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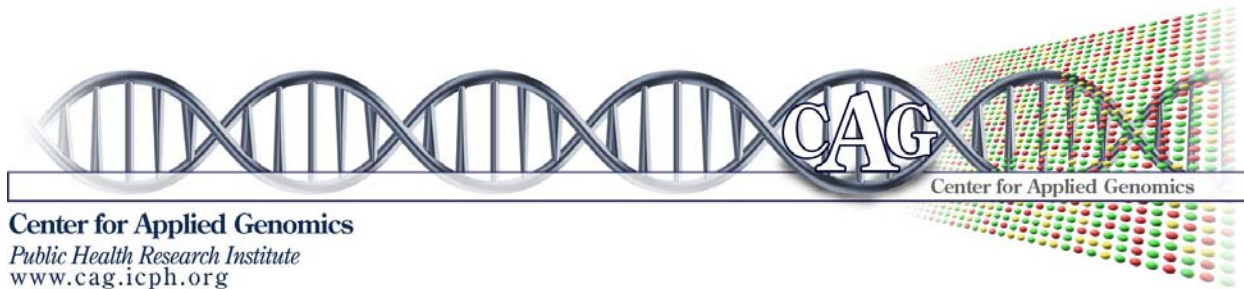
## PROTOCOLS FOR AFFYMETRIX GENE CHIPS

### Synthesis of Double-Stranded cDNA from mRNA

#### First Strand cDNA Synthesis

<u>Reagent</u>	<u>Volume</u>	<u>Final</u>
DEPC-treated H <sub>2</sub> O	--- $\mu$ l	
mRNA (0.2 to 5 $\mu$ g)	--- $\mu$ l	0.2 to 5 $\mu$ g
T7-(dT) <sub>24</sub> Primer (100 pmol/ $\mu$ l)	1 $\mu$ l	100 pmoles
Mix. Incubate at 70°C for 10 min. Quick spin and put on ice.		
5X first strand buffer	4 $\mu$ l	1X
0.1M DTT	2 $\mu$ l	10 mM
10 mM dNTP mix	1 $\mu$ l	500 $\mu$ M each
Mix. Incubate at 37°C for 2 min.		
SSII RT (200 U/ $\mu$ l)	--- $\mu$ l (1 $\mu$ l/ $\mu$ g RNA)	200 to 1000 U
Mix.		
Final Volume	20 $\mu$ l	

Incubate at 37°C for 1 h. Place on ice.



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## Second Strand cDNA Synthesis

<u>Reagent</u>	<u>Volume</u>	<u>Final</u>
DEPC-treated H <sub>2</sub> O	91 $\mu$ l	
5X second strand buffer	30 $\mu$ l	1X
10 mM dNTP mix	3 $\mu$ l	200 $\mu$ M each
10 U/ $\mu$ l DNA Ligase	1 $\mu$ l	10 U
10 U/ $\mu$ l DNA Polymerase I	4 $\mu$ l	40 U
2 U/ $\mu$ l RNase H	1 $\mu$ l	2 U
Volume	130 $\mu$ l	

Mix. Add to first strand synthesis tube.

Final Volume                      150  $\mu$ l

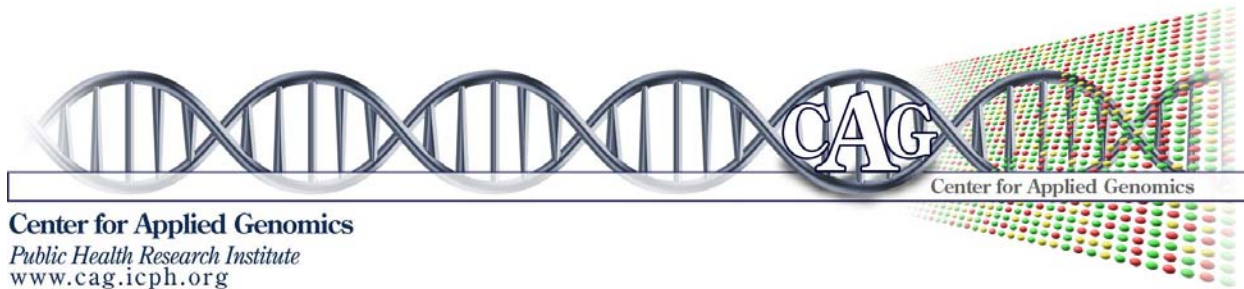
Incubate at 16 °C for 2 h.

Add 2  $\mu$ l (10 U) T4 DNA Polymerase.

Incubate at 16°C for 5 min.

Add 10  $\mu$ l 0.5 M EDTA.

