Primer B** (**Pink-labeled** Vial **– B, Lot #**), thoroughly mix, and spin down.
14. Add **1.0 µL** of **Primer B** at **4°C**. Mix thoroughly by flicking the tube and spin down.
15. Incubate sample at **95°C** for **2 minutes**, then chill and maintain the sample at **4°C** for **at least 2 minutes**.
16. Place **2nd Strand Components** (**White-labeled Vials**) **on ice**. ***2nd Strand master mix must be thawed, thoroughly mixed with all solids dissolved, and maintained at 4°C until used***. 2nd Strand Enzyme Mix does not require thawing and can be placed directly on ice. **Mix enzyme thoroughly by inverting several times**. **Spin briefly**.
17. Add **2nd Strand Synthesis components** separately in the order listed in the following table. If you are performing several amplifications, you may wish to prepare a Complete 2nd Strand Synthesis Mix based on the following table, and **add 30 µL Complete 2nd Strand Synthesis Mix to each sample**. Mix **thoroughly by flicking the tube** and **spin down briefly**. **Store at 4°C until use**

Complete 2nd Strand Synthesis Mix

Components	**Volume of One Reaction**		**White Vial #**
2nd Strand Master Mix (**Lot #** **)**	**29 µL**		**1**
2nd Strand Enzyme Mix (**Lot #** **)**	**1 µL**		**2**
Complete 2nd Strand Synthesis Mix	**30 µL**		

18. Incubate the sample as follows:
    - 25°C 5 minutes
    - 37°C 10 minutes
    - 70°C 5 minutes
    - 4°C Hold until ready to proceed (**up to a maximum of 30 minutes**).

⚠ ***Place components back onto ice or refreeze immediately after dispensing the reagent. Do not leave reagents at room temperature.***

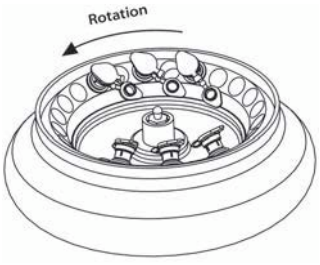
Round 1: cDNA Purification

1. Add **250 µL** of **DNA Binding Buffer** (**DB, Lot #**) to a new purification column seated in the collection tube provided.
2. Incubate for **five minutes** at room temperature. Centrifuge at **16,000 x g** for **one minute**.
3. At meantime, **label** and **color-code 1.5-mL RNase-free microfuge tubes** according to RNA samples. Set tubes on a rack.
4. Add **200 µL** of **DB** to each of the tubes prepared in step 3.
5. After **2 minutes at 4°C**, transfer tubes from the thermocycler to a rack at room temperature.
6. Pipet the entire volume (~**52 µL)** of of the **2nd Strand Synthesis solution to tubes at step 4**, mix well, and pipette the entire volume into the purification column.
7. To bind cDNA, centrifuge at **100 x g (or lowest speed setting available)** for **two minutes**, immediately followed by a centrifugation at **10,000 x g** for **1 minute**.
8. Add **250 µL** of **DNA Wash Buffer** (**DW, Lot #**) to the column and centrifuge at **16,000 x g** for **two minutes**. *Note: Check the purification column for any residual wash buffer. If any wash buffer remains, recentrifuge at 16000 x g for 1 minute. I found the membrane is always dry after centrifugation.*
9. Discard the collection tube and flowthrough. *Optional: Retain collection tubes and use to support 0.5 ml tubes during elution steps. See Section IV.D.3 for details.*
10. Place the column into the **provided 0.5 mL microcentrifuge tube** or **1.5-mL microfuge tube** (*I use it*) and carefully add **11 µL** of **DNA Elution Buffer** (**DE**) or **DEPC'd water** (*I use it*) onto the center of the purification column membrane. Gently touch the tip of the pipette to the surface of the membrane while dispensing **DE** to ensure maximum absorption of **DE** into the membrane. Gently tap the purification column to distribute the buffer, if necessary.
11. Incubate for **one minute** at room temperature.
12. Centrifuge at **1000 x g** for **one minute**, **followed immediately** by **16,000 x g** for **one minute**. Discard the column and retain the elution containing the cDNA.
13. Place **tubes containing purified cDNA solutions on ice**.

