

Synthesis of Double-Stranded cDNA from Total RNA

This protocol is a supplement to instructions provided in the Invitrogen Life Technologies SuperScript Choice system. Please note the following before proceeding:

- Read all information and instructions that come with reagents and kits.
- Use the GeneChip T7-Oligo(dT) Promoter Primer Kit¹ for priming first-strand cDNA synthesis in place of the oligo(dT) or random primers provided with the SuperScript Choice kit. The GeneChip T7-Oligo(dT) Promoter Primer Kit provides high-quality HPLC-purified T7-oligo(dT) primer, which is essential for this reaction.

T7-oligo(dT) primer

5' - GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-(dT)₂₄ - 3'

Step 1: First-strand cDNA Synthesis

Starting material: High-quality total RNA (5.0 µg - 20.0 µg)



Note

For smaller amounts of starting material, please refer to the alternative protocol for target labeling described in Small Sample Target Labeling Assay Version II, available at www.affymetrix.com.



Note

When using the GeneChip Sample Cleanup Module for the cDNA and IVT cRNA cleanup steps, there is a potential risk of overloading the columns if greater than the recommended amount of starting material is used.

After purification, the RNA concentration is determined by absorbance at 260 nm on a spectrophotometer (one absorbance unit = 40 µg/mL RNA). The A_{260}/A_{280} ratio should be approximately 2.0, with ranges between 1.9 to 2.1 considered acceptable. We recommend checking the quality of the RNA by running it on an agarose gel prior to starting the assay. The rRNA bands should be clear without any obvious smearing patterns from degradation.

Before starting cDNA synthesis, the correct volumes of DEPC-treated H₂O and Reverse Transcriptase (RT) must be determined. These volumes will depend on both the concentration and total volume of RNA that is being added to the reaction.



IMPORTANT

Use Table 2.1.1 and Table 2.1.2 for variable component calculations. Determine the volumes of RNA and SuperScript II RT required in Table 2.1.1, then calculate the amount of DEPC-treated H₂O needed in Step 1 Table 2.1.2 to bring the final First-Strand Synthesis volume to 20 µL.

1. Users who do not purchase the GeneChip T7-Oligo(dT) Promoter Primer Kit may be required to obtain a license under U.S. Patent Nos. 5,569,584, 5,716,785, 5,891,636, 6,291,170, and 5,545,522 or to purchase another licensed kit.

Table 2.1.1
Reverse Transcriptase Volumes for First-Strand cDNA Synthesis Reaction

Total RNA (μg)	SuperScript II RT (μL), 200U/ μL
5.0 to 8.0	1.0
8.1 to 16.0	2.0
16.1 to 20.0	3.0



Note

The combined volume of RNA, DEPC-treated H₂O and SuperScript II RT should not exceed 11 μL as indicated in Table 2.1.2.

Table 2.1.2
First-Strand cDNA Synthesis Components

	Reagents in reaction	Volume	Final concentration or amount in reaction
1: Primer Hybridization Incubate at 70°C for 10 minutes Quick spin and put on ice	DEPC-treated H ₂ O (variable) T7-oligo(dT) primer, 50 μM RNA (variable)	for final reaction volume of 20 μL 2 μL 5.0 to 20 μg	100 pmol 5.0 to 20 μg
2: Temperature Adjustment Add to the above tube and mix well Incubate at 42°C for 2 minutes	5X First-Strand cDNA buffer 0.1 M DTT 10 mM dNTP mix	4 μL 2 μL 1 μL	1X 10 mM DTT 500 μM each
3: First-Strand Synthesis Add to the above tube and mix well Incubate at 42°C for 1 hour	SuperScript II RT (variable) (200 U/ μL)	See Table 2.1.1	200 U to 1000 U
Total Volume		20 μL	



Note

The above incubations have been changed from the SuperScript protocols and are done at 42°C.

Step 2: Second-Strand cDNA Synthesis

1. Place First-Strand reactions on ice. Centrifuge briefly to bring down condensation on sides of tube.
2. Add to the First-Strand synthesis tube the reagents listed in the following Second-Strand Final Reaction Composition Table (Table 2.1.3).