Proceed to clean-up procedure or store at -20°C.



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## Cleanup of Double Stranded cDNA

### Phase Lock Gel - Phenol/Chloroform Extraction

Pellet the PLG (1.5 ml tube with PLG I-light) in a microfuge at 12,000xg for 20 to 30 sec.

Add 162  $\mu$ l of Phenol:chloroform:isoamyl (25:24:1, saturated with Tris-HCl, pH 8.0, 1 mM EDTA) to the cDNA synthesis preparation. Vortex.

Transfer the cDNA-phenol mixture to the PLG tube.

Microcentrifuge at 12,000xg for 2 min.

Transfer the aqueous upper phase to a fresh 1.5 ml tube.

### Ethanol Precipitation

Add 0.5 volumes of 7.5 M  $\text{NH}_4\text{Ac}$  and 2.5 volumes of absolute ethanol (stored at  $-20^\circ\text{C}$ ) to sample and vortex. Precipitate at  $-20^\circ\text{C}$  for at least 1 h (O/N precipitation may result in a better yield). Centrifuge at 12,000xg at room temp for 20 min.

Wash pellet with 0.5 ml 80% ethanol (stored at  $-20^\circ\text{C}$ ). Centrifuge at 12,000xg at room temp for 5 min.

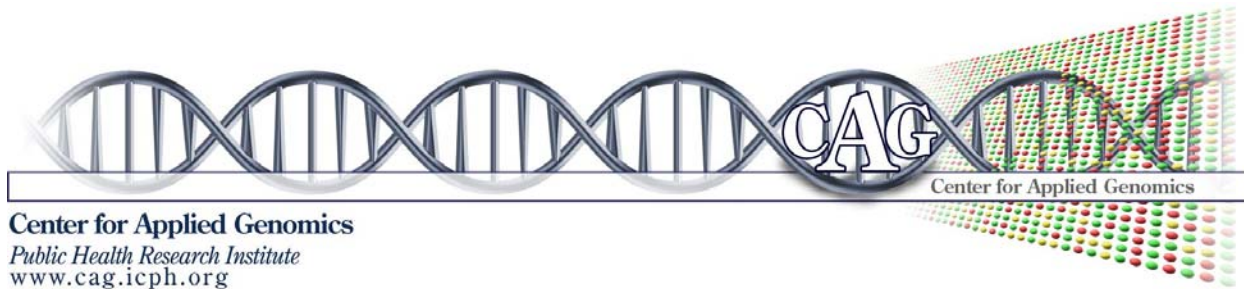
Repeat 80% ethanol wash.

Dry the pellet in speedvac.

Resuspend the dried pellet in 11  $\mu$ l RNase-free water.

Analyze 1  $\mu$ l on a gel.

Proceed to cRNA synthesis or store at  $-20^\circ\text{C}$ .



IVT Reaction

<u>Reagent</u>	<u>Volume</u>
cDNA	--- $\mu$ l*
DEPC-treated H <sub>2</sub> O	--- $\mu$ l
10X HY Reaction Buffer	4 $\mu$ l
10X Biotin Labeled Ribonucleotides	4 $\mu$ l
10X DTT	4 $\mu$ l
10X RNase Inhibitor Mix	4 $\mu$ l
20X T7 RNA Polymerase	2 $\mu$ l
Total Volume	40 $\mu$ l

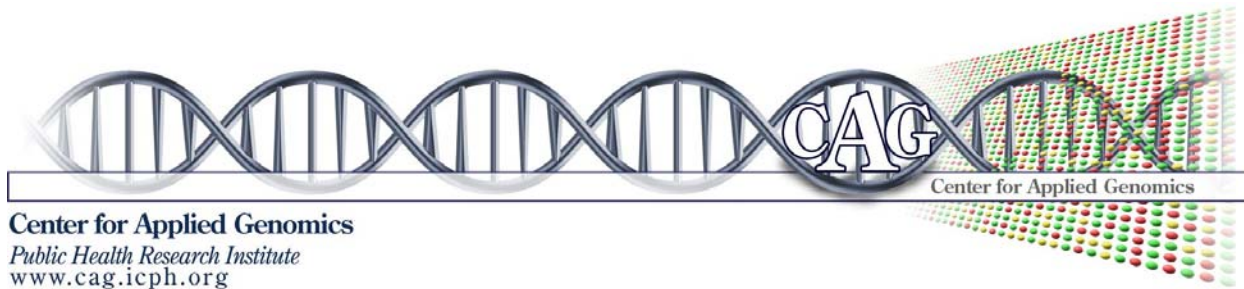
Mix well. Place tube at 37°C. Incubate for 4-5 hours, gently mixing the contents of the tube every 30-45 min.