OK ***It is safe to stop at this point in the protocol. You may store the sample overnight at –20°C.***

DNA Binding Buffer (DB) must be at room temperature and thoroughly mixed before use. A precipitate may form during long term storage. Dissolve precipitate prior to use by mixing. If necessary, warm the DB vial to redissolve.

Avoid splashing flowthrough in the collection tube onto the column. If flowthrough waste liquid wets the outside of the purification column, recentrifuge the column at 16,000 x g to remove liquid.



Tubes must be properly oriented in the rotor during elution. See Section IV.D.3 for details.

Round One: *In Vitro* Transcription (IVT)

1. Place all **IVT components** (**Blue-labeled Vials**) on ice. **IVT Buffer and Master Mix must be thawed to room temperature (22 – 25°C) and thoroughly mixed with all solids dissolved**. **IVT Enzyme Mix** does not require thawing and can be put directly on ice. Mix enzyme thoroughly by inverting several times. Spin briefly.
2. Label and color-code a set of 0.2-mL RNase-free microfuge tubes. Set tubes on a rack on ice.
3. Transfer **8-10 µL** of **purified cDNA solution** from 1.5-mL tubes to 0.2-mL tubes. ***Caution: pay attention to labels and color-code to prevent mixing up samples***.
4. Add **IVT components** in the order listed in the following table. If you are performing several amplifications, you may wish to prepare a Complete IVT Reaction. Mix according to the following table, and **add 12 µL** of the **Complete IVT Reaction Mix** to **each sample**. **Mix** thoroughly **by flicking the tube** and **spin down**.

Complete IVT Reaction Mix

Component	Volume	Blue Vial #	Master Mix (x____ rxns)
IVT Buffer (Lot #)	**4 µL**	**1**	
IVT Master Mix (Lot #)	**6 µL**	**2**	
IVT Enzyme Mix (Lot #)	**2 µL**	**3**	
Complete IVT Reaction Mix	**12 µL**		

5. Incubate at **42°C** for **3 hours** using **"42deg3hrs"** profile (42°C, 60 min, x3 cycles/4°C, ∞) on the MyCycler. Chill the sample(s) to 4°C.

OK ***At this point in the protocol, you may hold the reaction mixture at 4°C in the thermal cycler overnight.***

6. Add **1 µL DNase Mix** (**Blue-labeled Vial 4,** Lot #). Mix thoroughly and spin down.
7. Incubate at **37°C** for **15 minutes** using "**37deg15min"** profile (**37°C, 15 min/4°C, 5 min**). **Chill the sample(s) to 4°C**. **Proceed immediately to aRNA purification**.

***Place components back onto ice or refreeze immediately after dispensing the reagent. Do not leave* reagents at room temperature.**

DNase Mix *must be thoroughly mixed and held at 4°C until used.*
RNA may be adversely affected if not purified immediately after DNase treatment.

Round 1: Antisense RNA (aRNA) Purification

1. Add **250 µL** of **RNA Binding Buffer** (**RB**) to a new purification column.
2. Incubate for **5 minutes** at room temperature. Centrifuge at **16,000 x g** for **1 minute**.
3. Add **120 µL** of **RB** to the **IVT reaction** sample and **mix thoroughly**. Pipette the entire volume into the purification column.
4. To bind aRNA, centrifuge at **100 x g (or lowest speed setting available)** for **two minutes**, immediately followed by a centrifugation at **10,000 x g** for **one minute** to remove flowthrough.
5. Add **200 µL** of **RNA Wash Buffer** (**RW**) to the purification column and centrifuge at **10,000 x g** for **one minute**.
6. Add **200 µL** of fresh **RW** to the purification column, and centrifuge at **16,000 x g** for **two minutes**. *Check the purification column for any residual wash buffer. If any wash buffer remains, re-centrifuge at 16,000 x g for one minute.*
7. Discard the collection tube and flowthrough.
8. Place the purification column into a new **1.5 mL microcentrifuge** tube or 0.5 mL tube provided in the Kit and carefully add **12 µL** of **RNA Elution Buffer** (**RE**) or **DEPC'd water** (*I used it*) directly to the center of the purification column membrane. Gently touch the tip of the pipette to the surface of the membrane while dispensing **RE** to ensure maximum absorption of **RE** into the membrane. Gently tap the purification column to distribute the buffer, if necessary.
9. Incubate at **room temperature** for **one minute**.
10. Place each column-tube assembly into the centrifuge rotor with the 1.5 ml tube cap trailing the tube.
11. Centrifuge at **1,000 x g** for **one minute**, immediately followed by **16,000 x g** for **one minute**. Discard the purification column and retain the elution containing the aRNA.

