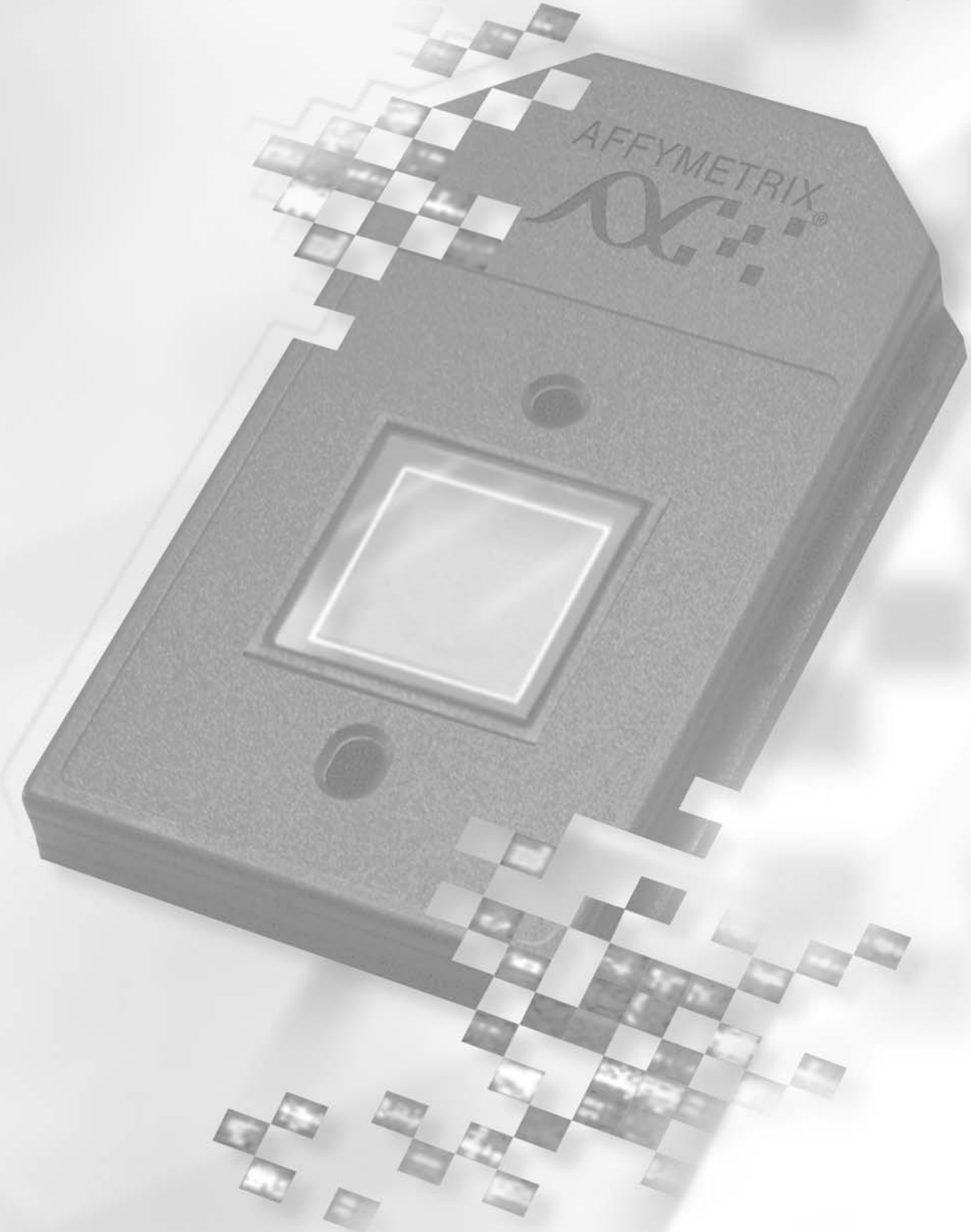
