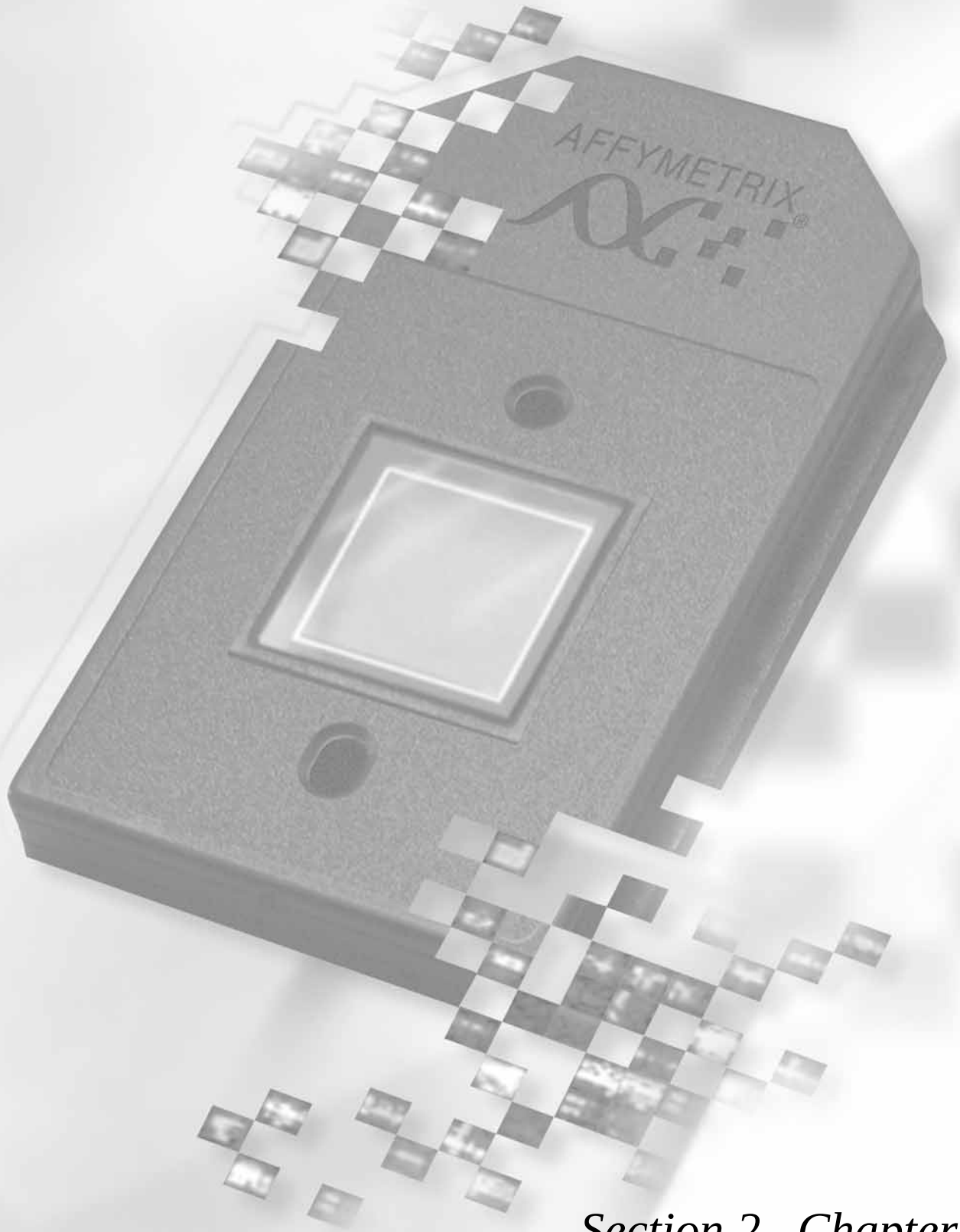

Section 2, Chapter 1

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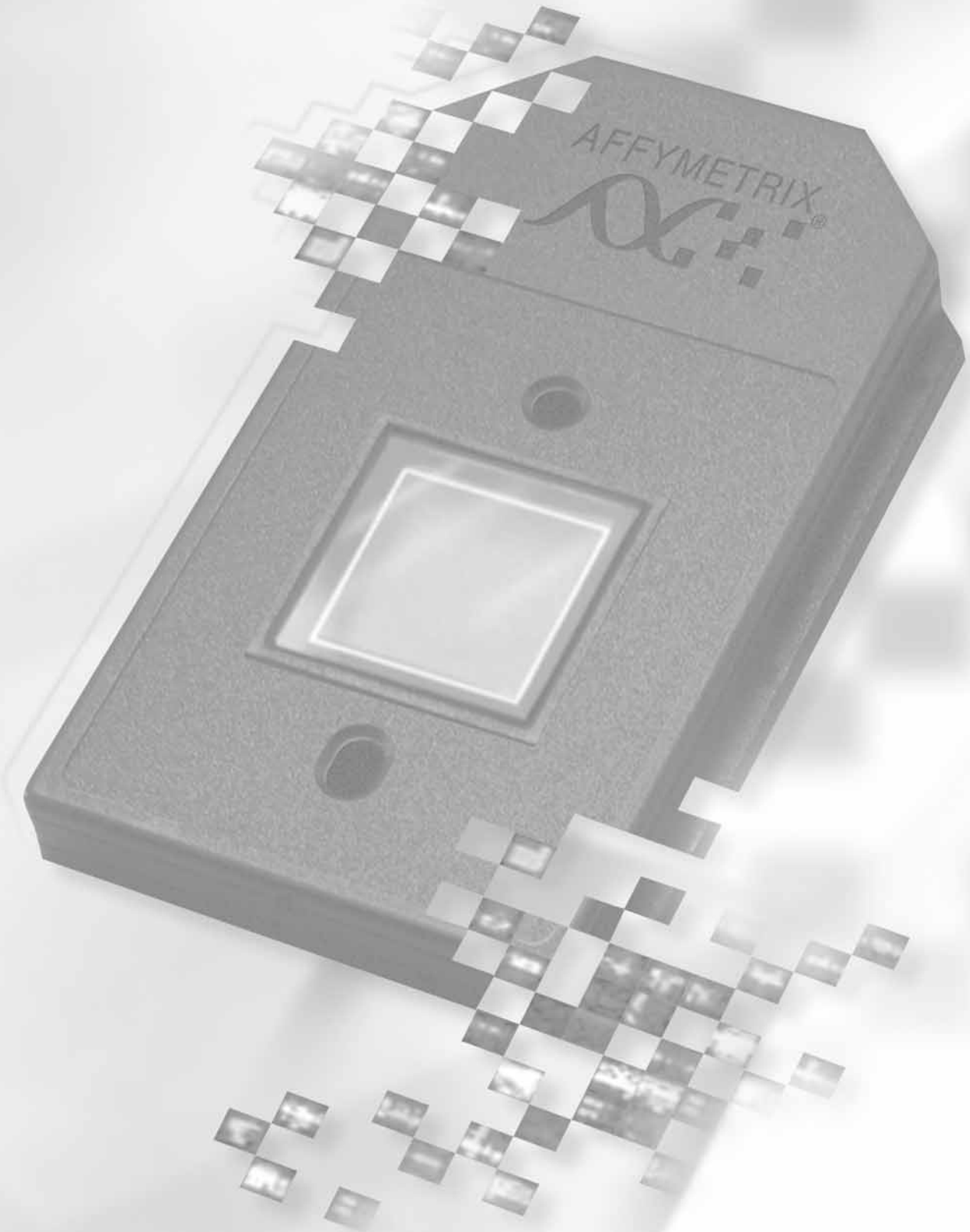

Eukaryotic Target Preparation

This Chapter Contains:

- Complete One-Cycle Target Labeling Assay with 1 to 15 µg of total RNA or 0.2 to 2 µg of poly-A mRNA
- Complete Two-Cycle Target Labeling Assay with 10 to 100 ng of total RNA

Introduction

This chapter describes the assay procedures recommended for eukaryotic target labeling in expression analysis using GeneChip® brand probe arrays. Following the protocols and using high-quality starting materials, a sufficient amount of biotin-labeled cRNA target can be obtained for hybridization to at least two arrays in parallel. The reagents and protocols have been developed and optimized specifically for use with the GeneChip system.

Depending on the amount of starting material, two procedures are described in detail in this manual. Use the following table to select the most appropriate labeling protocol for your samples:

Total RNA as Starting Material	*mRNA as Starting Material*	*Protocol*
1 µg – 15 µg	0.2 µg – 2 µg	One-Cycle Target Labeling
10 ng – 100 ng	N/A	Two-Cycle Target Labeling

The One-Cycle Eukaryotic Target Labeling Assay experimental outline is represented in Figure 2.1.1. Total RNA (1 µg to 15 µg) or mRNA (0.2 µg to 2 µg) is first reverse transcribed using a T7-Oligo(dT) Promoter Primer in the first-strand cDNA synthesis reaction. Following RNase H-mediated second-strand cDNA synthesis, the double-stranded cDNA is purified and serves as a template in the subsequent *in vitro* transcription (IVT) reaction. The IVT reaction is carried out in the presence of T7 RNA Polymerase and a biotinylated nucleotide analog/ribonucleotide mix for complementary RNA (cRNA) amplification and biotin labeling. The biotinylated cRNA targets are then cleaned up, fragmented, and hybridized to GeneChip expression arrays.