Avoid splashing flow-through in the collection tube onto the purification column. If flowthrough waste liquid wets the outside of the purification column, recentrifuge the column at 16,000 x g to remove the liquid.

12. Place tubes containing amplified RNA on **ice** or store at **4°C**.
13. Determine RNA concentration using Fluorescent Nanodrop spectrophotometer (see its protocol)
    a. Prepare **1.5 mL** of **1x TE buffer** from **20x TE buffer** with **DEPC'd water**.
    b. Remove an aliquot of 5 µL of Ribogreen solution stock from the -20°C RNA freezer in room 2911. *Note: Ribogreen dye is light sensitive.*
    c. Pipet 995 µL of of **1x TE buffer** to **5 µL of aliquoted Ribogreen solution**. Vortex briefly. Spin briefly. Keep the tube in a Revco box.
    d. Label 1.5-mL **Amber** tubes.
    e. Pipet **3 µL of 1x TE buffer** inside of the tube cap of the labeled tube.
    f. Pipet **1 µL of amplified RNA solution** using a **P-2 pipetman** to the 1xTE solution in step e and mix by pipetting up and down 3 times.
    g. Pipet **4 µL of 200-fold diluted Ribogreen solution to** the **amplified RNA and TE mixture** and mix by pipetting. *Note: the **dilution factor** is **8**.*
    h. Spin tubes briefly to bring solution to the bottom of the tubes.
    i. Incubate at room temperature for **5 minutes**.
    j. Read **RNA concentration** for RNA samples (two readings per sample) using the Fluorescent Nanodrop.
    k. Calculate the **average RNA concentration with dilution factor** of **8** and the **yield of RNA**.
14. Proceed to Round 2 for biotinylated cRNA synthesis or store the purified aRNA at –70°C overnight.

Round 2: Generating First-Strand Amplified cDNA

Round2 1st Strand Ribo
65°C, 5 min/ 4°C, 5 min/
25°C, 10 min/
37°C, 45 min/ 4°C, 5 min/

Round2 2nd strand Ribo
95°C, 2 min/ 4°C, 5 min/
37°C, 15 min/ 70°C, 5 min/ 4°C, for up to 30 min

The RiboAmp OA Kit is formatted to insert alternate validated methods for transcript labeling conveniently during a second round of amplification. After the first round of amplification, purified aRNA is converted to ds cDNA and purified as described in the following protocol section. This ds cDNA becomes template in a second amplification with simultaneous labeling using either commercial kits, such as the ENZO® BioArray™ High Yield™ RNA Transcript Labeling Kit (Affymetrix) or the Fluorescent Linear Amplification Kit (Agilent), or other protocols provided by the user. Next, labeled aRNA is purified using MiraCol Purification Columns included in the RiboAmp OA Kit.
The following protocol should only be used after performing the first round of amplification. Before proceeding, please note that the ds cDNA synthesis protocol in this section differs from that used in the first cDNA synthesis in the following ways: 1) Primer A is a component of 2nd Strand Synthesis and Primer B is a component of 1st Strand Synthesis; 2) Some reaction temperatures and times have changed; and 3) No 1st Strand Nuclease Mix is used.