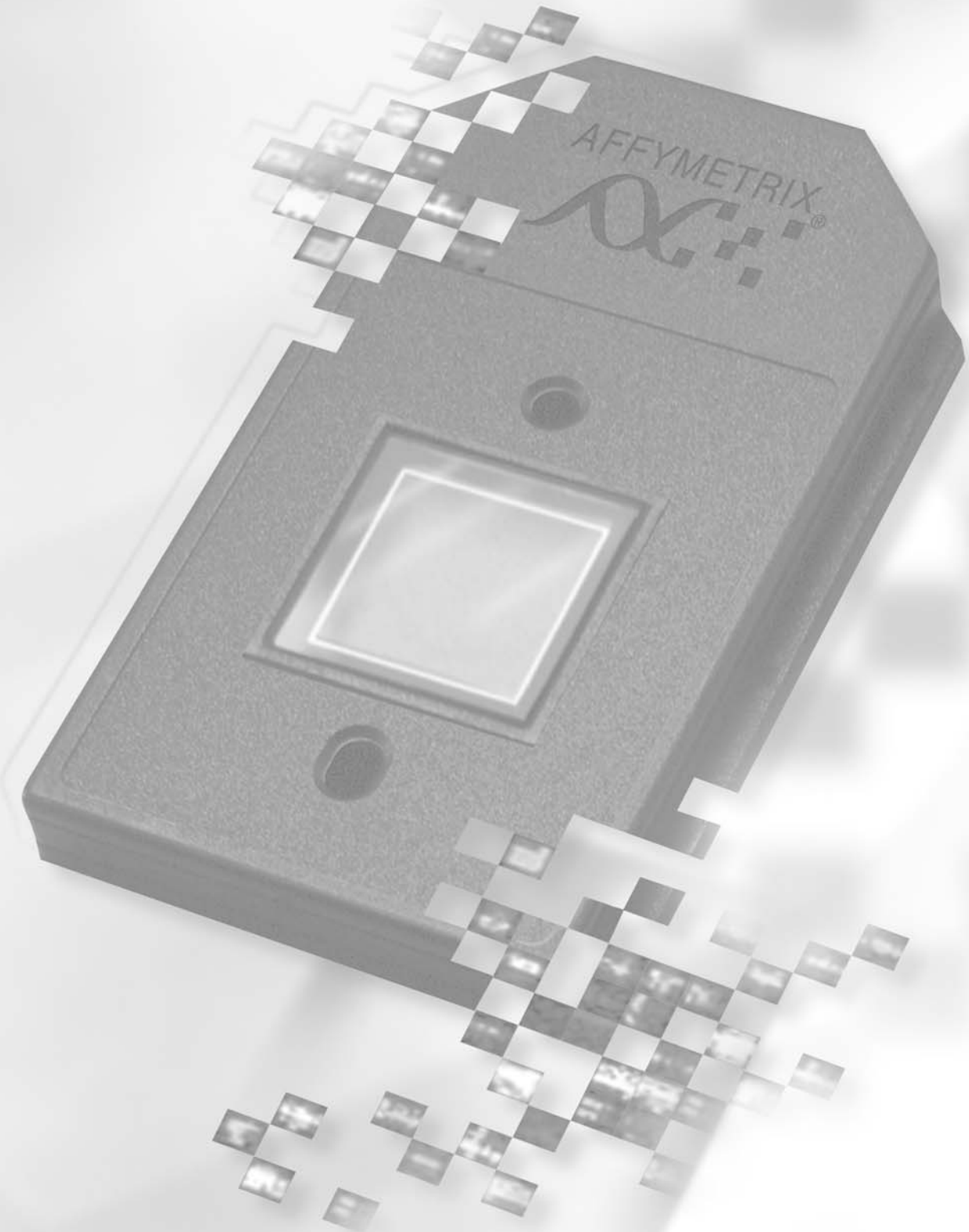
Section 2:

Eukaryotic Sample and Array Processing



Section 2



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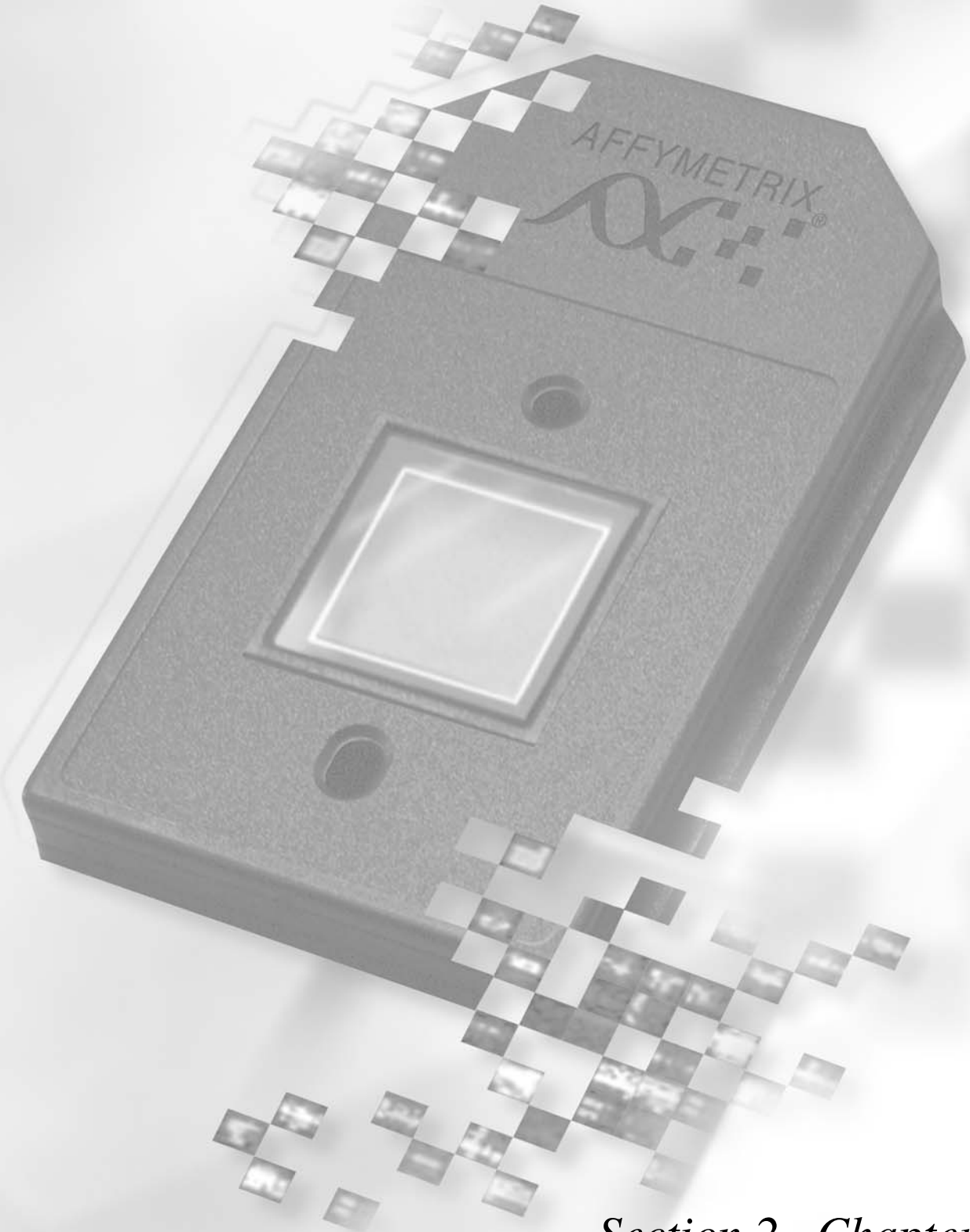