For smaller amounts of starting total RNA, in the range of 10 ng to 100 ng, an additional cycle of cDNA synthesis and IVT amplification is required to obtain sufficient amounts of labeled cRNA target for analysis with arrays. The Two-Cycle Eukaryotic Target Labeling Assay experimental outline is also represented in Figure 2.1.1. After cDNA synthesis in the first cycle, an unlabeled ribonucleotide mix is used in the first cycle of IVT amplification. The unlabeled cRNA is then reverse transcribed in the first-strand cDNA synthesis step of the second cycle using random primers. Subsequently, the T7-Oligo(dT) Promoter Primer is used in the second-strand cDNA synthesis to generate double-stranded cDNA template containing T7 promoter sequences. The resulting double-stranded cDNA is then amplified and labeled using a biotinylated nucleotide analog/ribonucleotide mix in the second IVT reaction. The labeled cRNA is then cleaned up, fragmented, and hybridized to GeneChip expression arrays.

Alternative One-Cycle cDNA Synthesis protocols are also included at the end of this chapter for reference.

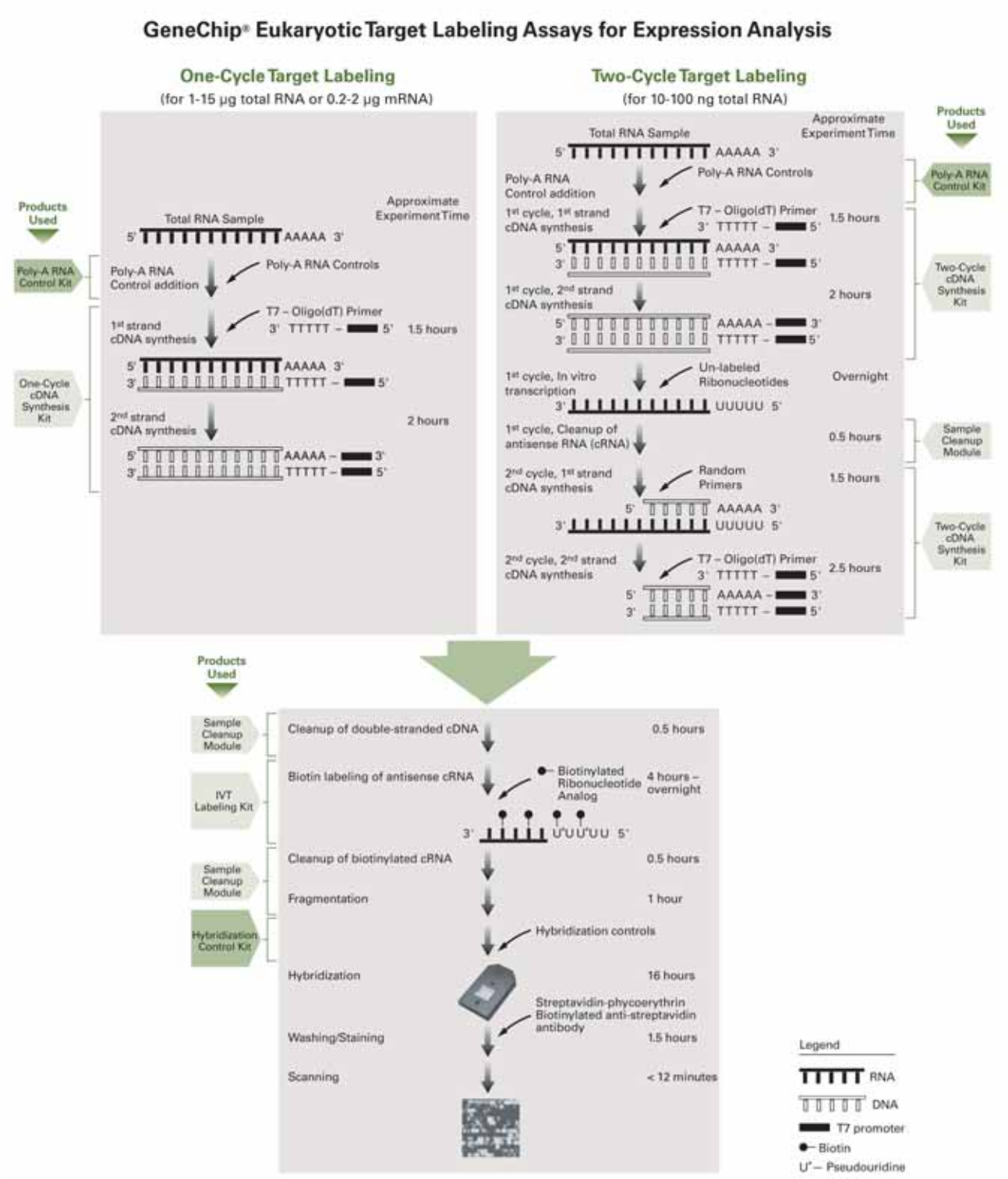

Figure 2.1.1
GeneChip Eukaryotic Labeling Assays for Expression Analysis

Reagents and Materials Required

The following reagents and materials are recommendations and have been tested and evaluated by Affymetrix scientists. For supplier phone numbers in the U.S. and Europe, please refer to the Supplier and Reagent Reference List, Appendix A, of this manual. Information and part numbers listed are based on U.S. catalog information. Additional reagents needed for the complete analysis are listed in the appropriate chapters. Appendix A contains a master list of all reagents used in this manual.

IMPORTANT *Do not store enzymes in a frost-free freezer.*

Total RNA Isolation

- TRIzol Reagent, Invitrogen Life Technologies, P/N 15596-018
- RNeasy Mini Kit, QIAGEN, P/N 74104

Poly-A mRNA Isolation

- Oligotex Direct mRNA Kit (isolation of mRNA from whole cells), QIAGEN, P/N 72012, 72022, or 72041
- Oligotex mRNA Kit (isolation of mRNA from total RNA), QIAGEN, P/N 70022, 70042, or 70061
- QIAshredder, QIAGEN, P/N 79654 (Required only for use with QIAGEN Oligotex Direct Kit)
- DEPC-Treated Water, Ambion, P/N 9920

One-Cycle Target Labeling and Control Reagents

- One-Cycle Target Labeling and Control Reagents, Affymetrix, P/N 900493
  A convenient package containing all required labeling and control reagents to perform 30 one-cycle labeling reactions.

  Contains 1 IVT labeling Kit, 1 One-Cycle cDNA Synthesis Kit, 1 Sample Cleanup Module, 1 Poly-A RNA Control Kit, and 1 Hybridization Controls. Each of these components may be ordered individually (described below) as well as in this complete kit.

Two-Cycle Target Labeling and Control Reagents

- Two-Cycle Target Labeling and Control Reagents, Affymetrix, P/N 900494
  A convenient package containing all required labeling and control reagents to perform 30 two-cycle labeling reactions.

  Contains 1 IVT labeling Kit, 1 Two-Cycle cDNA Synthesis Kit, 2 Sample Cleanup Modules, 1 Poly-A RNA Control Kit, and 1 Hybridization Controls. Each of these components may be ordered individually (described below) as well as in this complete kit.

One-Cycle cDNA Synthesis

- GeneChip® Expression 3'-Amplification Reagents One-Cycle cDNA Synthesis Kit, 30 reactions, Affymetrix, P/N 900431
- GeneChip® Eukaryotic Poly-A RNA Control Kit, Affymetrix, P/N 900433

Two-Cycle cDNA Synthesis

- GeneChip® Expression 3'-Amplification Reagents Two-Cycle cDNA Synthesis Kit, 30 reactions, Affymetrix, P/N 900432
- GeneChip® Eukaryotic Poly-A RNA Control Kit, Affymetrix, P/N 900433
- MEGAscript® High Yield Transcription Kit, Ambion Inc, P/N 1334

Cleanup of Double-Stranded cDNA

- GeneChip® Sample Cleanup Module, 30 eukaryotic reactions, Affymetrix, P/N 900371

Synthesis of Biotin-Labeled cRNA

- GeneChip® Expression 3'-Amplification Reagents for IVT Labeling, 30 reactions, Affymetrix, P/N 900449

IVT cRNA Cleanup and Quantification

- GeneChip Sample Cleanup Module, Affymetrix, P/N 900371
- 10X TBE, Cambrex, P/N 50843

cRNA Fragmentation

- GeneChip Sample Cleanup Module, Affymetrix, P/N 900371

Miscellaneous Reagents

- Absolute ethanol (stored at -20°C for RNA precipitation; store ethanol at room temperature for use with the GeneChip Sample Cleanup Module)
- 80% ethanol (stored at -20°C for RNA precipitation; store ethanol at room temperature for use with the GeneChip Sample Cleanup Module)
- SYBR Green II, Cambrex, P/N 50523; or Molecular Probes, P/N S7586 (optional)
- Pellet Paint, Novagen, P/N 69049-3 (optional)
- Glycogen, Ambion, P/N 9510 (optional)
- 3M Sodium Acetate (NaOAc), Sigma-Aldrich, P/N S7899
- Ethidium Bromide, Sigma-Aldrich, P/N E8751
- 1N NaOH
- 1N HCl

Miscellaneous Supplies

- Sterile, RNase-free, microcentrifuge vials, 1.5 mL, USA Scientific, P/N 1415-2600 (or equivalent)
- Micropipettors, (P-2, P-20, P-200, P-1000), Rainin Pipetman or equivalent
- Sterile-barrier, RNase-free pipette tips (Tips must be pointed, not rounded, for efficient use with the probe arrays) Beveled pipette tips may cause damage to the array septa and cause leakage.
- Mini agarose gel electrophoresis unit with appropriate buffers
- UV spectrophotometer
- Bioanalyzer
- Non-stick RNase-free microfuge tubes, 0.5 mL and 1.5 mL, Ambion, P/N12350 and P/N 12450, respectively

Alternative Protocol for One-Cycle cDNA Synthesis

- GeneChip T7-Oligo(dT) Promoter Primer Kit,
  5´ - GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-$(dT)_{24}$ - 3´
  50 µM, HPLC purified, Affymetrix, P/N 900375
- SuperScript™ II, Invitrogen Life Technologies, P/N 18064-014 or SuperScript Choice System for cDNA Synthesis, Invitrogen Life Technologies, P/N 18090-019

✔ Note

SuperScript Choice System contains, in addition to SuperScript II Reverse Transcriptase, other reagents for cDNA synthesis. However, not all components provided in the Choice System are used in the GeneChip cDNA synthesis protocol.

