

PROTOCOLS FOR AFFYMETRIX GENECHIPS

Synthesis of Double-Stranded cDNA from total RNA

First Strand cDNA Synthesis

<u>Reagent</u>	<u>Volume</u>	<u>Final</u>
DEPC-treated H ₂ O	--- μ l	
Total RNA (5 to 40 μ g)	--- μ l	5 to 40 μ g
T7-(dT) ₂₄ Primer (100 pmol/ μ l)	1 μ l	100 pmoles

Mix. Incubate at 70°C for 10 min. Quick spin and put on ice.

5X first strand buffer	4 μ l	1X
0.1M DTT	2 μ l	10 mM
10 mM dNTP mix	1 μ l	500 μ M each

Mix. Incubate at 42°C for 2 min.

SSII RT (200 U/ μ l)	--- μ l *(see chart below)	200 to 1000 U
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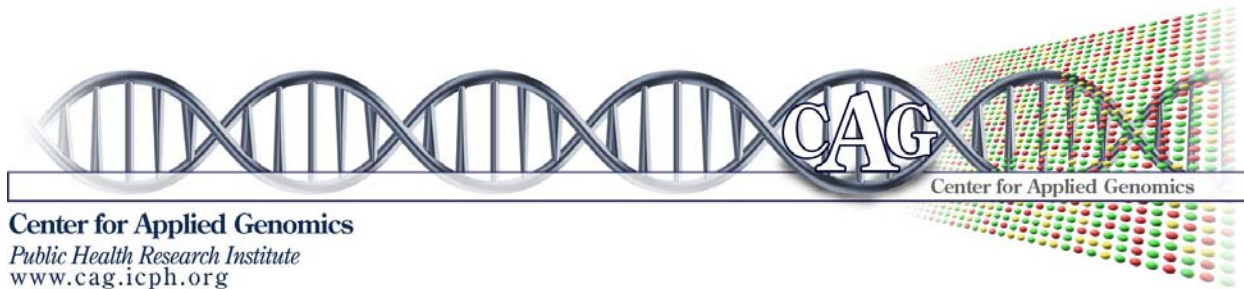
Mix.

Final Volume	20 μ l
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Incubate at 42°C for 1 h. Place on ice.

*Volume of SSII RT to use

<u>Total RNA (μg)</u>	<u>Superscript II RT (μl)</u>
5.0 – 8.0	1.0
8.1 – 16.0	2.0



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Second Strand cDNA Synthesis

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

Mix. Add to first strand synthesis tube.

Final Volume 150 μ l

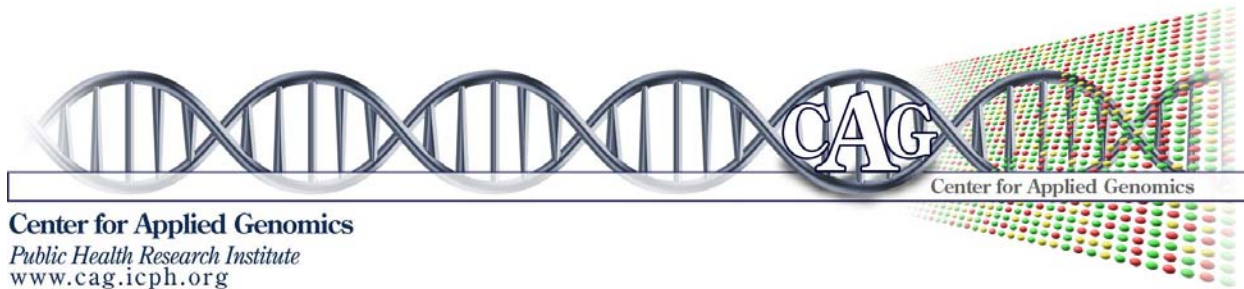
Incubate at 16 °C for 2 h.

Add 2 μ l (10 U) T4 DNA Polymerase.

Incubate at 16°C for 5 min.

Add 10 μ l 0.5 M EDTA.

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



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 μ 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 at room temperature 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 NH_4Ac and 2.5 volumes of absolute ethanol (stored at -20°C) to sample and vortex. Precipitate at -20°C for at least 1 h (O/N precipitation may result in a better yield). Centrifuge at 12,000xg at 5°C for 20 min.

Wash pellet with 0.5 ml 80% ethanol (stored at -20°C). Centrifuge at 12,000xg at 5°C for 5 min.

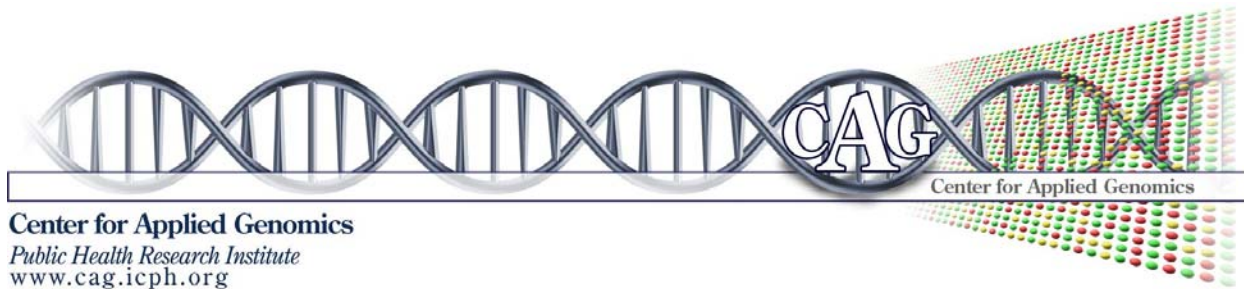
Repeat 80% ethanol wash.