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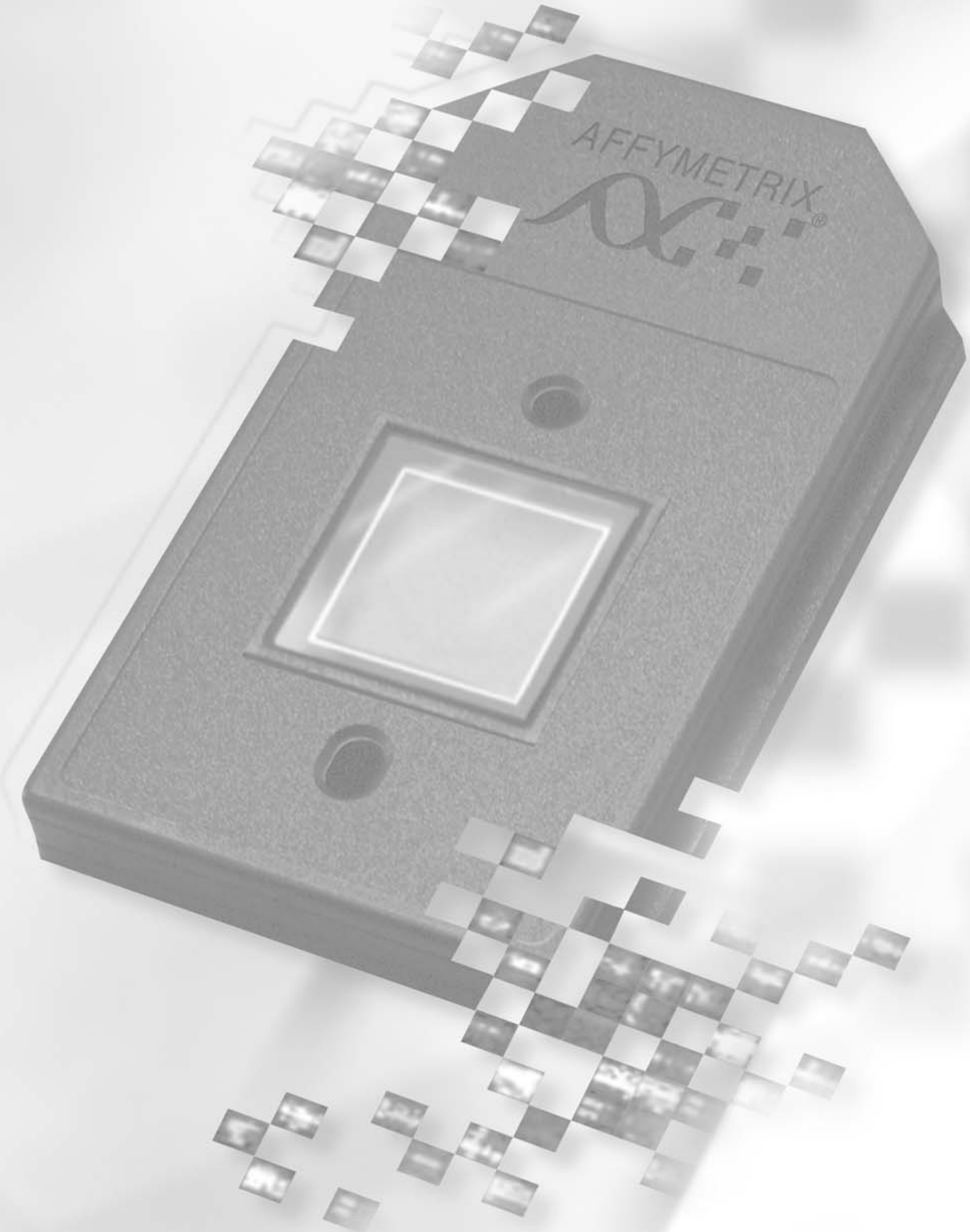


Eukaryotic Target Preparation

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.