Table 2.1.3
Second-Strand Final Reaction Composition

Component	Volume	Final Concentration or Amount in Reaction
DEPC-treated water	91 μ L	
5X Second-Strand Reaction Buffer	30 μ L	1X
10 mM dNTP mix	3 μ L	200 μ M each
10 U/ μ L <i>E. coli</i> DNA Ligase	1 μ L	10 U
10 U/ μ L <i>E. coli</i> DNA Polymerase I	4 μ L	40 U
2 U/ μ L <i>E. coli</i> RNase H	1 μ L	2 U
Final Volume	150 μ L	

3. Gently tap tube to mix. Then, briefly spin in a microcentrifuge to remove condensation and incubate at 16°C for 2 hours in a cooling waterbath.
4. Add 2 μ L [10 U] T4 DNA Polymerase.
5. Return to 16°C for 5 minutes.
6. Add 10 μ L 0.5M EDTA.
7. Proceed to cleanup procedure for cDNA, *Cleanup of Double-Stranded cDNA* on page 2.1.15, or store at -20°C for later use.

Synthesis of Double-Stranded cDNA from Purified Poly-A mRNA

This protocol is a supplement to instructions provided in the Invitrogen Life Technologies SuperScript Choice system. Please note the following before proceeding:

- Read all information and instructions that come with reagents and kits.
- Use the GeneChip T7-Oligo(dT) Promoter Primer Kit² for priming first-strand cDNA synthesis in place of the oligo(dT) or random primers provided with the SuperScript Choice kit. The GeneChip T7-Oligo(dT) Promoter Primer Kit provides high-quality HPLC-purified T7-oligo(dT) primer, which is essential for this reaction.
- It is recommended that each step of this protocol is checked by gel electrophoresis.

T7-oligo(dT) primer

5' - GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-(dT)₂₄ - 3'

Step 1: First-Strand cDNA Synthesis

Starting material: High-quality poly-A mRNA (0.2 µg to 2.0 µg).



Note

When using the GeneChip Sample Cleanup Module for the cDNA and IVT cRNA cleanup steps, there is a potential risk of overloading the columns if greater than the recommended amount of starting material is used.

Before starting cDNA synthesis, the correct volumes of DEPC-treated H₂O and Reverse Transcriptase (RT) must be determined. These volumes will depend on both the concentration and total volume of mRNA that is being added to the reaction. For every µg of mRNA, you will need to add 1 µL of SuperScript II RT (200 U/µL). For mRNA quantity ≤ 1 µg, use 1 µL of SuperScript II RT. Synthesis reactions should be done in a polypropylene tube (RNase-free).



IMPORTANT

*Use Table 2.1.4 for variable component calculations. Determine volumes of mRNA and SuperScript II RT required, and then calculate the amount of DEPC-treated H₂O needed in the **Primer Hybridization Mix** step to bring the final First-Strand Synthesis reaction volume to 20 µL.*

2. Users who do not purchase the GeneChip T7-Oligo(dT) Promoter Primer Kit may be required to obtain a license under U.S. Patent Nos. 5,569,584, 5,716,785, 5,891,636, 6,291,170, and 5,545,522 or to purchase another licensed kit.

Table 2.1.4

First-Strand cDNA Synthesis Components

	Reagents in Reaction	Volume	Final Concentration or Amount in Reaction
1: Primer Hybridization Incubate at 70°C for 10 minutes Quick spin and put on ice	DEPC-treated H ₂ O (variable) T7-oligo(dT) primer, 50 µM mRNA (variable)	for final reaction volume of 20 µL 2 µL 0.2 to 2 µg	100 pmol 0.2 to 2 µg
2: Temperature Adjustment Add to the above tube and mix well Incubate at 37°C for 2 minutes	5X First-Strand cDNA buffer 0.1 M DTT 10 mM dNTP mix	4 µL 2 µL 1 µL	1X 10 mM 500 µM each
3: First-Strand Synthesis Add to the above tube and mix well Incubate at 37°C for 1 hour	SuperScript II RT (variable) (200 U/µL)	1 µL per µg mRNA	200 U to 1000 U
Total Volume		20 µL	

Step 2: Second-Strand cDNA Synthesis

- Place First-Strand reactions on ice. Centrifuge briefly to bring down condensation on sides of tube.
- Add to the First-Strand synthesis tube the reagents listed in the following Second-Strand Final Reaction Composition Table (Table 2.1.5).