Dry the pellet in speedvac.

Resuspend the dried pellet in 11 μ l RNase-free water.

Analyze 1 μ l on a gel.

Proceed to cRNA synthesis or store at -20°C .



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IVT Reaction

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

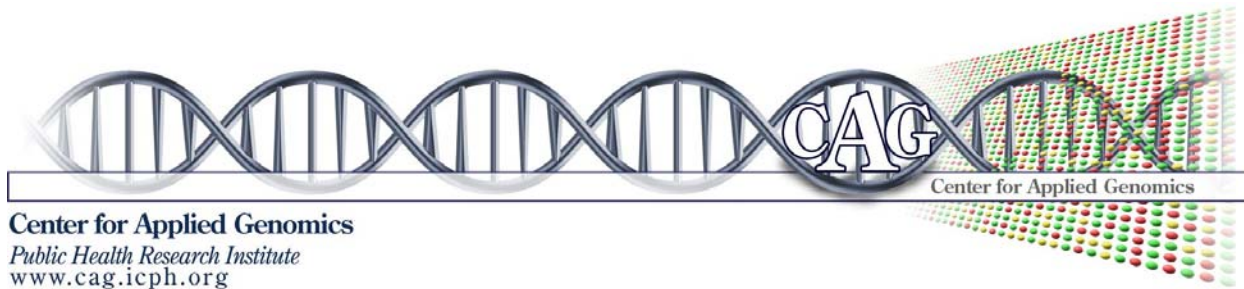
Mix well. Place tube at 37°C. Incubate for 4-5 hours, gently mixing the contents of the tube every 30-45 min.

Purify RNA immediately or store at -20°C.

Approximately 50-100 μg of cRNA are usually produced from 1 μg of template.

*Volume of cDNA to use in IVT

<u>Starting total RNA(μg)</u>	<u>Volume of cDNA to use (μl)</u>
5.0 – 8.0	10.0
8.1 – 16.0	5.0



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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 μg to the column.

Do not DNase treat the IVT reactions.

Do not add β -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 μl with RNase-free water. Add 350 μl of Buffer RLT to the sample. Mix thoroughly.

Add 250 μl 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 μl of Buffer RPE. Centrifuge at 8000xg (10,000 rpm) for 15 sec. Pour off flow through.

Add 500 μl 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 μ 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 NH_4Ac and 2.5 volumes of absolute ethanol (stored at -20°C) to sample and mix. Precipitate at -20°C for at least 1 hour (O/N precipitation may result in a better yield). Centrifuge at 12,000xg at 5°C for 20 min.

Wash pellet with 0.5 ml 80% ethanol (stored at -20°C). Centrifuge at 12,000xg at 5°C for 5 min.

Repeat 80% ethanol wash.

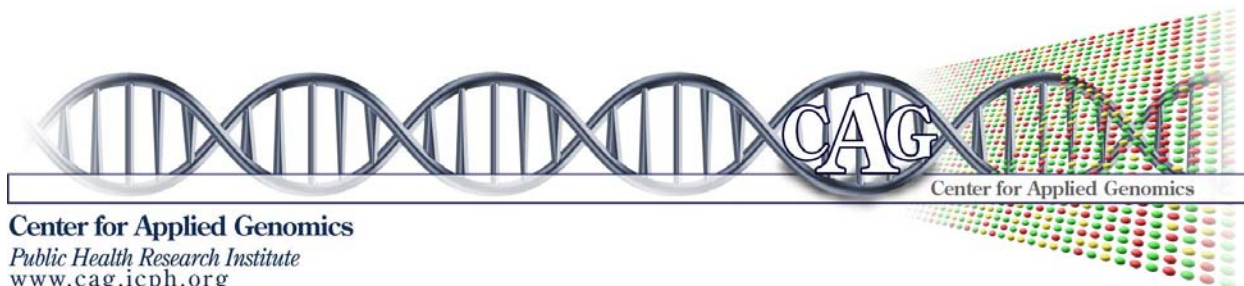
Dry the pellet in speedvac.

Resuspend the dried pellet in 12 - 20 μ l RNase-free water.

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

Analyze 1 μ l on a gel.

Store at -80°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) ₂₄ Primer (100pmol/ul)	Sequence is as follows: 5'-GGCCAGTGAATTGTAATACGAC TCACTATAGGGAGGCGG-(dT) ₂₄ -3'	IDT or Qiagen	IDT or Qiagen	n/a
Phase Lock Gel Light, 1.5 ml	200 Extractions	Eppendorf	Eppendorf	0032 007.961
*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	Biowhitaker/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