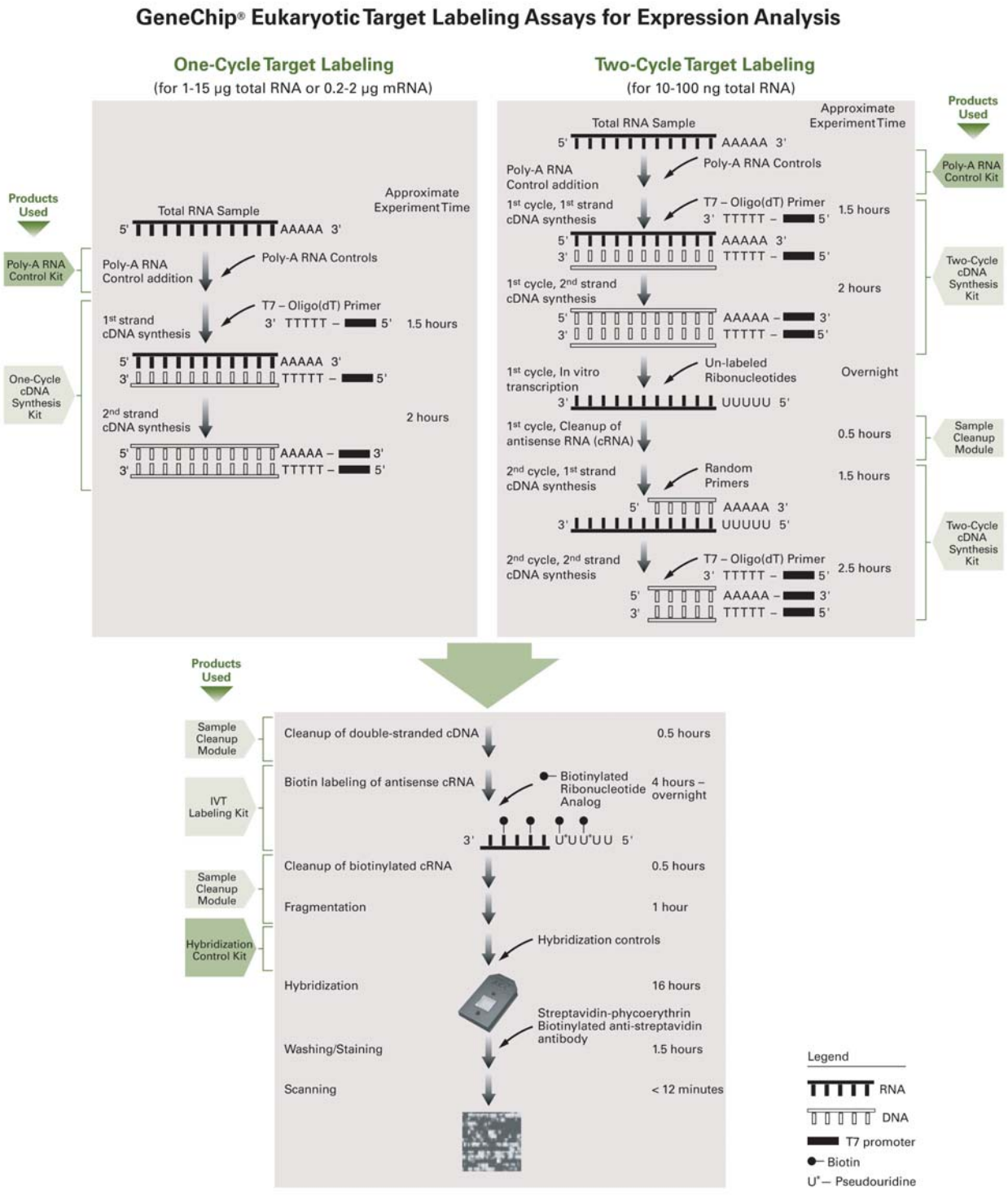


Figure 2.1.1
GeneChip Eukaryotic Labeling Assays for Expression Analysis

Reagents and Materials Required

The following reagents and materials are recommendations and have been tested and evaluated by Affymetrix scientists. Information and part numbers listed are based on U.S. catalog information. For supplier information, please refer to the Supplier Reference List in Appendix A of 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