Table 2.1.5

Second-Strand Final Reaction Composition

Component	Volume	Final Concentration or Amount in Reaction
DEPC-treated water	91 µL	
5X Second-Strand Reaction Buffer	30 µL	1X
10 mM dNTP mix	3 µL	200 µM each
10 U/µL <i>E. coli</i> DNA Ligase	1 µL	10 U
10 U/µL <i>E. coli</i> DNA Polymerase I	4 µL	40 U
2 U/µL <i>E. coli</i> RNase H	1 µL	2 U
Final Volume	150 µL	

- Gently tap tube to mix. Then, briefly spin in a microcentrifuge to remove condensation and incubate at 16°C for 2 hours in a cooling waterbath.
- Add 2 µL [10 U] T4 DNA Polymerase.
- Return to 16°C for 5 minutes.
- Add 10 µL 0.5M EDTA.
- Proceed to cleanup procedure for cDNA, *Cleanup of Double-Stranded cDNA* on page 2.1.15, or store at -20°C for later use.

Cleanup of Double-Stranded cDNA

Reagents to be Supplied by User

- Ethanol, 96-100% (v/v)

All other components needed for cleanup of double-stranded cDNA are supplied with the GeneChip Sample Cleanup Module.

IMPORTANT

BEFORE STARTING, please note:

- cDNA Wash Buffer is supplied as a concentrate. Before using for the first time, add 24 mL of ethanol (96-100%), as indicated on the bottle, to obtain a working solution, and checkmark the box on the left-hand side of the bottle label to avoid confusion.
- All steps of the protocol should be performed at room temperature. During the procedure, work without interruption.
- If cDNA synthesis was performed in a reaction tube smaller than 1.5 mL, transfer the reaction mixture into a 1.5 or 2 mL microfuge tube (not supplied) prior to addition of cDNA Binding Buffer.

1. Add 600 μ L cDNA Binding Buffer to the 162 μ L final double-stranded cDNA synthesis preparation (page 2.1.10 or 2.1.13). Mix by vortexing for 3 seconds.
2. Check that the color of the mixture is yellow (similar to cDNA Binding Buffer without the cDNA synthesis reaction).

✓ Note

If the color of the mixture is orange or violet, add 10 μ L of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

3. Apply 500 μ L of the sample to the cDNA Cleanup Spin Column sitting in a 2 mL Collection Tube, and centrifuge for 1 minute at $\geq 8,000 \times g$ ($\geq 10,000$ rpm). Discard flow-through.
4. Reload the spin column with the remaining mixture (262 μ L) and centrifuge as above. Discard flow-through and Collection Tube.
5. Transfer spin column into a new 2 mL Collection Tube (supplied). Pipet 750 μ L cDNA Wash Buffer onto the spin column. Centrifuge for 1 minute at $\geq 8,000 \times g$ ($\geq 10,000$ rpm). Discard flow-through.

✓ Note

cDNA Wash Buffer is supplied as a concentrate. Ensure that ethanol is added to the cDNA Wash Buffer before use (see **IMPORTANT** note above before starting).

6. Open the cap of the spin column and centrifuge for 5 minutes at maximum speed ($\leq 25,000 \times g$). Discard flow-through and Collection Tube.
Place columns into the centrifuge using every second bucket. Position caps over the adjoining bucket so that they are oriented in the opposite direction to the rotation (i.e., if the microcentrifuge rotates in a clockwise direction, orient the caps in a counter-clockwise direction). This avoids damage of the caps.
Centrifugation with open caps allows complete drying of the membrane.

