

from TotRNA labeling to Affymetrix GeneChip array hybridization

Step 1. TotRNA clean up after extraction

RNA quality is possibly the most crucial factor for the success of the experiment and should not be underestimated.

Whatever protocol has been used for RNA extraction is strongly suggested to perform a clean-up using QIAGEN RNeasy columns before proceeding to sample labeling.

Step 2. Spectrophotometric QC

A quality assessment is necessary to verify that the samples are of sufficient molecular weight and purity to proceed with full labeling and hybridization.

Affymetrix Microarray Unit recommends the use of an Agilent Bioanalyzer and a Nano-drop spectrophotometer for these QC measurements, because these instruments require very small sample amounts.

Verify that all samples meet the following requirements for acceptance:

- Concentration > 0.5mg/ml
- A260/A280 >1.7
- Minimum total yield per sample of 3µg

Step 3. Denaturing gel/Bioanalyzer QC

Is always strongly suggested to check for absence of RNA degradation by agarose denaturing gel or by using the Bioanalyzer 2100 if available.

Samples exhibiting degradation should not be carried through labeling and hybridization, because there is an unacceptable risk of poor results.

Step 4. Sample Labeling

In our experience,if starting from 3µg of TotRNA, enough biotinilated cRNA is obtained to hybridize at least single GeneChip array while is suggested to start from 5µg of TotRNA if multiple GeneChip arrays should be processed with aliquotes of the same target.

Important !

The protocol describes the procedure of sample labeling starting from 3 µg of TotRNA; if starting from higher quantities of TotRNA all reagents should be scaled accordingly.

- First strand synthesis -

1) To an RNase free tube add:

0.5 µl	T7-(T)24 primer (100µM)
3 µg	total RNA
<i>to a total volume of 5.5 µl</i>	
<i>DEPC-treated H2O</i>	

2) Mix and incubate at 70°C for 10 minutes. Chill on ice, spin.

3) In the meanwhile on ice premix for EACH sample to process:

2 µl	5x first strand buffer
1 µl	0.1M DTT
0.5 µl	dNTP mix [10mM]
3.5 µl	Total Volume* <i>*remember to add extra volume</i>

4) add 3.5 µl of 1st strand premix to each annealed primer-RNA mix

5) incubate at 37°C for 2 minutes

6) add to each target 0.5µl of SuperScript II RT

7) incubate at 42°C for 1 hour total RNA, 37°C for mRNA.

8) set water bath at 16°C for 2nd strand synthesis.

- Second strand synthesis -

1) Place 1st strand synthesis reactions on ice - spin.

2) On ice, premix the following reagents:

45.5 µl	DEPC-treated H ₂ O
15 µl	5x Second strand buffer
1.5 µl	dNTP mix [10mM]
0.5 µl	E.Coli DNA ligase [10U/µl]
2 µl	E.Coli DNA Polymerase 1 [10U/µl]
0.5 µl	RNaseH [2U/µl]
65 µl	Total Volume* <i>*remember to add extra volume</i>

- 3) add 2nd strand mix to each 1st strand reaction, mix well, spin.
 - 4) incubate at 16°C for 2 hours.
 - 5) add 1µl T4 DNA Polymerase [10U/µl] to the RNA sample, lightly vortex, spin.
 - 6) incubate at 16°C for 5 minutes.
 - 7) add 5µl of 0.5M EDTA.
- Store ds cDNA at -20°C, or proceed directly to the next step.

- Cleanup of ds cDNA -

- 1) add 80 µl of Phenol: chloroform: isoamyl alcohol [48:1:1] (saturated with 10mM Tris pH8) to each ds cDNA sample and vortex.
- 2) take a 1.5 ml Phase Lock Gel Light tube (PLG) for each sample, and pellet the gel in a microcentrifuge for 20-30 seconds;
- 3) transfer the entire cDNA-PCI mixture to the PLG tube and microcentrifuge for 2 minutes at top speed.
- 4) transfer the aqueous phase to a fresh tube and precipitate adding:
 - . 2 µl Glycogen
 - . 80 µl NH₄OAc 5M
 - . 400µl EtOH 100%
- 5) store at -80°C for 60 minutes to increase cDNA recovery
- 6) centrifuge at 4°C for 30 minutes and wash with 70% EtOH
- 7) centrifuge at 4°C for 10 minutes; air dry pellet for 10 minutes.
- 8) resuspend pellet in 1.5µl DEPC-treated H₂O

- In Vitro transcription reaction (using biotin-NTPs) -

- 1) prepare the 1X NTP labeling mix as follows:

2 µl	10x ATP [75 mM]
2 µl	10x GTP [75 mM]
1.5 µl	10x CTP [75 mM]
1.5 µl	10x UTP [75 mM]
3.75 µl	Bio-11-CTP [10 mM]
3.75 µl	Bio-11-UTP [10 mM]
14.5 µl	Total Volume* <i>*remember to add extra volume</i>

- 2) for each 1.5µl cDNA, combine the following reagents at ROOM TEMPERATURE:

14.5 µl	NTP labeling mix
2 µl	10x T7 transcription buffer
1.5 µl	ds cDNA (obtained from 3 µg TotRNA)
2 µl	10x T7 enzyme mix
20 µl	Total Volume

Important !