- *E. coli* DNA Ligase, Invitrogen Life Technologies, P/N 18052-019
- *E. coli* DNA Polymerase I, Invitrogen Life Technologies, P/N 18010-025
- *E. coli* RNaseH, Invitrogen Life Technologies, P/N 18021-071
- T4 DNA Polymerase, Invitrogen Life Technologies, P/N 18005-025
- 5X Second-strand buffer, Invitrogen Life Technologies, P/N 10812-014
- 10 mM dNTP, Invitrogen Life Technologies, P/N 18427-013
- 0.5M EDTA

Total RNA and mRNA Isolation for One-Cycle Target Labeling Assay

Protocols are provided for preparing labeled cRNA from either total RNA or purified poly-A mRNA. It was found that results obtained from samples prepared by both of these methods are similar, but not identical. Therefore, to get the best results, it is suggested to only compare samples prepared using the same type of RNA material.

Please review precautions and interfering conditions in Section 1.

IMPORTANT

The quality of the RNA is essential to the overall success of the analysis. Since the most appropriate protocol for the isolation of RNA can be source dependent, we recommend using a protocol that has been established for the tissues or cells being used. In the absence of an established protocol, using one of the commercially available kits designed for RNA isolation is suggested.

When using a commercial kit, follow the manufacturer's instructions for RNA isolation.

Isolation of RNA from Yeast

Total RNA

Good-quality total RNA has been isolated successfully from yeast cells using a hot phenol protocol described by Schmitt, *et al. Nucl Acids Res* **18**:3091-3092 (1990).

Poly-A mRNA

Affymetrix recommends first purifying total RNA from yeast cells before isolating poly-A mRNA from total RNA. Good-quality mRNA has been successfully isolated from total RNA using QIAGEN's Oligotex mRNA Kit. A single round of poly-A mRNA selection provides mRNA of sufficient purity and yield to use as a template for cDNA synthesis. Two rounds of poly-A mRNA selection will result in significantly reduced yield and are not generally recommended.

Isolation of RNA from Arabidopsis

Total RNA

TRIzol Reagent from Invitrogen Life Technologies have been used to isolate total RNA from Arabidopsis. Follow the instructions provided by the supplier and, when necessary, use the steps outlined specifically for samples with high starch and/or high lipid content.

Poly-A mRNA

Arabidopsis poly-A mRNA has been successfully isolated using QIAGEN's Oligotex products. However, other standard isolation products are likely to be adequate.

Isolation of RNA from Mammalian Cells or Tissues

Total RNA

High-quality total RNA has been successfully isolated from mammalian **cells** (such as cultured cells and lymphocytes) using the RNeasy Mini Kit from QIAGEN.

If mammalian **tissue** is used as the source of RNA, it is recommended to isolate total RNA with a commercial reagent, such as TRIzol.

IMPORTANT

If going directly from TRIzol-isolated total RNA to cDNA synthesis, it may be beneficial to perform a second cleanup on the total RNA before starting. After the ethanol precipitation step in the TRIzol extraction procedure, perform a cleanup using the QIAGEN RNeasy Mini Kit. Much better yields of labeled cRNA are obtained from the in vitro transcription-labeling reaction when this second cleanup is performed.

Poly-A mRNA

Good-quality mRNA has been successfully isolated from mammalian **cells** (such as cultured cells and lymphocytes) using QIAGEN's Oligotex Direct mRNA kit and from total RNA using the Oligotex mRNA kit. If mammalian **tissue** is used as the source of mRNA, total RNA should be first purified using a commercial reagent, such as TRIzol, and then using a poly-A mRNA isolation procedure or a commercial kit.

Precipitation of RNA

Total RNA

It is not necessary to precipitate total RNA following isolation or cleanup with the RNeasy Mini Kit. Adjust elution volumes from the RNeasy column to prepare for cDNA synthesis based upon expected RNA yields from your experiment. Ethanol precipitation is required following TRIzol isolation and hot phenol extraction methods; see methods on page 2.1.11 for details.

Poly-A mRNA

Most poly-A mRNA isolation procedures will result in dilution of RNA. It is necessary to concentrate mRNA prior to the cDNA synthesis.

Precipitation Procedure

1. Add 1/10 volume 3M NaOAc, pH 5.2, and 2.5 volumes ethanol.*
2. Mix and incubate at -20°C for at least 1 hour.
3. Centrifuge at ≥ 12,000 x g in a microcentrifuge for 20 minutes at 4°C.
4. Wash pellet twice with 80% ethanol.
5. Air dry pellet. Check for dryness before proceeding.
6. Resuspend pellet in DEPC-treated H_2O. The appropriate volume for resuspension depends on the expected yield and the amount of RNA required for the cDNA synthesis. Please read ahead to the cDNA synthesis protocol in order to determine the appropriate resuspension volume at this step.

*Addition of Carrier to Ethanol Precipitations

Adding carrier material has been shown to improve the RNA yield of precipitation reactions.

- *Pellet Paint*

  Addition of 0.5 µL of Pellet Paint per tube to nucleic acid precipitations makes the nucleic acid pellet easier to visualize and helps reduce the chance of losing the pellet during washing steps. The pellet paint does not appear to affect the outcome of subsequent steps in this protocol; however, it can contribute to the absorbance at 260 nm when quantifying the mRNA.
- *Glycogen*

  Addition of 0.5 to 1 µL of glycogen (5 mg/mL) to nucleic acid precipitations aids in visualization of the pellet and may increase recovery. The glycogen does not appear to affect the outcome of subsequent steps in this protocol.

Quantification of RNA

Quantify RNA yield by spectrophotometric analysis using the convention that 1 absorbance unit at 260 nm equals 40 µg/mL RNA.

- The absorbance should be checked at 260 and 280 nm for determination of sample concentration and purity.
- The A_{260}/A_{280} ratio should be close to 2.0 for pure RNA (ratios between 1.9 and 2.1 are acceptable).
- Integrity of total RNA samples can also be assessed qualitatively on an Agilent 2100 Bioanalyzer. Refer to Figure 2.1.2 for an example of good-quality total RNA sample.

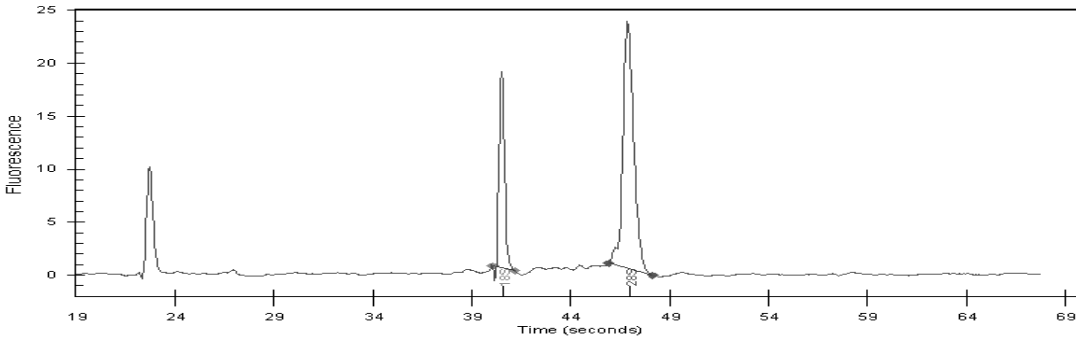

Figure 2.1.2
Electropherogram (from the Agilent 2100 Bioanalyzer) for HeLa Total RNA. For a high-quality total RNA sample, two well-defined peaks corresponding to the 18S and 28S ribosomal RNAs should be observed, similar to a denaturing agarose gel, with ratios approaching 2:1 for the 28S to 18S bands.

Total RNA Isolation for Two-Cycle Target Labeling Assay

Several commercial kits and protocols are currently available for total RNA isolation from small samples (tissues, biopsies, LCM samples, etc.). Select the one that is suitable for processing of your samples and follow the vendor-recommended procedures closely since high-quality and high-integrity starting material is essential for the success of the assay.

One-Cycle cDNA Synthesis[1]

Step 1: Preparation of Poly-A RNA Controls for One-Cycle cDNA Synthesis (Spike-in Controls)

Eukaryotic Poly-A RNA Control Kit is used for this step.

Designed specifically to provide exogenous positive controls to monitor the entire eukaryotic target labeling process, a set of poly-A RNA controls is supplied in the GeneChip Eukaryotic Poly-A RNA Control Kit.

Each eukaryotic GeneChip probe array contains probe sets for several *B. subtilis* genes that are absent in eukaryotic samples (*lys, phe, thr,* and *dap*). These poly-A RNA controls are *in vitro* synthesized, and the polyadenylated transcripts for the *B. subtilis* genes are pre-mixed at staggered concentrations. The concentrated **Poly-A Control Stock** can be diluted with the **Poly-A Control Dil Buffer** and spiked directly into RNA samples to achieve the final concentrations (referred to as a ratio of copy number) summarized below in Table 2.1.1.