7. Transfer spin column into a 1.5 mL Collection Tube, and pipet 14 μ L of cDNA Elution Buffer directly onto the spin column membrane. Incubate for 1 minute at room temperature and centrifuge 1 minute at maximum speed ($\leq 25,000 \times g$) to elute. Ensure that the cDNA Elution Buffer is dispensed directly onto the membrane. The average volume of eluate is 12 μ L from 14 μ L Elution Buffer.

✓ **Note**

We do not recommend RNase treatment of the cDNA prior to the in vitro transcription and labeling reaction; the carry-over ribosomal RNA does not seem to inhibit the reaction.

8. An aliquot of the cDNA prepared from isolated poly-A RNA can be analyzed for size distribution and yield on a 1% agarose gel. One microliter of double-stranded cDNA should be sufficient to detect on an agarose gel stained with ethidium bromide. A representative gel is shown in **Figure 2.1.1** on page 2.1.22. We do not recommend gel analysis for cDNA prepared from total RNA.

✓ **Note**

Quantifying the amount of double-stranded cDNA by absorbance at 260 nm is not recommended. The primer can contribute significantly to the absorbance. Subtracting the theoretical contribution of the primer based on the amount added to the reaction is not practical.

9. After cleanup, please proceed to *Synthesis of Biotin-Labeled cRNA* on page 2.1.17.

Synthesis of Biotin-Labeled cRNA

✓ Note

The purity and quality of template cDNA is important for high yields of biotin-labeled RNA.

Use only RNase-free water, buffers, and pipette tips.

▶ IMPORTANT

Store all reagents at -20°C, in a freezer that is not self-defrosting.

Prior to use, centrifuge all reagents briefly to ensure that the components remain at the bottom of the tube.

The product should be used only until the expiration date stated on the label.

1. Enzo® BioArray™ HighYield™ RNA Transcript Labeling Kit³ (Affymetrix, P/N 900182) is used for generating labeled cRNA target. Use the following tables to determine the amount of cDNA used for each IVT reaction. Done properly, each reaction should produce sufficient biotin-labeled target to hybridize to at least two Standard Format (49 Format) GeneChip expression probe arrays in parallel.

Table 2.1.6
cDNA in IVT (Total RNA)

Total RNA (µg)	Volume of cDNA to use in IVT*
5.0 to 8.0	10 µL
8.1 to 16.0	5 µL
16.1 to 20.0	3.3 µL

* assuming 12 µL was eluted from the column, as previously described.

Table 2.1.7
cDNA in IVT (Poly-A mRNA)

Poly-A mRNA (µg)	Volume cDNA to use in IVT*
0.2 - 0.5	10 µL
0.6 - 1.0	8 µL
1 - 2	5 µL

* assuming 12 µL was eluted from the column, as previously described.

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IMPORTANT

Each GeneChip® Sample Cleanup Module contains 30 cDNA cleanup columns and 30 IVT cRNA cleanup columns. If more than one IVT is carried out from a single cDNA sample and is purified on separate IVT cRNA cleanup columns, there will not be sufficient IVT cRNA columns in each kit for 30 samples.

- Add to RNase-free microfuge tubes template cDNA and additions of other reaction components in the order indicated in the following table. Keep reactions at room temperature while additions are made to avoid precipitation of DTT.

Table 2.1.8
IVT cRNA Labeling

Reagent	Volume
Template cDNA	Variable. Refer to Table 2.1.6 and Table 2.1.7.
Distilled or deionized water	Variable (to give a final reaction volume of 40 μ L).
10X HY Reaction Buffer (Vial 1)	4 μ L
10X Biotin-Labeled Ribonucleotides (Vial 2)	4 μ L
10X DTT (Vial 3)	4 μ L
10X RNase Inhibitor Mix (Vial 4)	4 μ L
20X T7 RNA Polymerase (Vial 5)	2 μ L
Total Volume	40 μL

- Carefully mix the reagents and collect the mixture in the bottom of the tube by brief (5 second) microcentrifugation.
- Immediately place the tube in a 37°C water bath. Incubate for 4 to 5 hours, gently mixing the contents of the tube every 30-45 minutes during the incubation.

✓ Note

Overnight incubation may produce shorter products, which is less desirable.