- 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. Each of these components may be ordered individually (described below) as well as in this complete kit.
  Contains:
  - 1 IVT Labeling Kit (Affymetrix, P/N 900449)
  - 1 One-Cycle cDNA Synthesis Kit (Affymetrix, P/N 900431)
  - 1 Sample Cleanup Module (Affymetrix, P/N 900371)
  - 1 Poly-A RNA Control Kit (Affymetrix, P/N 900433)
  - 1 Hybridization Control Kit (Affymetrix, P/N 900454)

Two-Cycle Target Labeling

- Two-Cycle Target Labeling and Control Reagents, Affymetrix, P/N 900494
  A convenient package containing required labeling and control reagents to perform 30 two-cycle labeling reactions. Each of these components may be ordered individually (described below) as well as in this complete kit.
  Contains:
  - 1 IVT Labeling Kit (Affymetrix, P/N 900449)
  - 1 Two-Cycle cDNA Synthesis Kit (Affymetrix, P/N 900432)
  - 2 Sample Cleanup Modules (Affymetrix, P/N 900371)
  - 1 Poly-A RNA Control Kit (Affymetrix, P/N 900433)
  - 1 Hybridization Control Kit (Affymetrix, P/N 900454)
- MEGAscript® High Yield Transcription Kit, Ambion Inc, P/N 1334

Miscellaneous Reagents

- 10X TBE, Cambrex, P/N 50843
- 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 has 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.

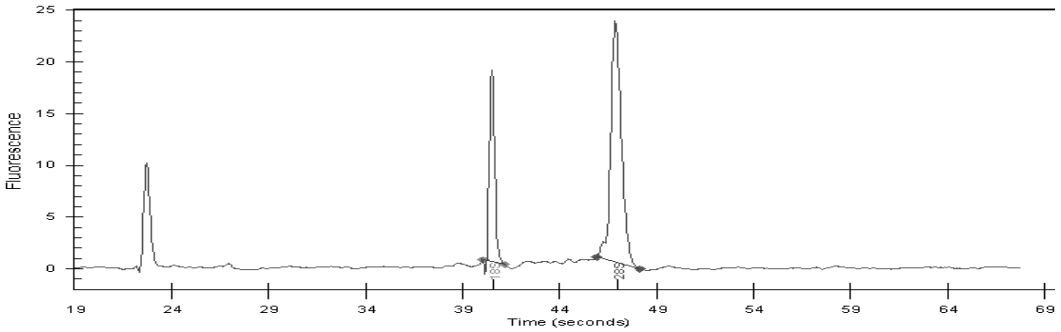


Figure 2.1.2
Electropherogram (from the Agilent 2100 Bioanalyzer) for HeLa Total RNA. For a high-quality total RNA sample, two well-defined peaks corresponding to the 18S and 28S ribosomal RNAs should be observed, similar to a denaturing agarose gel, with ratios approaching 2:1 for the 28S to 18S bands.

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	1:6,667

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.