Table 2.1.1
Final Concentrations of Poly-A RNA Controls in Samples

Poly-A RNA Spike	*Final Concentration (ratio of copy number)*
lys	1:100,000
phe	1:50,000
thr	1:25,000
dap	1:7,500

The controls are then amplified and labeled together with the samples. Examining the hybridization intensities of these controls on GeneChip arrays helps to monitor the labeling process independently from the quality of the starting RNA samples. Typical GeneChip array results from these poly-A spike-in controls are shown in Figure 2.1.3.

✔ Note

For Drosophila Genome Arrays (P/N 900335 and 900336) and Yeast Genome S98 Arrays (P/N 900256 and 900285), the 3′ AFFX-r2-Bs probe sets are not available. Note that the data shown here may not be representative of those obtained using the previous generation AFFX-(Spike-in transcript name) X probe sets on the GeneChip arrays listed above.

1. Users who do not purchase this Kit may be required to obtain a license under U.S. Patent Nos. 5,716,785, 5,891,636, 6,291,170, and 5,545,522 or to purchase another licensed kit.

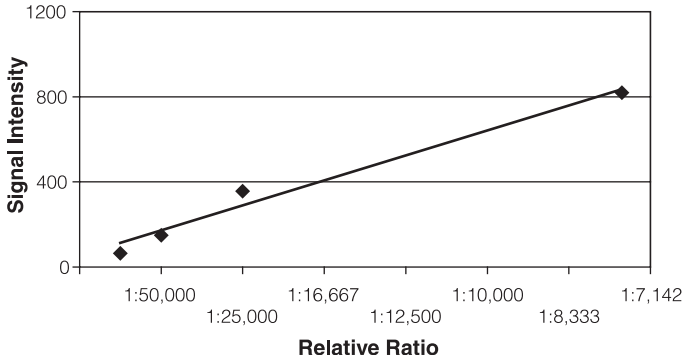

Figure 2.1.3
Poly-A RNA spikes amplified using a complex human Jurkat total RNA sample. Evaluation was performed using Affymetrix® Microarray Suite (MAS) 5.0 software.

The **Poly-A RNA Control Stock** and **Poly-A Control Dil Buffer** are provided with the kit to prepare the appropriate serial dilutions based on Table 2.1.2. This is a guideline when 1, 5, or 10 µg of total RNA or 0.2 µg of mRNA is used as starting material. For starting sample amounts other than those listed here, calculations are needed in order to perform the appropriate dilutions to arrive at the same proportionate final concentration of the spike-in controls in the samples.

IMPORTANT *Use non-stick RNase-free microfuge tubes to prepare all of the dilutions.*

Table 2.1.2
Serial Dilutions of Poly-A RNA Control Stock

Starting Amount		*Serial Dilutions*			*Spike-in Volume*
Total RNA	*mRNA*	*First*	*Second*	*Third*	
1 µg		1:20	1:50	1:50	2 µL
5 µg		1:20	1:50	1:10	2 µL
10 µg	0.2 µg	1:20	1:50	1:5	2 µL

Recommendation *Avoid pipetting solutions less than 2 µL in volume to maintain precision and consistency when preparing the dilutions.*

For example, to prepare the poly-A RNA dilutions for 5 µg of total RNA:

1. Add 2 µL of the **Poly-A Control Stock** to 38 µL of **Poly-A Control Dil Buffer** for the First Dilution (1:20).
2. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.
3. Add 2 µL of the First Dilution to 98 µL of **Poly-A Control Dil Buffer** to prepare the Second Dilution (1:50).
4. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.
5. Add 2 µL of the Second Dilution to 18 µL of **Poly-A Control Dil Buffer** to prepare the Third Dilution (1:10).

6. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.
7. Add 2 µL of this Third Dilution to 5 µg of sample total RNA.

✔ Note

The First Dilution of the poly-A RNA controls can be stored up to six weeks in a non-frost-free freezer at -20°C and frozen-thawed up to eight times.

Step 2: First-Strand cDNA Synthesis

One-Cycle cDNA Synthesis Kit is used for this step.

✔ Note

1. Briefly spin down all tubes in the Kit before using the reagents.

2. Perform all of the incubations in thermal cyclers. The following program can be used as a reference to perform the first-strand cDNA synthesis reaction in a thermal cycler; the 4°C holds are for reagent addition steps:

70°C	*10 minutes*
4°C	*hold*
42°C	*2 minutes*
42°C	*1 hour*
4°C	*hold*

1. Mix RNA sample, diluted poly-A RNA controls, and T7-Oligo(dT) Primer.

Table 2.1.3
RNA/T7-Oligo(dT) Primer Mix Preparation for 1 to 8 µg of total RNA, or 0.2 to 1 µg of mRNA

Component	*Volume*
Sample RNA	variable
Diluted poly-A RNA controls	2 µL
T7-Oligo(dT) Primer, 50 µM	2 µL
RNase-free Water	variable
Total Volume	*12 µL*

Table 2.1.4
RNA/T7-Oligo(dT) Primer Mix Preparation for 8.1 to 15 µg of total RNA, or > 1 µg of mRNA

Component	*Volume*
Sample RNA	variable
Diluted poly-A RNA controls	2 µL
T7-Oligo(dT) Primer, 50 µM	2 µL
RNase-free Water	variable
Total Volume	*11 µL*

a. Place total RNA (1 µg to 15 µg) or mRNA sample (0.2 µg to 2 µg) in a 0.2 mL PCR tube.

b. Add 2 µL of the appropriately diluted poly-A RNA controls (See *Step 1: Preparation of Poly-A RNA Controls for One-Cycle cDNA Synthesis (Spike-in Controls)* on page 2.1.13).

c. Add 2 µL of 50 µM **T7-Oligo(dT) Primer**.

d. Add **RNase-free Water** to a final volume of 11 or 12 µL (see Table 2.1.3 and Table 2.1.4).

e. Gently flick the tube a few times to mix, and then centrifuge briefly (~5 seconds) to collect the reaction at the bottom of the tube.

f. Incubate the reaction for 10 minutes at 70°C.

g. Cool the sample at 4°C for at least 2 minutes.

h. Centrifuge the tube briefly (~5 seconds) to collect the sample at the bottom of the tube.

2. In a separate tube, assemble the First-Strand Master Mix.

   a. Prepare sufficient **First-Strand Master Mix** for all of the RNA samples. When there are more than 2 samples, it is prudent to include additional material to compensate for potential pipetting inaccuracy or solution lost during the process. The following recipe, in Table 2.1.5, is for a single reaction.

Table 2.1.5
Preparation of First-Strand Master Mix

Component	*Volume*
5X 1st Strand Reaction Mix	4 µL
DTT, 0.1M	2 µL
dNTP, 10 mM	1 µL
Total Volume	*7 µL*

   b. Mix well by flicking the tube a few times. Centrifuge briefly (~5 seconds) to collect the master mix at the bottom of the tube.

3. Transfer 7 µL of **First-Strand Master Mix** to each RNA/T7-Oligo(dT) Primer mix for a final volume of 18 or 19 µL. Mix thoroughly by flicking the tube a few times. Centrifuge briefly (~5 seconds) to collect the reaction at the bottom of the tube, and immediately place the tubes at 42°C.

4. Incubate for 2 minutes at 42°C.

5. Add the appropriate amount of **SuperScript II** to each RNA sample for a final volume of 20 µL.
   - For 1 to 8 µg of total RNA: 1 µL *SuperScript II*
   - For 8.1 to 15 µg of total RNA: 2 µL *SuperScript II*
   - For every µg of mRNA add 1 µL *SuperScript II*.
   - For mRNA quantity less than 1 µg, use 1 µL *SuperScript II*.

   Mix thoroughly by flicking the tube a few times. Centrifuge briefly (~5 seconds) to collect the reaction at the bottom of the tube, and immediately place the tubes at 42°C.

6. Incubate for 1 hour at 42°C; then cool the sample for at least 2 minutes at 4°C.

IMPORTANT

Cooling the samples at 4°C is required before proceeding to the next step. Adding the Second-Strand Master Mix directly to solutions that are at 42°C will compromise enzyme activity.

After incubation at 4°C, centrifuge the tube briefly (~5 seconds) to collect the reaction at the bottom of the tube and immediately proceed to *Step 3: Second-Strand cDNA Synthesis*.

Step 3: Second-Strand cDNA Synthesis

One-Cycle cDNA Synthesis Kit is used for this step.

✔ Note

The following program can be used as a reference to perform the second-strand cDNA synthesis reaction in a thermal cycler.

16°C	*2 hours*
4°C	*hold*
16°C	*5 minutes*
4°C	*hold*

1. In a separate tube, assemble Second-Strand Master Mix.

✔ Note

It is recommended to prepare Second-Strand Master Mix immediately before use.

   a. Prepare sufficient **Second-Strand Master Mix** for all of the samples. When there are more than 2 samples, it is prudent to include additional material to compensate for potential pipetting inaccuracy or solution lost during the process. The following recipe, in Table 2.1.6, is for a single reaction.

Table 2.1.6
Preparation of Second-Strand Master Mix

Component	*Volume*
RNase-free Water	91 µL
5X 2nd Strand Reaction Mix	30 µL
dNTP, 10 mM	3 µL
E. coli DNA ligase	1 µL
E. coli DNA Polymerase I	4 µL
RNase H	1 µL
Total Volume	*130 µL*

   b. Mix well by gently flicking the tube a few times. Centrifuge briefly (~5 seconds) to collect the solution at the bottom of the tube.