1. Thaw **Primer B** (Pink-labeled Vial B), thoroughly mix, and spin down.
2. **Label** and **color-code 0.2-mL RNase-free PCR tubes** according to the earlier color-code for each RNA sample. Set tubes on ice.
3. Into eluted **aRNA product from Round One**, add **1.0 µL of Primer B**, mix well and spin down.
4. Incubate the PCR tube at **65°C** for **5 minutes** then chill the samples to **4°C** for **at least 1 minute**.
5. Place **1st Strand Synthesis components** (Red-labeled Vials) 1st on ice. Strand Master Mix must be thawed, thoroughly mixed with all solids dissolved, and maintained at 4°C until used. 1st Strand Enzyme Mix does not require thawing and can be placed directly on ice. Mix enzyme thoroughly by inverting several times. Spin briefly.
6. Add 1st Strand Synthesis components separately in the order listed in the following table. If you are performing several amplifications, you may wish to prepare a Complete 1st Strand Synthesis Mix based on the following table, and **add 9.0 µL Complete 1st Strand Synthesis Mix** to each sample. Mix thoroughly by flicking the tube and spin down. **DO NOT VORTEX**.

   Complete 1st Strand Synthesis Mix

Component	Volume of One Reaction	Volume of Master mix (x)	Red Vial #
1st Strand Master Mix	**7 µL**		**1**
1st Strand Enzyme Mix	**2 µL**		**2**
Complete 1st Strand Synthesis Mix	**9 µL**		

7. Incubate the sample(s) at **25°C** for **10 minutes** then at **37°C** for **45 minutes**.

8. Chill the sample(s) to **4°C** for **at least one minute**, **but less than 30 minutes**, then spin down briefly.

OK ***It is okay to stop at this point in the protocol. Sample(s) may be stored overnight at –20°C.***

Place components back onto ice or refreeze immediately after dispensing the reagent. Do not leave reagents at room temperature.

Generating Amplified cDNA: 2nd Strand Synthesis

1. Thaw **Primer A** (Beige-labeled Vial - 2), thoroughly mix, and spin down.
2. Add **1.0 µL** of **Primer A** at **4°C**. Mix thoroughly by flicking the tube and spin down.
3. Incubate sample at **95°C** for **2 minutes**, then chill and maintain the sample at **4°C** for **at least 1 minute**.
4. Place **2nd Strand Components** (White-labeled Vials) **on ice**. **2nd Strand master mix** must be thawed, thoroughly mixed with all solids dissolved, and maintained at 4°C until used. **2nd Strand Enzyme Mix** does not require thawing and can be placed directly on ice. Mix enzyme thoroughly by inverting several times. Spin briefly.
5. Add 2nd Strand Synthesis components separately in the order listed in the following table. If you are performing several amplifications, you may wish to prepare a Complete 2nd Strand Synthesis Mix based on the following table, and **add 30 µL Complete 2nd Strand Synthesis Mix** to each sample. Mix thoroughly by flicking the tube and spin down.

Complete 2nd Strand Synthesis Mix

Component	Volume of One Rxn	Volume of Master Mix (x)	White Vial #
2nd Strand Master Mix	**29 µL**		**1**
2nd Strand Enzyme Mix	**1 µL**		**2**
Complete 2nd Strand Synthesis Mix	**30 µL**		
(Store at 4°C until use)			

6. Incubate the sample(s) as follows:
   - 37°C 15 minutes
   - 70°C 5 minutes
   - 4°C Hold until ready to proceed (***up to a maximum of 30 minutes***).

Place components back onto ice or refreeze immediately after dispensing the reagent. Do not leave reagents at room temperature.