- Store labeled cRNA at -20°C, or -70°C (long-term storage) if not purifying immediately.
- Proceed to cRNA cleanup procedure, *Cleanup and Quantification of Biotin-Labeled cRNA* on page 2.1.19

Cleanup and Quantification of Biotin-Labeled cRNA

Reagents to be Supplied by User

- Ethanol, 96-100% (v/v)
- Ethanol, 80% (v/v)

All other components needed for cleanup of biotin-labeled cRNA are supplied with the GeneChip Sample Cleanup Module.

Step 1: Cleanup of Biotin-Labeled cRNA

IMPORTANT

BEFORE STARTING please note:

- It is essential to remove unincorporated NTPs, so that the concentration and purity of cRNA can be accurately determined by 260 nm absorbance.
- DO NOT extract biotin-labeled RNA with phenol-chloroform. The biotin will cause some of the RNA to partition into the organic phase. This will result in low yields.
- Save an aliquot of the unpurified IVT product for analysis by gel electrophoresis.
- IVT cRNA Wash Buffer is supplied as a concentrate. Before using for the first time, add 20 mL of ethanol (96-100%) as indicated on the bottle to obtain a working solution, and checkmark the box on the left-hand side of the bottle label to avoid confusion.
- IVT cRNA Binding Buffer may form a precipitate upon storage. If necessary, redissolve by warming in a water bath at 30°C, and then place the buffer at room temperature.
- All steps of the protocol should be performed at room temperature. During the procedure, work without interruption.

1. Add 60 μL of RNase-free water to the *in vitro* transcription reaction and mix by vortexing for 3 seconds.
2. Add 350 μL IVT cRNA Binding Buffer to the sample and mix by vortexing for 3 seconds.
3. Add 250 μL ethanol (96-100%) to the lysate, and mix well by pipetting. Do not centrifuge.
4. Apply sample (700 μL) to the IVT cRNA Cleanup Spin Column sitting in a 2 mL Collection Tube. Centrifuge for 15 seconds at $\geq 8,000 \times g$ ($\geq 10,000$ rpm). Discard flow-through and Collection Tube.
5. Transfer the spin column into a new 2 mL Collection Tube (supplied). Pipet 500 μL IVT cRNA Wash Buffer onto the spin column. Centrifuge for 15 seconds at $\geq 8,000 \times g$ ($\geq 10,000$ rpm) to wash. Discard flow-through.

✓ Note

IVT cRNA Wash Buffer is supplied as a concentrate. Ensure that ethanol is added to the IVT cRNA Wash Buffer before use (see **IMPORTANT** note above before starting).

6. Pipet 500 μL 80% (v/v) ethanol onto the spin column and centrifuge for 15 seconds at $\geq 8,000 \times g$ ($\geq 10,000$ rpm). Discard flow-through.

7. Open the cap of the spin column and centrifuge for 5 minutes at maximum speed ($\leq 25,000 \times g$). Discard flow-through and Collection Tube.
Place columns into the centrifuge using every second bucket. Position caps over the adjoining bucket so that they are oriented in the opposite direction to the rotation (i.e., if the microcentrifuge rotates in a clockwise direction, orient the caps in a counter-clockwise direction). This avoids damage of the caps. Centrifugation with open caps allows complete drying of the membrane.
8. Transfer spin column into a new 1.5 mL Collection Tube (supplied), and pipet 11 μL of RNase-free water directly onto the spin column membrane. Ensure that the water is dispensed directly onto the membrane. Centrifuge 1 minute at maximum speed ($\leq 25,000 \times g$) to elute.
9. Pipet 10 μL of RNase-free water directly onto the spin column membrane. Ensure that the water is dispensed directly onto the membrane. Centrifuge 1 minute at maximum speed ($\leq 25,000 \times g$) to elute.
For subsequent photometric quantification of the purified cRNA, we recommend dilution of the eluate between 1:100 fold and 1:200 fold.

IMPORTANT

The minimum concentration for purified cRNA is 0.6 $\mu\text{g}/\mu\text{L}$ before starting the following fragmentation reaction in "Fragmenting the cRNA for Target Preparation" on page 2.1.21.