2. Add 130 µL of **Second-Strand Master Mix** to each first-strand synthesis sample from *Step 2: First-Strand cDNA Synthesis* for a total volume of 150 µL.

   Gently flick the tube a few times to mix, and then centrifuge briefly (~5 seconds) to collect the reaction at the bottom of the tube.

3. Incubate for 2 hours at 16°C.

4. Add 2 µL of **T4 DNA Polymerase** to each sample and incubate for 5 minutes at 16°C.

5. After incubation with **T4 DNA Polymerase** add 10 µL of **EDTA, 0.5M** and proceed to *Cleanup of Double-Stranded cDNA for Both the One-Cycle and Two-Cycle Target Labeling Assays* on page 2.1.32.

   Do not leave the reactions at 4°C for long periods of time.

Two-Cycle cDNA Synthesis[2]

Step 1: Preparation of Poly-A RNA Controls for Two-Cycle cDNA Synthesis (Spike-in Controls)

Eukaryotic Poly-A RNA Control Kit is used for this step.

Designed specifically to provide exogenous positive controls to monitor the entire eukaryotic target labeling process, a set of poly-A RNA controls are supplied in the GeneChip Eukaryotic Poly-A RNA Control Kit.

Each eukaryotic GeneChip probe array contains probe sets for several *B. subtilis* genes that are absent in eukaryotic samples (*lys, phe, thr,* and *dap*). These poly-A RNA controls are *in vitro* synthesized, and the polyadenylated transcripts for these *B. subtilis* genes are pre-mixed at staggered concentrations. The concentrated **Poly-A Control Stock** can be diluted with the **Poly-A Control Dil Buffer** and spiked directly into the RNA samples to achieve the final concentrations (referred to as a ratio of copy number) summarized below:

Table 2.1.7
Final Concentrations of Poly-A RNA Controls in Samples

Poly-A RNA Spike	*Final Concentration (ratio of copy number)*
lys	1:100,000
phe	1:50,000
thr	1:25,000
dap	1:7,500

The controls are then amplified and labeled together with the samples. Examining the hybridization intensities of these controls on GeneChip arrays helps to monitor the labeling process independently from the quality of the starting RNA samples. Typical GeneChip array results from these poly-A Spike-in Controls are shown in Figure 2.1.4.

Note

For Drosophila Genome Arrays (P/N 900335 and 900336) and Yeast Genome S98 Arrays (P/N 900256 and 900285), the 3′ AFFX-r2-Bs probe sets are not available. Note that the data shown here may not be representative of those obtained using the previous generation AFFX-(Spike-in transcript name) X probe sets on the GeneChip arrays listed above.

2. Users who do not purchase this Kit may be required to obtain a license under U.S. Patent Nos. 5,716,785, 5,891,636, 6,291,170, and 5,545,522 or to purchase another licensed kit.

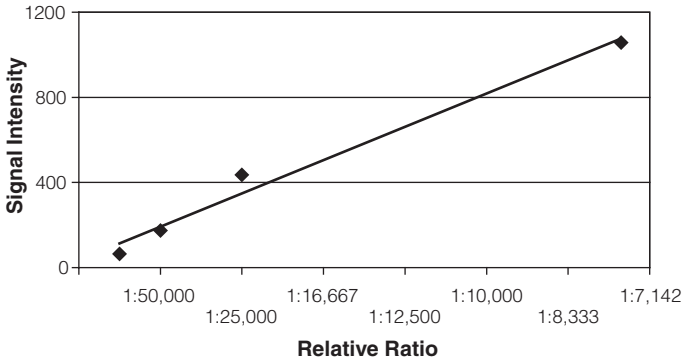

Figure 2.1.4
Poly-A RNA spikes amplified using a complex human Jurkat total RNA sample. Evaluation was performed using MAS 5.0 software.

The **Poly-A RNA Control Stock** and **Poly-A Control Dil Buffer** are provided with the kit to prepare the appropriate serial dilutions based on Table 2.1.8. This is a guideline when 10, 50, or 100 ng of total RNA is used as starting material. For other intermediate starting sample amounts, calculations are needed in order to perform the appropriate dilutions to arrive at the same proportionate final concentration of the spike-in controls in the samples.

IMPORTANT

- *The dilution scheme outlined below is different from the previous protocol developed for the Small Sample Target Labeling vII. Closely adhere to the recommendation below to obtain the desired final concentrations of the controls.*
- *Use non-stick RNase-free microfuge tubes to prepare the dilutions.*

Table 2.1.8
Serial Dilutions of Poly-A RNA Control Stock

Starting Amount of Total RNA	*Serial Dilutions*				*Volume to Add into 50 µM T7-Oligo(dT) Primer*
	First	*Second*	*Third*	*Fourth*	
10 ng	1:20	1:50	1:50	1:10	2 µL
50 ng	1:20	1:50	1:50	1:2	2 µL
100 ng	1:20	1:50	1:50		2 µL

Recommendation

Avoid pipetting solutions less than 2 µL in volume to maintain precision and consistency when preparing the dilutions.

For example, to prepare the poly-A RNA dilutions for 10 ng of total RNA:

1. Add 2 µL of the **Poly-A Control Stock** to 38 µL of **Poly-A Control Dil Buffer** to prepare the First Dilution (1:20).
2. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.
3. Add 2 µL of the First Dilution to 98 µL of **Poly-A Control Dil Buffer** to prepare the Second Dilution (1:50).
4. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.

5. Add 2 µL of the Second Dilution to 98 µL of **Poly-A Control Dil Buffer** to prepare the Third Dilution (1:50).
6. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.
7. Add 2 µL of the Third Dilution to 18 µL of **Poly-A Control Dil Buffer** to prepare the Fourth Dilution (1:10).
8. Use the Fourth Dilution to prepare the solution described next.

✔ Note

The first dilution of the poly-A RNA controls (1:20) can be stored in a non-frost-free freezer at -20°C up to six weeks and frozen-thawed up to eight times.

Preparation of T7-Oligo(dT) Primer/Poly-A Controls Mix

Prepare a fresh dilution of the **T7-Oligo(dT) Primer** from 50 µM to 5 µM. The diluted poly-A RNA controls should be added to the concentrated **T7-Oligo(dT) Primer** as follows, using a non-stick RNase-free microfuge tube. The following recipe is sufficient for 10 samples.

Table 2.1.9
Preparation of T7-Oligo(dT) Primer/Poly-A Controls Mix

Component	*Volume*
T7-Oligo(dT) Primer, 50 µM	2 µL
Diluted Poly-A RNA controls (See Table 2.1.8)	2 µL
RNase-free Water	16 µL
Total Volume	*20 µL*

Step 2: First-Cycle, First-Strand cDNA Synthesis

Two-Cycle cDNA Synthesis Kit is used for this step.

✔ Note

1. Briefly spin down all tubes in the Kit before using the reagents.

2. Perform all of the incubations in thermal cyclers. The following program can be used as a reference to perform the First-Cycle, First-Strand cDNA synthesis reaction in a thermal cycler; the 4°C holds are for reagent addition steps:

70°C 6 minutes
4°C hold
42°C 1 hour
70°C 10 minutes
4°C hold

1. Mix total RNA sample and the T7-Oligo(dT) Primer/Poly-A Controls Mix.

Table 2.1.10
Preparation of Total RNA Sample/T7-Oligo(dT) Primer/Poly-A Controls Mix

Component	*Volume*
Total RNA sample	variable (10 – 100 ng)
T7-Oligo(dT) Primer/Poly-A Controls Mix	2 µL
RNase-free Water	variable
Total Volume	*5 µL*

   a. Place total RNA sample (10 to 100 ng) in a 0.2 mL PCR tube.

   b. Add 2 µL of the T7-Oligo(dT) Primer/Poly-A Controls Mix (See *Step 1: Preparation of Poly-A RNA Controls for Two-Cycle cDNA Synthesis (Spike-in Controls)* on page 2.1.19).

   c. Add **RNase-free Water** to a final volume of 5 µL.

   d. Gently flick the tube a few times to mix, then centrifuge the tubes briefly (~5 seconds) to collect the solution at the bottom of the tube.

   e. Incubate for 6 minutes at 70°C.

   f. Cool the sample at 4°C for at least 2 minutes. Centrifuge briefly (~5 seconds) to collect the sample at the bottom of the tube.

2. In a separate tube, assemble the First-Cycle, First-Strand Master Mix.

   a. Prepare sufficient **First-Cycle, First-Strand Master Mix** for all of the total RNA samples. When there are more than 2 samples, it is prudent to include additional material to compensate for potential pipetting inaccuracy or solution lost during the process. The following recipe, in Table 2.1.11, is for a single reaction.

Table 2.1.11
Preparation of First-Cycle, First-Strand Master Mix

Component	*Volume*
5X 1st Strand Reaction Mix	2.0 µL
DTT, 0.1M	1.0 µL
RNase Inhibitor	0.5 µL
dNTP, 10 mM	0.5 µL
SuperScript II	1.0 µL
Total Volume	*5.0 µL*

b. Mix well by gently flicking the tube a few times. Centrifuge briefly (~5 seconds) to collect the solution at the bottom of the tube.

3. Transfer 5 µL of **First-Cycle, First-Strand Master Mix** to each total RNA sample/ T7-Oligo(dT) Primer/Poly-A Controls Mix (as in Table 2.1.10) from the previous step for a final volume of 10 µL.

   Mix thoroughly by gently flicking the tube a few times. Centrifuge briefly (~5 seconds) to collect the reaction at the bottom of the tube, and immediately place the tubes at 42°C.

4. Incubate for 1 hour at 42°C.

5. Heat the sample at 70°C for 10 minutes to inactivate the RT enzyme, then cool the sample for at least 2 minutes at 4°C.