Generating Amplified cDNA: cDNA Purification

1. Add **250 µL** of **DNA Binding Buffer** (**DB, Lot #**) to a new purification column seated in the collection tube provided.
2. Incubate for **five minutes** at room temperature. Centrifuge at **16,000 x g** for **one minute.**
3. At meantime, **label** and **color-code 1.5-mL RNase-free microfuge tubes** according to RNA samples. Set tubes on a rack.
4. Add **200 µL** of **DB** to each of the tubes prepared in step 3.
5. After **2 minutes at 4°C**, transfer tubes from the thermocycler to a rack at room temperature.
6. Pipet the **entire volume** (~**52 µL)** of the **2nd Strand Synthesis solution** to **tubes at step 4**, mix well, and pipette the entire volume into the purification column.
7. To bind cDNA, centrifuge at **100 x g (**or **lowest speed setting available)** for **two minutes**, immediately followed by a centrifugation at **10,000 x g** for **1 minute**.
8. Add **250 µL** of **DNA Wash Buffer** (**DW, Lot #**) to the column and centrifuge at **16,000 x g** for **two minutes**. *Note: Check the purification column for any residual wash buffer. If any wash buffer remains, recentrifuge at 16000 x g for 1 minute. I found the membrane is always dry after centrifugation.*
9. Discard the collection tube and flowthrough. *Optional: Retain collection tubes and use to support 0.5 ml tubes during elution steps. See Section IV.D.3 for details.*
10. Place the column into either the **provided 0.5 mL microcentrifuge tube** or **1.5-mL microfuge tube** (*I use it*) and carefully add **22 µL (for Enzo biotin labeling reactions) or 11 µL (for any other reactions)** of **DNA Elution Buffer** (**DE**) or **DEPC'd water** (*I use it*) onto the center of the purification column membrane. Gently touch the tip of the pipette to the surface of the membrane while dispensing **DE** to ensure maximum absorption of **DE** into the membrane. Gently tap the purification column to distribute the buffer, if necessary.
11. Incubate for **one minute** at room temperature.
12. Centrifuge at **1000 x g** for **one minute**, **followed immediately** by **16,000 x g** for **one minute**. Discard the column and retain the elution containing the cDNA.
13. Place **tubes containing purified cDNA solutions on ice**.

OK ***It is okay to stop at this point in the protocol. Sample may be stored overnight at –20°C.***

DNA Binding Buffer (DB) must be at room temperature and thoroughly mixed before use. A precipitate may form during long-term storage. Dissolve precipitate by mixing. If necessary, warm the DB vial to redissolve.

Avoid splashing flow-through in the collection tube onto the purification column. If flowthrough waste liquid wets the outside of the purification column, recentrifuge at 16,000 x g to remove liquid.

22 µl DE can be used if proceeding to ENZO® BioArray™ HighYield™ RNA Transcript *Labeling Kit, or the GeneChip® Expression 3' Amplification Reagent for IVT Labeling (Affymetrix).*

SYNTHESIS OF BIOTIN-LABELED cRNA (IN VITRO TRANSCRIPTION) USING BIOARRAY HIGHYIELD RNA TRANSCRIPT LABELING KIT (T7, ENZO LIFE SCIENCES, INC.)

1. **Label** and **color-code 0.2 mL RNase-free PCR tubes** on their lids and side the sample number/name.
2. Remove a **plastic box of Enzo High Yield RNA Labeling kit** containing FIVE tubes of components listed in the table below from the -20°C RNA freezer in room LS 2918.
3. Keep **tubes #4 and 5 in the Stratagene cooler**. Spin tubes briefly to bring down any liquid on the sidewall to the bottom of the tubes.
4. **Thaw tubes #1-3** (10X HY reaction buffer, 10X Biotin labeled ribonucleotides, and 10X DTT) **at room temperature for a few minutes**. Once solutions thawed, vortex tubes briefly and spin them briefly. Keep the 10X DTT solution on a microfuge-tube rack at room temperature and two other solutions on ice.
5. Prepare a master mix according to the number of samples (or reactions). *Note: work with a master mix to avoid mistakes and errors. Also, calculate the master mix with an extra volume, such as 4.5 reactions for the actual 4 reactions. Lot# on the label of the kit box.*

	Volume of One Rxn	**Master Mix (x)**
cDNA solution	**22 μL**	**------**
10X HY reaction buffer (Lot#)	4 μL	
10X Biotin labeled ribonucleotides (Lot#)	4 μL	
10X DTT (Lot#)	4 μL	
10X RNase inhibitor mix (Lot#)	4 μL	
20X T7 RNA polymerase (Lot#)	2 μL	
Total volume	**40 μL**	