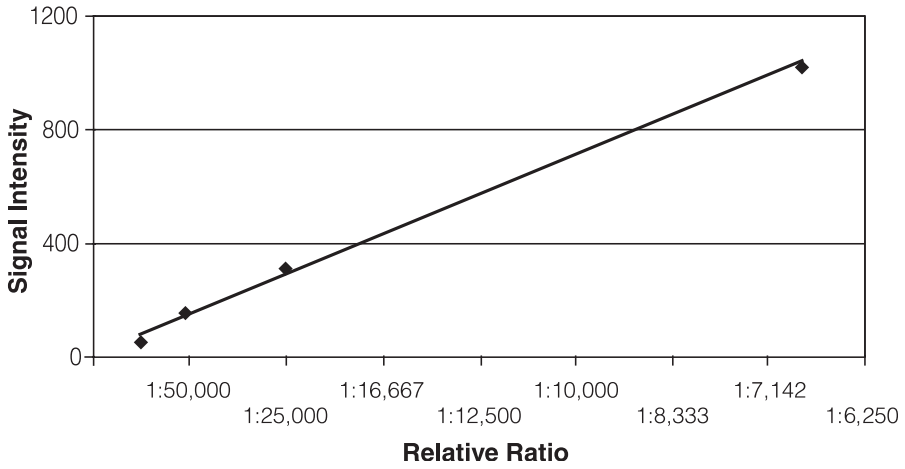


Figure 2.1.3
Poly-A RNA spikes amplified using 5 µg of a complex human HeLa total RNA sample.
Labeled target was hybridized onto a GeneChip® Human Genome U133A 2.0 Array. Signal intensities of the 3' AFFX-r2-Bs probe set of the control transcripts were obtained using GeneChip Operating Software version 1.1.1 and the average values from the triplicate experiments were plotted.

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	1:6,667

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.

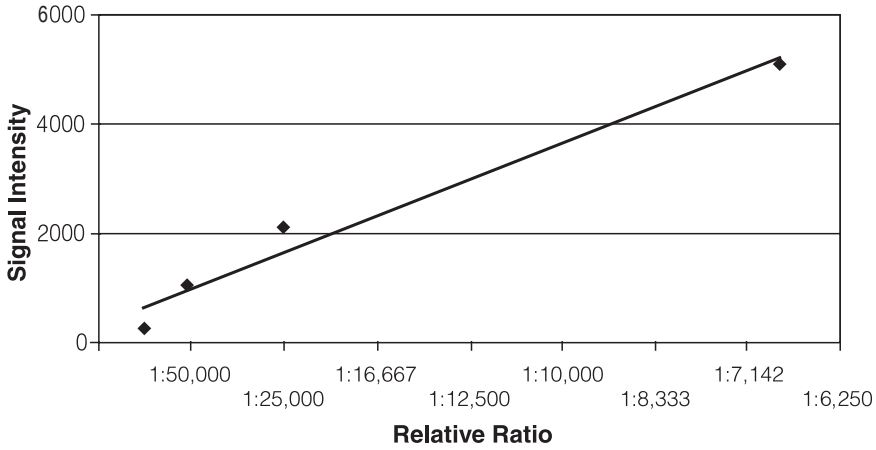


Figure 2.1.4
Poly-A RNA spikes amplified using 10 ng of a complex human brain total RNA sample.
Labeled target was hybridized onto a GeneChip® Human Genome U133 Plus 2.0 Array. Signal intensities of the 3' AFFX-r2-Bs probe set of the control transcripts were obtained using GeneChip Operating Software version 1.1.1 and the average values from the triplicate experiments were plotted.

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 or spectrophotometric quantitation for cDNA prepared from total RNA. This is due to the presence of other nucleic acid species in the sample that can interfere with the results.

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 for this step.

✔ 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 2.1.16)
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.
10. Store cRNA at -20°C, or -70°C if not quantitated immediately. Alternatively, proceed to *Step 2: Quantification of the cRNA*.

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:

$$\text{adjusted cRNA yield} = RNA_m - (\text{total } RNA_i)\ (y)$$

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 *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.