   After the 2 minute incubation at 4°C, centrifuge the tube briefly (~5 seconds) to collect the reaction at the bottom of the tube and immediately proceed to *Step 3: First-Cycle, Second-Strand cDNA Synthesis* on page 2.1.24.

IMPORTANT

Cooling the sample at 4°C is required before proceeding to the next step. Adding the First-Cycle, Second-Strand Master Mix directly to solutions that are at 70°C will compromise enzyme activity.

Step 3: First-Cycle, Second-Strand cDNA Synthesis

Two-Cycle cDNA Synthesis Kit is used for this step.

✔ Note

The following program can be used as a reference to perform the First-cycle, Second-strand cDNA synthesis reaction in a thermal cycler. For the 16°C incubation, turn the heated lid function off. If the heated lid function cannot be turned off, leave the lid open. Use the heated lid for the 75°C incubation.

16°C 2 hours
75°C 10 minutes
4°C hold

1. In a separate tube, assemble the First-Cycle, Second-Strand Master Mix.

Recommendation

It is recommended to prepare this First-Cycle, Second-Strand Master Mix immediately before use. Prepare this First-Cycle, Second-Strand Master Mix for at least 4 reactions at one time for easier and more accurate pipetting.

a. Prepare sufficient **First-Cycle, Second-Strand Master Mix** for all samples. When there are more than 2 samples, it is prudent to include additional material to compensate for potential pipetting inaccuracy or solution lost during the process. The following recipe, in Table 2.1.12, is for a single reaction.

Table 2.1.12
Preparation of First-Cycle, Second-Strand Master Mix

Component	*Volume*
RNase-free Water	4.8 µL
Freshly diluted $MgCl_2$, 17.5 mM*	4.0 µL
dNTP, 10 mM	0.4 µL
E.coli DNA Polymerase I	0.6 µL
RNase H	0.2 µL
Total Volume	*10.0 µL*

* Make a fresh dilution of the $MgCl_2$ each time. Mix 2 µL of $MgCl_2$, 1M with 112 µL of RNase-free Water.

b. Mix well by gently flicking the tube a few times. Centrifuge briefly (~5 seconds) to collect the solution at the bottom of the tube.

2. Add 10 µL of the **First-Cycle, Second-Strand Master Mix** to each sample from *Step 2: First-Cycle, First-Strand cDNA Synthesis* reaction for a total volume of 20 µL.

   Gently flick the tube a few times to mix, and then centrifuge briefly (~5 seconds) to collect the reaction at the bottom of the tube.

3. Incubate for 2 hours at 16°C, then 10 minutes at 75°C and cool the sample at least 2 minutes at 4°C. Turn the heated lid function off only for the 16°C incubation.

   After the 2 minute incubation at 4°C, centrifuge the tube briefly (~5 seconds) to collect the reaction at the bottom of the tube. Proceed to *Step 4: First-Cycle, IVT Amplification of cRNA* on page 2.1.25.

✔ Note

No cDNA cleanup is required at this step.

Step 4: First-Cycle, IVT Amplification of cRNA

MEGAscript® T7 Kit (Ambion, Inc.) is used for this step.

✔ Note

The following program can be used as a reference to perform the First-cycle, IVT Amplification of cRNA reaction in a thermal cycler.

37°C 16 hours
4°C hold

1. In a separate tube, assemble the First-Cycle, IVT Master Mix at room temperature.
   a. Prepare sufficient **First-Cycle, IVT Master Mix** for all of the samples. When there are more than 2 samples, it is prudent to include additional material to compensate for potential pipetting inaccuracy or solution lost during the process. The following recipe, in Table 2.1.13, is for a single reaction.

Table 2.1.13
Preparation of First-Cycle, IVT Master Mix

Component	*Volume*
10X Reaction Buffer	5 µL
ATP Solution	5 µL
CTP Solution	5 µL
UTP Solution	5 µL
GTP Solution	5 µL
Enzyme Mix	5 µL
Total Volume	*30 µL*

   b. Mix well by gently flicking the tube a few times. Centrifuge briefly (~5 seconds) to collect the solution at the bottom of the tube.
2. Transfer 30 µL of **First-Cycle, IVT Master Mix** to each cDNA sample.

   At room temperature, add 30 µL of the **First-Cycle, IVT Master Mix** to each 20 µL of cDNA sample from *Step 3: First-Cycle, Second-Strand cDNA Synthesis* on page 2.1.24 for a final volume of 50 µL.

   Gently flick the tube a few times to mix, then centrifuge briefly (~5 seconds) to collect the reaction at the bottom of the tube.
3. Incubate for 16 hours at 37°C.

   After the 16 hour incubation at 37°C, centrifuge the tube briefly (~5 seconds) to collect the reaction at the bottom of the tube.
   The sample is now ready to be purified in *Step 5: First-Cycle, Cleanup of cRNA* on page 2.1.26. Alternatively, samples may be stored at -20°C for later use.

Step 5: First-Cycle, Cleanup of cRNA

Sample Cleanup Module is used for this step.

Reagents to be Supplied by User

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

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

IMPORTANT

***BEFORE STARTING** please note:*

- *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 50 µL of **RNase-free Water** to the IVT 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 ≥ 8,000 x g (≥ 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 ≥ 8,000 x g (≥ 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 ≥ 8,000 x g (≥ 10,000 rpm). Discard flow-through.
7. Open the cap of the spin column and centrifuge for 5 minutes at maximum speed (≤ 25,000 x 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 counterclockwise direction). This avoids damage of the caps.

Recommendation

Label the collection tubes with the sample name. During centrifugation some column caps may break, resulting in loss of sample information.

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 13 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 (≤ 25,000 x g) to elute. The average volume of eluate is 11 µL from 13 µL RNase-free Water.

9. To determine cRNA yield for samples starting with 50 ng or higher, remove 2 µL of the cRNA, and add 78 µL of water to measure the absorbance at 260 nm. Use 600 ng of cRNA in the following *Step 6: Second-Cycle, First-Strand cDNA Synthesis Reaction.*

   For starting material less than 50 ng, or if the yield is less than 600 ng, use the entire eluate for the Second-Cycle, First-Strand cDNA Synthesis Reaction.

   Samples can be stored at -20°C for later use, or proceed to *Step 6: Second-Cycle, First-Strand cDNA Synthesis* described next.

Step 6: Second-Cycle, First-Strand cDNA Synthesis

Two-Cycle cDNA Synthesis Kit is used for this step.

✔ Note

The following program can be used as a reference to perform the Second-Cycle, First-Strand cDNA synthesis reaction in a thermal cycler; the 4°C holds are for reagent addition steps:

70°C	*10 minutes*
4°C	*hold*
42°C	*1 hour*
4°C	*hold*
37°C	*20 minutes*
95°C	*5 minutes*
4°C	*hold*

1. Mix cRNA and diluted random primers.
   a. Make a fresh dilution of the **Random Primers** (final concentration 0.2 µg/µL). Mix 2 µL of **Random Primers, 3 µg/µL,** with 28 µL **RNase-free Water**.
   b. Add 2 µL of diluted random primers to purified cRNA from *Step 5: First-Cycle, Cleanup of cRNA*, substep 9 on page 2.1.27 and add **RNase-free Water** for a final volume of 11 µL.
   c. Incubate for 10 minutes at 70°C.
   d. Cool the sample at 4°C for at least 2 minutes. Centrifuge briefly (~5 seconds) to collect the sample at the bottom of the tube.
2. In a separate tube, assemble the Second-Cycle, First-Strand Master Mix.
   a. Prepare sufficient **Second-Cycle, First-Strand Master Mix** for all of the samples. When there are more than two samples, it is prudent to include additional material to compensate for potential pipetting inaccuracy or solution lost during the process. The following recipe, in Table 2.1.14, is for a single reaction.

Table 2.1.14
Preparation of Second-Cycle, First-Strand Master Mix

Component	*Volume*
5X 1st Strand Reaction Mix	4 µL
DTT, 0.1M	2 µL
RNase Inhibitor	1 µL
dNTP, 10 mM	1 µL
SuperScript II	1 µL
Total Volume	*9 µL*

   b. Mix well by gently flicking the tube a few times. Centrifuge briefly (~5 seconds) to collect the solution at the bottom of the tube.

3. Transfer 9 µL of **Second-Cycle, First-Strand Master Mix** to each cRNA/random primer sample from *Step 6: Second-Cycle, First-Strand cDNA Synthesis* on page 2.1.28, substep 1, for a final volume of 20 µL.

   Mix thoroughly by gently flicking the tube a few times. Centrifuge briefly (~5 seconds) to collect the reaction at the bottom of the tube and place the tubes at 42°C immediately.

4. Incubate for 1 hour at 42°C, then cool the sample for at least 2 minutes at 4°C.

   After the incubation at 4°C, centrifuge briefly (~5 seconds) to collect the reaction at the bottom of the tube.

5. Add 1 µL of **RNase H** to each sample for a final volume of 21 µL.

   Mix thoroughly by gently flicking the tube a few times. Centrifuge briefly (~5 seconds) to collect the reaction at the bottom of the tube and incubate for 20 minutes at 37°C.

6. Heat the sample at 95°C for 5 minutes. Cool the sample for at least 2 minutes at 4°C; then, proceed directly to *Step 7: Second-Cycle, Second-Strand cDNA Synthesis* on page 2.1.30.

Step 7: Second-Cycle, Second-Strand cDNA Synthesis

Two-Cycle cDNA Synthesis Kit is used for this step.