Step 2: Quantification of the cRNA

Use spectrophotometric analysis to determine the cRNA yield. Apply the convention that 1 absorbance unit at 260 nm equals 40 $\mu\text{g}/\text{mL}$ RNA.

- Check the absorbance at 260 nm and 280 nm to determine sample concentration and purity.
- Maintain the A_{260}/A_{280} ratio close to 2.0 for pure RNA (ratios between 1.9 and 2.1 are acceptable).

For quantification of cRNA when using total RNA as starting material, an adjusted cRNA yield must be calculated to reflect carryover of unlabeled total RNA. Using an estimate of 100% carryover, use the formula below to determine adjusted cRNA yield:

$$\text{adjusted cRNA yield} = \text{RNA}_m - (\text{total RNA}_i)(y)$$

RNA_m = amount of cRNA measured after IVT (μg)

total RNA_i = starting amount of total RNA (μg)

y = fraction of cDNA reaction used in IVT

Example: Starting with 10 μg total RNA, 50% of the cDNA reaction is added to the IVT, giving a yield of 50 μg cRNA. Therefore, adjusted cRNA yield = 50 μg cRNA - (10 μg total RNA) (0.5 cDNA reaction) = 45.0 μg .

Use adjusted yield in *Eukaryotic Target Hybridization* on page 2.3.3.

✓ Note

Please refer to Table 2.3.1 on page 2.3.7 for the amount of cRNA required for one array hybridization experiment. The amount varies depending on the array format. Please refer to the specific probe array package insert for information on the array format.

Step 3: Checking Unfragmented Samples by Gel Electrophoresis

Gel electrophoresis of the IVT product is done to estimate the yield and size distribution of labeled transcripts. Parallel gel runs of unpurified and purified IVT product can help determine the extent of a loss of sample during the cleanup process.

- Run 1% of each sample on a 1% agarose gel.
- Mix RNA (samples or markers) with loading dye and heat to 65°C for 5 minutes before loading on the gel.
- Ethidium bromide can be used to visualize the RNA in the gel. Alternatively, gels can be stained with SYBR Green II at a 1:10,000 dilution in 1X TBE buffer. SYBR Green II stains single-stranded RNA with greater sensitivity than ethidium bromide, but it requires a special photographic filter available from Molecular Probes to photograph stained bands.
- As an option, run a denaturing gel to obtain a more accurate estimation of the RNA size distribution. Please refer to Figure 2.1.1 for the typical size distribution of unfragmented cRNA.

Fragmenting the cRNA for Target Preparation

5X Fragmentation Buffer is supplied with the GeneChip Sample Cleanup Module.

Fragmentation of cRNA target before hybridization onto GeneChip probe arrays has been shown to be critical in obtaining optimal assay sensitivity.

Affymetrix recommends that the cRNA used in the fragmentation procedure be sufficiently concentrated to maintain a small volume during the procedure. This will minimize the amount of magnesium in the final hybridization cocktail. The cRNA must be at a minimum concentration of 0.6 µg/µL. Fragment an appropriate amount of cRNA for hybridization cocktail and gel analysis (see Section 2, Chapter 3, Table 2.3.1).

1. Add 2 µL of 5X Fragmentation Buffer for every 8 µL of RNA plus H₂O. The fragmentation buffer has been optimized to break down full-length cRNA to 35 to 200 base fragments by metal-induced hydrolysis.

The final concentration of RNA in the fragmentation mix can range from 0.5 µg/µL to 2 µg/µL. The following table shows an example of a fragmentation mix for a 20 µg cRNA sample at a final concentration of 0.5 µg/µL.

For fragmentation, use **adjusted** cRNA concentration, as described in *Step 2: Quantification of the cRNA* on page 2.1.20.

Example for 0.5 µg/µL final concentration:

Table 2.1.9
Example of Fragmentation Reaction

Component	Volume
20 µg cRNA	1 to 21 µL
5X Fragmentation Buffer	8 µL
RNase-free water	to 40 µL

2. Incubate at 94°C for 35 minutes. Put on ice following the incubation.