6. Add the following components in order at room temperature to avoid DTT precipitation. Mix well by flicking the tube. Spin briefly in a microfuge.
7. Dispense **18 μL** of **a master mix** containing all components to each tube of samples.
8. **Pipet 22 μL** of cDNA solution to appropriate tube.
9. Mix the content by flicking the tube for 5-10 times. Spin tubes briefly in a microfuge if there are lots of bubbles present.
10. Incubate at **37°C** in a **PCR thermocycler** for **16 hours** (Affymetrix Gene Expression Manual November 2004 version suggested 16 hours instead of 4 hours as in the original version of

1999-2003). *Note: you can set the PCR thermocycler to a profile of **16 cycles of 37˚C, 60 min, followed by 4˚C indefinitely**.*

11. Put the tubes of **cRNA solution on ice**.
12. Proceed with cRNA purification.

CLEANING UP AND QUANTIFYING IN VITRO TRANSCRIPTION PRODUCTS USING RNEASY PLANT MINI KIT (QIAGEN)

Use RNeasy mini spin-column (Qiagen, cat#) to clean up one-half volume of cRNA reaction (**20 μL**) as recommended by Affymetrix Genechip Expression Analysis. Rationale is that (1) this volume contains enough cRNA for the Genechip hybridization and (2) if one loses cRNA for some technical problem, he/she can get cRNA from the second half volume. However, if you are familiar well with this method, you can purify the whole volume (**40 μL**), instead of (**20 μL**). Note: although the RiboAmp protocol suggests that the MiraCol columns can be used to purify biotin-labeled cRNA solutions, we recommend NOT to use these columns because Julie (Harada lab) experienced very low yield (~6%) of biotin-labeled cRNA.

1. Label and color-code 1.5-mL RNase-free microfuge tubes according to RNA samples. Set tubes on a rack at room temperature.
2. Transfer **~40 μL** of **biotinylated cRNA solution** from 0.2-mL PCR tubes to newly labeled 1.5-mL microfuge tube according to RNA samples and color-code.
3. Adjust the reaction volume to **100 μL** with RNase-free water

	Full		Half
cRNA solution	**40 μL**	or	**20 μL**
RNase-free water (Qiagen)	**60 μL**	or	**80 μL**

4. Prepare RLT buffer (Qiagen) just before use.

   For each **100 μL** of cRNA mixture 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 (in the fumehood) **8 μL**

   Vortex briefly to mix the content well. Spin briefly to bring down liquid.
5. Add **350 μL** of **RLT buffer** in step 2 to each sample. Mix by pipetting the mixture several times.
6. Add **250 μL** of **100% ethanol solution** to each sample. Mix by pipetting the mixture several times.

7. **Label** and **color-code RNeasy mini spin-column**s according to RNA samples in step 1.
8. Apply **700 µL** of the **mixture** to an **RNeasy mini spin-column** sitting in a collection tube. Spin the tube at **10,000 rpm** for **15 seconds**.
9. Transfer the column to a new collection tube.
10. **Add the flow-through in the first collection tube to the same column**. 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. Spin the tube at **10,000 rpm** for **15 seconds**. Discard the flow-through.
13. **Repeat step 12**.
14. After discarding the flow-through, spin the column at **10,000 rpm** for **2 minutes** to get rid of ethanol.
15. Label and color-code **1.5-mL RNase-free microfuge tubes** according to RNA samples. Set tubes on a rack at room temperature.
16. Transfer the column to the labeled **RNase-free 1.5-mL microfuge tube**.
17. Elute cRNA by pipetting **30 µL** of **RNase-free water** (Qiagen) or **DEPC'd water** to the membrane of the column. Wait for **1 min**. Spin at **10,000 rpm** for **1 min**.
18. Add **another 20 µL** of **RNase-free water** or **DEPC'd water** to the membrane of the column. Wait for **1 min**. Spin at **10,000 rpm** for **1 min**. The total volume is **~50 ml**.
19. Determine the **recovery volume** with a P-100 or P-200 pipetman.
20. Determine the **concentration** of cRNA solutions using a spectrophotometer, i.e. Nanodrop.