✔ Note

The following program can be used as a reference to perform the Second-Cycle, Second-Strand cDNA Synthesis reaction in a thermal cycler. For the 16°C incubations turn the heated lid function off. If the heated lid function cannot be turned off, leave the lid open. The 4°C holds are for reagent addition steps:

70°C	*6 minutes*
4°C	*hold*
16°C	*2 hours*
4°C	*hold*
16°C	*10 minutes*
4°C	*hold*

1. Add 4 µL of diluted T7-Oligo(dT) Primer to each sample.
   a. Make a fresh dilution of the T7-Oligo(dT) Primer (final concentration 5 µM). Mix 2 µL of **T7-Oligo(dT) Primer, 50 µM**, with 18 µL of **RNase-free Water**.
   b. Add 4 µL of diluted T7-Oligo(dT) Primer to the sample from *Step 6: Second-Cycle, First-Strand cDNA Synthesis*, substep 6 on page 2.1.29 for a final volume of 25 µL.
   c. Gently flick the tube a few times to mix, and then centrifuge briefly (~5 seconds) to collect the reaction at the bottom of the tube.
   d. Incubate for 6 minutes at 70°C.
   e. Cool the sample at 4°C for at least 2 minutes. Centrifuge briefly (~5 seconds) to collect sample at the bottom of the tube.

IMPORTANT

Cooling the samples at 4°C is required before proceeding to the next step. Adding the Second-Strand Master Mix directly to solutions that are at 70°C will compromise enzyme activity.

Recommendation

It is recommended to prepare the Second-Cycle, Second-Strand Master Mix immediately before use.

2. In a separate tube, assemble the Second-Cycle, Second-Strand Master Mix.
   a. Prepare sufficient **Second-Cycle, Second-Strand Master Mix** for all of the samples. When there are more than two samples, it is prudent to include additional material to compensate for potential pipetting inaccuracy or solution lost during the process. The following recipe, in Table 2.1.15, is for a single reaction.

Table 2.1.15
Preparation of Second-Cycle, Second-Strand Master Mix

Component	*Volume*
RNase-free Water	88 µL
5X 2nd Strand Reaction Mix	30 µL
dNTP, 10 mM	3 µL
E.coli DNA Polymerase I	4 µL
Total Volume	*125 µL*

b. Mix well by gently flicking the tube a few times. Centrifuge briefly (~5 seconds) to collect the master mix at the bottom of the tube.

3. Add 125 µL of the **Second-Cycle, Second-Strand Master Mix** to each sample from *Step 7: Second-Cycle, Second-Strand cDNA Synthesis*, substep 1, for a total volume of 150 µL.

   Gently flick the tube a few times to mix, then centrifuge briefly (~5 seconds) to collect the reaction at the bottom of tube.

4. Incubate for 2 hours at 16°C.

5. Add 2 µL of **T4 DNA Polymerase** to the samples for a final volume of 152 µL. Gently flick the tube a few times to mix, and then centrifuge briefly (~5 seconds) to collect the reaction at the bottom of the tube.

6. Incubate for 10 minutes at 16°C, then cool the sample at 4°C for at least 2 minutes. Centrifuge briefly (~5 seconds) to collect sample at the bottom of the tube.

   After the incubation at 4°C, centrifuge the tube briefly (~5 seconds) to collect the reaction at the bottom of the tube. Proceed to *Cleanup of Double-Stranded cDNA for Both the One-Cycle and Two-Cycle Target Labeling Assays* on page 2.1.32. Alternatively, immediately freeze the sample at –20°C for later use. Do not leave the reaction at 4°C for long periods of time.

Cleanup of Double-Stranded cDNA for Both the One-Cycle and Two-Cycle Target Labeling Assays

Sample Cleanup Module is used for cleaning up the 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 µL of **cDNA Binding Buffer** to the double-stranded cDNA synthesis preparation. 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 µL of 3M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

3. Apply 500 µL of the sample to the **cDNA Cleanup Spin Column** sitting in a **2 mL Collection Tube** (supplied), and centrifuge for 1 minute at ≥ 8,000 x g (≥ 10,000 rpm). Discard flow-through.
4. Reload the spin column with the remaining mixture and centrifuge as above. Discard flow-through and Collection Tube.
5. Transfer spin column into a new **2 mL Collection Tube** (supplied). Pipet 750 µL of the **cDNA Wash Buffer** onto the spin column. Centrifuge for 1 minute at ≥ 8,000 x g (≥ 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 (≤ 25,000 x g). Discard flow-through and Collection Tube.

Recommendation

Label the collection tubes with the sample name. During centrifugation some column caps may break, resulting in loss of sample information.

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 counterclockwise 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 µL of **cDNA Elution Buffer** directly onto the spin column membrane. Incubate for 1 minute at room temperature and centrifuge 1 minute at maximum speed (≤ 25,000 x g) to elute.

   Ensure that the cDNA Elution Buffer is dispensed directly onto the membrane. The average volume of eluate is 12 µL from 14 µL 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.

We do not recommend gel analysis for cDNA prepared from total RNA.

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

8. After cleanup, please proceed to *Synthesis of Biotin-Labeled cRNA for Both the One-Cycle and Two-Cycle Target Labeling Assays* on page 2.1.34.

Synthesis of Biotin-Labeled cRNA for Both the One-Cycle and Two-Cycle Target Labeling Assays

GeneChip IVT Labeling Kit is used.

✔ Note

This kit is only used for the IVT labeling step for generating biotin-labeled cRNA. For the IVT amplification step using unlabeled ribonucleotides in the First Cycle of the Two-Cycle cDNA Synthesis Procedure, a separate kit is recommended (MEGAscript® T7 Kit, Ambion, Inc.). Use only nuclease-free water, buffers, and pipette tips.

IMPORTANT

Store all reagents in a -20°C freezer that is not self-defrosting. Prior to use, centrifuge all reagents briefly to ensure that the solution is collected at the bottom of the tube.

The Target Hybridizations and Array Washing protocols have been optimized specifically for this IVT Labeling Protocol. Closely follow the recommendations described below for maximum array performance.

1. Use the following table to determine the amount of cDNA used for each IVT reaction following the cDNA cleanup step.

Table 2.1.16
IVT Reaction Set Up

Starting Material	*Volume of cDNA to use in IVT*
Total RNA	
10 to 100 ng	all (~12 µL)
1.0 to 8.0 µg	all (~12 µL)
8.1 to 15 µg	6 µL
mRNA	
0.2 to 0.5 µg	all (~12 µL)
0.6 to 1.0 µg	9 µL
1 to 2.0 µg	6 µL

2. Transfer the needed amount of template cDNA to RNase-free microfuge tubes and add the following reaction components in the order indicated in the table below. If more than one IVT reaction is to be performed, a master mix can be prepared by multiplying the reagent volumes by the number of reactions. Do not assemble the reaction on ice, since spermidine in the **10X IVT Labeling Buffer** can lead to precipitation of the template cDNA.

Table 2.1.17
IVT Reaction

Reagent	*Volume*
Template cDNA*	variable (see table above)
RNase-free Water	variable (to give a final reaction volume of 40 µL)
10X IVT Labeling Buffer	4 µL
IVT Labeling NTP Mix	12 µL
IVT Labeling Enzyme Mix	4 µL
Total Volume	*40 µL*

*0.5 to 1 µg of the 3'-Labeling Control can be used in place of the template cDNA sample in this reaction as a positive control for the IVT components in the kit.

3. Carefully mix the reagents and collect the mixture at the bottom of the tube by brief (~5 seconds) microcentrifugation.
4. Incubate at 37°C for 16 hours. To prevent condensation that may result from water bath-style incubators, incubations are best performed in oven incubators for even temperature distribution, or in a thermal cycler.

✔ Note

Overnight IVT reaction time has been shown to maximize the labeled cRNA yield with high-quality array results. Alternatively, if a shorter incubation time (4 hours) is desired, 1 µL (200 units) of cloned T7 RNA polymerase (can be purchased directly from Ambion, P/N 2085) can be added to each reaction and has been shown to produce adequate labeled cRNA yield within 4 hours. The two different incubation protocols generate comparable array results, and users are encouraged to choose the procedure that best fits their experimental schedule and process flow.

5. Store labeled cRNA at -20°C, or -70°C if not purifying immediately. Alternatively, proceed to *Cleanup and Quantification of Biotin-Labeled cRNA* on page 2.1.36.

Cleanup and Quantification of Biotin-Labeled cRNA

Sample Cleanup Module is used for cleaning up the 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 IVT 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 ≥ 8,000 x g (≥ 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 ≥ 8,000 x g (≥ 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 ≥ 8,000 x g (≥ 10,000 rpm). Discard flow-through.
7. Open the cap of the spin column and centrifuge for 5 minutes at maximum speed (≤ 25,000 x 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 counterclockwise direction). This avoids damage of the caps.

Recommendation *Label the collection tubes with the sample name. During centrifugation some column caps may break, resulting in loss of sample information.*

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 (≤ 25,000 x 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 (≤ 25,000 x 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.

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 µg/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:

adjusted cRNA yield = RNA_m - (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 *Fragmenting the cRNA for Target Preparation* on page 2.1.39.

✔ Note *Please refer to the 'Eukaryotic Target Hybridization' chapter in Section 2 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. The following are examples of typical cRNA products examined on an Agilent 2100 Bioanalyzer.