- 3) run in a thermocycler for precisely 6 hours at 37°C, then leave on hold at 4°C.

DO NOT incubate longer than 6 hours as this may reduce the yield of longer products.

Important !

DO NOT use phenol: chloroform to extract biotinylated RNA or DNA!!!

Important !

If possible, try not to dilute the cRNA to a concentration below 1µg/µl for the fragmentation step.

Important !

Remember to store 1µg of unfragmented cRNA for gel analysis.

- Cleanup of labeled cRNA -

- 1) adjust sample volume to 100µl with RNase free H₂O
- 2) add 350 µl Buffer RTL and mix thoroughly
- 3) add 250µl absolute EtOH and mix well by pipetting - do not centrifuge
- 4) apply the entire sample (700µl) to an RNeasy mini spin column sitting in a collection tube - centrifuge for 15 seconds at ~10,000 rpm
- 5) transfer RNeasy column into a new 2 ml collection tube, add 500µl Buffer RPE (previously diluted with 4 volumes of EtOH as indicated by the supplier!), centrifuge for 15 seconds at ~10,000 rpm
- 6) discard the flow-through
- 7) pipet 500µl Buffer RPE onto RNeasy column and centrifuge 2 minutes at maximum speed to dry the membrane.
- 8) discard the flow-through and spin dry at max speed for 2 minutes.
- 9) Transfer the RNeasy column into a new 1.5ml collection tube and pipet 30-50µl (see below) with RNase free H₂O directly on the membrane. Leave 1 minute, then centrifuge 1 minute at ~13,000 rpm to elute. Repeat elution step.
- 10) Quantitate cRNA with spectrophotometer (NanoDrop if available).

- Fragmentation of cRNA for target preparation -

First calculate the adjusted yield of cRNA:

Adjusted yield = amount of cRNA measured - starting amount of totalRNA

Adjusted concentration = adjusted yield/ volume of elution.

Fragmentation reaction

NB: Use 10µg (min 5 max 30) of fragmented cRNA for each 200µl of hybridization mix. For each chip set, you will need 250µl of hybridization mix. Before fragmenting, calculate how many replicates you want to perform, and therefore how much hybridization buffer you will need. Fragment only the cRNA necessary for your experiment, and store the remaining cRNA (if any) at -80°C.

Perform all calculations considering the adjusted yield and adjusted concentration.

For example, if you have 60µg of cRNA and you want to perform a duplicate of your chip set, you will need 500µl of hybridization solution, therefore 25 µg of cRNA.

Set up the fragmentation reaction in the minimum possible volume, considering the fragmentation buffer is a 5X.

Incubate for 35 minutes at 94°C.

Save an aliquot of fragmented cRNA for gel analysis.

At least 2µg of fragmented cRNA are necessary for gel analysis.

Store the undiluted, fragmented cRNA at -20 until ready to perform hybridization.

Run a denaturing agarose gel (1%), loading 1µg of unfragmented cRNA and 2µg of fragmented cRNA.

Marker (9.49 kb, 7.46 kb, 4.4 kb, 2.37 kb, 1.35 kb, 0.24 kb)

MK 1 2 3 4 5 6 MK



Unfragmented cRNA

Fragmented cRNA
(should be ~35 - 200 bp)

- Preparing the hybridization target -

Before preparing the pre-mix, the 20X GeneChip Eukaryotic Hybridization Control cocktail must be heated for 5 minutes at 65°C.

The composition of the premix is as follows:

Important !

It is convenient to premix all reagents before adding the master mix to the fragmented cRNA samples.

<i>Reagents</i>	<i>Single array</i>	<i>Two arrays</i>
Control B2 oligonucleotide	4.15 µl	8.3 µl
20X Eukaryotic Hybridization Controls	12.5 µl	25 µl
Herring Sperm DNA (10mg/ml)	2.5 µl	5 µl
Acetylated BSA (50mg/ml)	2.5 µl	5 µl
2x Hybridization buffer	125 µl	250 µl
fragmented cRNA in H ₂ O	12.5 µg to final volume of 250 µl	25 µg to final volume of 500 µl

- Target hybridization to GeneChip arrays -

- 1) heat the hybridization cocktail for 5 minutes to 95°C
- 2) spin in microfuge at maximum speed for 5 minutes to remove any insoluble material.

In the meanwhile prepare the GeneChip probe array as follows:

- 1) equilibrate probe arrays to room temperature before use
- 2) wet the array by filling it with the appropriate volume of 1X Hybridization buffer (80µl for Test Chips, 200µl for Standard arrays)
- 3) incubate for 10 minutes at 45°C with rotation at 60rpm.
- 4) remove the buffer and replace with appropriate volume of clarified hybridization cocktail
- 5) place the probe array in the rotisserie oven and hybridize for 16 hours at 45°C, with a rotation of 60 rpm.