Purify RNA immediately or store at -20°C.

Approximately 50-100  $\mu$ g of cRNA are usually produced from 1  $\mu$ g of template.

\*Volume of cDNA to use in IVT

<u>Starting mRNA(<math>\mu</math>g)</u>	<u>Volume of cDNA to use (<math>\mu</math>l)</u>
0.2 - 0.5	10.0
0.6 - 1.0	8.0
1.0 - 2.0	5.0
3.0 - 5.0	2.0



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### Cleaning up IVT Reaction

The IVT reaction is cleaned up using the Qiagen RNeasy Mini Protocol for RNA Cleanup.

Divide each reaction in half and purify each half on a separate column.

### Important notes before starting

Do not apply more than 50  $\mu\text{g}$  to the column.

Do not DNase treat the IVT reactions.

Do not add  $\beta$ -mercaptoethanol to Buffer RLT.

Buffer RPE is supplied as a concentrate. Before using for the first time add 4 volumes of 96-100% ethanol to obtain a working solution.

Perform all centrifugation steps at room temperature.

Adjust sample to a volume of 100  $\mu\text{l}$  with RNase-free water. Add 350  $\mu\text{l}$  of Buffer RLT to the sample. Mix thoroughly.

Add 250  $\mu\text{l}$  of 100% ethanol. Mix well.

Place an RNeasy mini spin column in a 1.5 ml collection tube. Apply sample to column. Centrifuge at 8000xg (10,000 rpm) for 15 sec.

Collect flow through and re-apply to column. Centrifuge at 8000xg (10,000 rpm) for 15 sec.

Transfer the RNeasy column to a new 2 ml collection tube. Add 500  $\mu\text{l}$  of Buffer RPE. Centrifuge at 8000xg (10,000 rpm) for 15 sec. Pour off flow through.

Add 500  $\mu\text{l}$  of Buffer RPE to the column. Centrifuge for 2 min at maximum speed.



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Place the column in a new 2 ml collection tube. Centrifuge for 1 min at maximum speed.

Transfer the column to a new 1.5 ml collection tube. Add 30-50  $\mu$ l of RNase-free water directly to the center of the membrane. Let sit for 1 min. Centrifuge at 8000xg (10,000 rpm) for 1 min. Repeat.

Concentrate the cRNA by ethanol precipitation.

#### Ethanol Precipitation

Add 0.5 volumes of 7.5 M  $\text{NH}_4\text{Ac}$  and 2.5 volumes of absolute ethanol (stored at  $-20^\circ\text{C}$ ) to sample and mix. Precipitate at  $-20^\circ\text{C}$  for at least one hour (O/N precipitation may result in a better yield). Centrifuge at 12,000xg at room temp for 20 min.

Wash pellet with 0.5 ml 80% ethanol (stored at  $-20^\circ\text{C}$ ). Centrifuge at 12,000xg at room temp for 5 min.

Repeat 80% ethanol wash.

Dry the pellet in speedvac.

Resuspend the dried pellet in 10 - 20  $\mu$ l RNase-free water.

Quantitate the cRNA and determine the O.D. 260/280.

Analyze 1  $\mu$ l on a gel.

Store at  $-80^\circ\text{C}$ .



## REAGENTS

Name	Stock Concentration/ number of units	Brand	Vendor	Catalog#
SuperScript Double-Stranded cDNA Synthesis Kit	10 Reactions	Invitrogen	Invitrogen	11917-010
T7-(dT) <sub>24</sub> Primer (100pmol/ul)	Sequence is as follows: 5'-GGCCAGTGAATTGTAATACGA CTCACTATAGGGAGGCGG-(dT) <sub>24</sub> -3'	IDT or Qiagen	IDT or Qiagen	n/a
Phase Lock Gel Light, 1.5 ml	200 Extractions	Eppendorf	Eppendorf	0032007.961
*0.5M EDTA	0.5M	Sigma	Sigma	E-7889
Phenol:Chloroform:IAA, 25:24:1	pH 6.6, raise to pH 7.9 with included buffer	Ambion	Ambion	9732
*Ammonium acetate solution 7.5 M	7.5 M	Sigma	Sigma	A2706
*Ethanol	Absolute and 80%	Any	Any	n/a
*Water, Molecular Biology Grade	Molecular Biology Grade	Biowhittaker /Accugene	Fisher	51200
BioArray HighYield RNA Transcription Kit	10 Reactions	Enzo	Enzo	42655-10
RNeasy Mini Kit (50)	50 RNeasy Mini Spin Columns	Qiagen	Qiagen	74104

\*substitute brand permissible