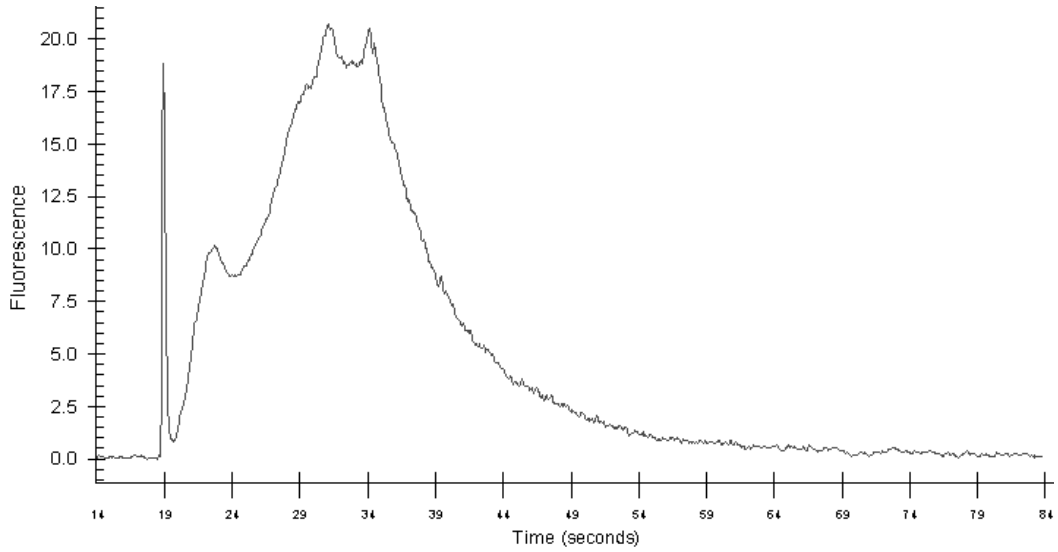

Figure 2.1.5
Biotin-labeled cRNA from One-Cycle cDNA Synthesis Kit. Bioanalyzer electropherogram for labeled cRNA from HeLa total RNA using the One-Cycle Kit. This electropherogram displays the nucleotide size distribution for 400 ng of labeled cRNA resulting from one round of amplification. The average size is approximately 1580 nt.

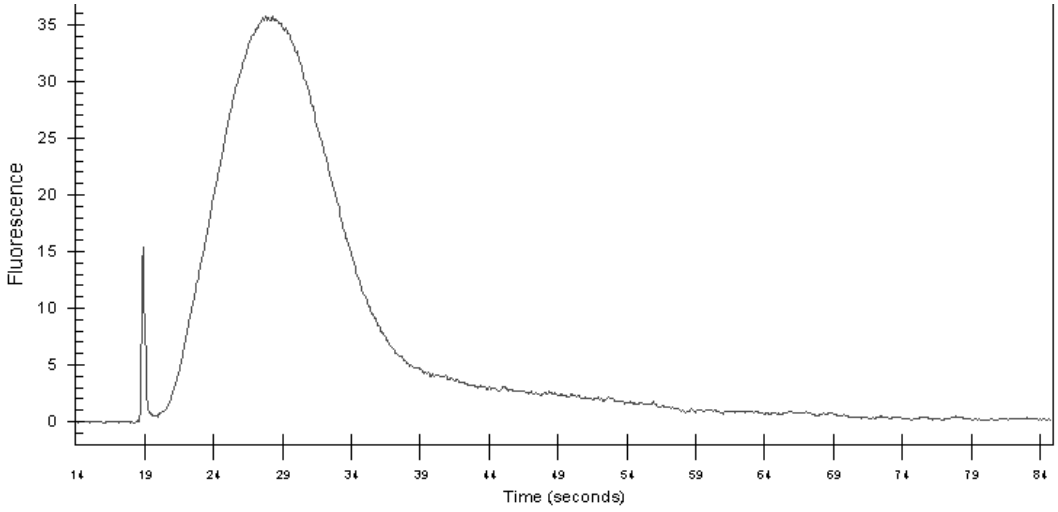

Figure 2.1.6
Biotin-labeled cRNA from Two-Cycle cDNA Synthesis Kit. Bioanalyzer electropherogram for labeled cRNA from HeLa total RNA using the Two-Cycle Kit. This electropherogram displays the nucleotide size distribution for 400 ng of labeled cRNA resulting from two rounds of amplification. The average size is approximately 850 nt.

Fragmenting the cRNA for Target Preparation

Sample Cleanup Module is used for this step.

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. Fragment an appropriate amount of cRNA for hybridization cocktail and gel analysis (refer to the *Eukaryotic Target Hybridization* chapter in Section 2).

1. The Fragmentation Buffer has been optimized to break down full-length cRNA to 35 to 200 base fragments by metal-induced hydrolysis.

   The following table shows suggested fragmentation reaction mix for cRNA samples at a final concentration of 0.5 µg/µL. Use **adjusted** cRNA concentration, as described in *Step 2: Quantification of the cRNA* on page 2.1.37. The total volume of the reaction may be scaled up or down dependent on the amount of cRNA to be fragmented.

Table 2.1.18
Sample Fragmentation Reaction by Array Format*

Component	*49/64 Format*	*100 Format*
cRNA	20 µg (1 to 21 µL)	15 µg (1 to 21 µL)
5X Fragmentation Buffer	8 µL	6 µL
RNase-free Water (variable)	to 40 µL final volume	to 30 µL final volume
Total Volume	*40 µL*	*30 µL*

*Please refer to specific probe array package insert for information on array format.

2. Incubate at 94°C for 35 minutes. Put on ice following the incubation.
3. Save an aliquot for analysis on the Bioanalyzer. A typical fragmented target is shown in Figure 2.1.7.

   The standard fragmentation procedure should produce a distribution of RNA fragment sizes from approximately 35 to 200 bases.
4. Store undiluted, fragmented sample RNA at -20°C until ready to perform the hybridization, as described in the *Eukaryotic Target Hybridization* chapter in Section 2.

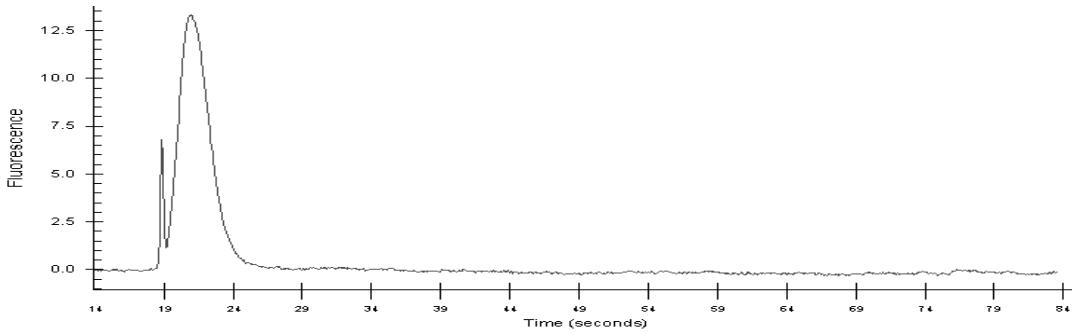

Figure 2.1.7
Fragmented cRNA. Bioanalyzer electropherogram for fragmented labeled cRNA from HeLa total RNA. This electropherogram displays the nucleotide size distribution for 150 ng of fragmented labeled cRNA resulting from one round of amplification. The average size is approximately 100 nt.

Alternative Protocol for One-Cycle cDNA Synthesis 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 Kit[3] 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 H_2O 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 2.1.19 and Table 2.1.20 for variable component calculations. Determine the volumes of RNA and SuperScript II RT required in Table 2.1.19, then calculate the amount of DEPC-treated H_2O needed in Step 1 Table 2.1.20 to bring the final First-Strand Synthesis volume to 20 µL.*

3. 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. 5,716,785, 5,891,636, 6,291,170, and 5,545,522 or to purchase another licensed kit.

Table 2.1.19
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 H_2O and SuperScript II RT should not exceed 11 µL as indicated in Table 2.1.20.

Table 2.1.20
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_2O (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 2.1.19	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 2.1.21).

Table 2.1.21
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 *E. coli* DNA Ligase	1 µL	10 U
10 U/µL *E. coli* DNA Polymerase I	4 µL	40 U
2 U/µL *E. coli* RNase H	1 µL	2 U
Final Volume	*150 µL*	

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 µL [10 U] T4 DNA Polymerase.
5. Return to 16°C for 5 minutes.
6. Add 10 µL 0.5M EDTA.
7. Proceed to cleanup procedure for cDNA, *Cleanup of Double-Stranded cDNA for Both the One-Cycle and Two-Cycle Target Labeling Assays* on page 2.1.32, or store at -20°C for later use.

Alternative Protocol for One-Cycle cDNA Synthesis 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 Kit[4] 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 H_2O 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 2.1.22 for variable component calculations. Determine volumes of mRNA and SuperScript II RT required, and then calculate the amount of DEPC-treated H_2O needed in the **Primer Hybridization Mix** step to bring the final First-Strand Synthesis reaction volume to 20 µL.*

4. 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. 5,716,785, 5,891,636, 6,291,170, and 5,545,522 or to purchase another licensed kit.

Table 2.1.22
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_2O (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

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 2.1.23).

Table 2.1.23
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 *E. coli* DNA Ligase	1 µL	10 U
10 U/µL *E. coli* DNA Polymerase I	4 µL	40 U
2 U/µL *E. coli* RNase H	1 µL	2 U
Final Volume	*150 µL*	

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 µL [10 U] T4 DNA Polymerase.
5. Return to 16°C for 5 minutes.
6. Add 10 µL 0.5M EDTA.
7. Proceed to cleanup procedure for cDNA, *Cleanup of Double-Stranded cDNA for Both the One-Cycle and Two-Cycle Target Labeling Assays* on page 2.1.32, or store at -20°C for later use.