***Note:** Write concentration on the side of the tubes.*

Recovery Volume (µL)				
RNA conc. (µg/µL)				
Total amount RNA (µg)				

21. Determine **cRNA quality** using either Experion (BioRad) or Lab-on-a chip (Agilent) or formaldehyde agarose gel (see below).
22. Store **tubes containing biotinylated cRNAs in a -20°C RNA Freezer or -70°C freezer** if fragmentation is not performed immediately.

FRAGMENTATION OF cRNA AND GEL-ANALYSIS

***Note:* It is important to keep the final concentration of sheared biotinylated cRNA (b-cRNA) solutions at least 0.5 μg/μL. That is, the concentration of unsheared b-cRNA must be at least 0.6 μg/μL. If the latter is 0.5 μg/μL or less, you concentrate either the whole volume of b-cRNA solution of the volume of 20 μg in an RNase-free Speedvac for 5-10 minutes.**

1. Add **2 μL of 5X Fragmentation buffer** (200 mM Tris-acetate, pH 8.1, 500 mM KOAc, 150 mM MgOAc) to **every 8 μL of cRNA solution**.

   Note: If the volume of the cRNA solution is less than a multiple of 8 μl, adjust the volume to the multiple of 8 μL with DEPC-treated water. Example, you have 28 μL of cRNA solution, you want to bring the total volume to 32 μL with DEPC-treated water.

   5X Fragmentation buffer

   (combine the following components to a total volume of 20 mL in a sterile 50-mL conical centrifuge tube)

   4.0 mL 1M Tris-acetate, pH 8.1 (Trizma base, pH adjusted with glacial acetic acid)

   0.64 g MgOAc

   0.98 g KOAc

   DEPC-treated water to 20 mL

   Mix thoroughly and filter through a **0.2 mm vacuum filter unit**. This reagent should be aliquoted and stored at room temperature.
2. Label **0.2-mL PCR tubes** according to the sample names.
3. Pipet volumes of b-cRNA, DEPC'd water and 5X Fragmentation solution according to the table below:

Biotinylated cRNA Solutions	Conc. of b-cRNA (μg/μL)	Volume of 20 μg b-cRNA (μL)	Vol. of DEPC'd water (μL)	Vol. of 5X Fragm't. Buffer (μL)	Final Conc. of cRNA (μg/μL)

Mix the contents in the tubes well.

i. **Incubate** at **94˚C** on a heat block for **35 minutes** or in the **MyCycler** thermocycler with the **Fragmentation** profile of **(94ºC, 36 min/4ºC, ∞)**.

ii. Quench tubes **on ice** for **at least 2 minutes**.

iii. Spin tubes briefly to bring down water condensation on the lids. Keep tubes on ice.

4. Label and color-code **1.5-mL tubes** according to the sample names.
5. Transfer fragmented cRNA solutions from the 0.2-mL PCR tubes to the 1.5-mL tubes.
6. Write concentration of the fragmented cRNA solutions on the side of the tubes.
7. Aliquot **1 µL** of **fragmented cRNA solution** into a **new set of tubes** for quality analysis using the Experion StdSens chip(s).
8. Store the fragmented cRNA solutions at **-20˚C** or **-70˚C** until needed.

GEL-ANALYSIS OF UNFRAGMENTED AND FRAGMENTED cRNA USING EITHER EXPERION STDSENS CHIP OR FORMALDEHYDE AGAROSE GEL

EXPERION STDSENS CHIP

1. Label **1.5-mL microfuge tubes** according to sheared (or fragmented) b-cRNA samples.
2. Pipet **1 µL** of unfragmented or fragmented cRNA (@ **~0.5-0.67 µg/µL**) into labeled microfuge tubes. Set tubes on ice.
3. Heat samples on a heat block at **70ºC** for **2 minutes**. Quench **on ice** for **>2 minutes**.
4. Spin tubes at full speed for **15 seconds**. Set tubes back **on ice**.
5. Prepare an Experion **StdSens** chip as usual.
6. Load **1 µL** of unfragmented or fragmented cRNA solution into each of 12 appropriate wells.
7. Run the chip on the Experion system.
8. Analyze data and copy the **gel picture** and **data**.