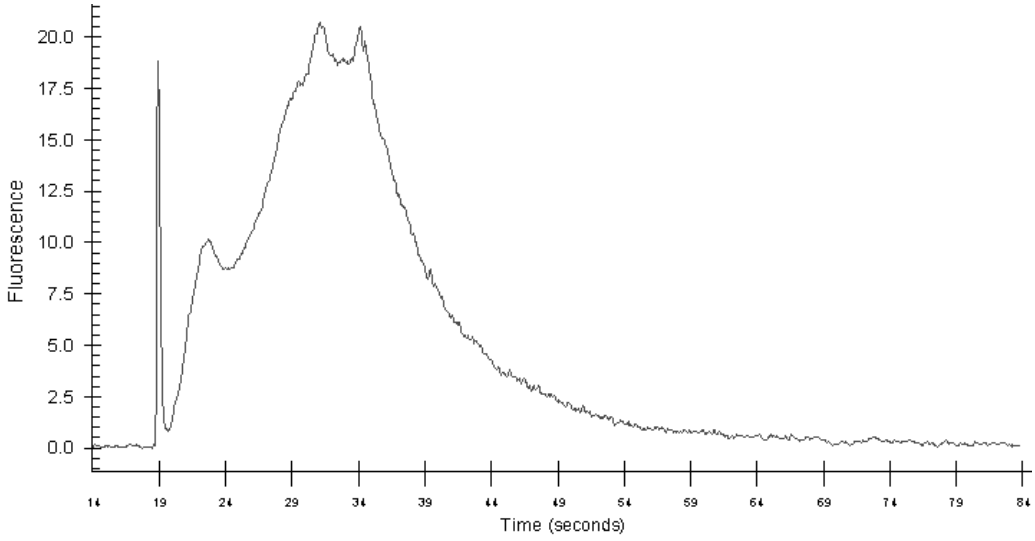


Figure 2.1.5
Biotin-labeled cRNA from One-Cycle cDNA Synthesis Kit. Bioanalyzer electropherogram for labeled cRNA from HeLa total RNA using the One-Cycle Kit. This electropherogram displays the nucleotide size distribution for 400 ng of labeled cRNA resulting from one round of amplification. The average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

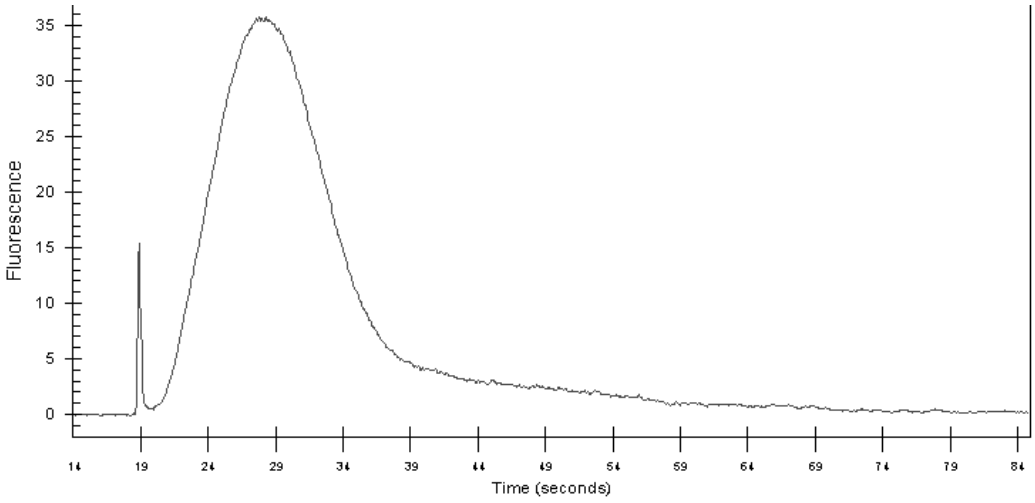


Figure 2.1.6
Biotin-labeled cRNA from Two-Cycle cDNA Synthesis Kit. Bioanalyzer electropherogram for labeled cRNA from HeLa total RNA using the Two-Cycle Kit. This electropherogram displays the nucleotide size distribution for 400 ng of labeled cRNA resulting from two rounds of amplification. The average 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 preparation 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 cRNA at -20°C (or -70°C for longer-term storage) until ready to perform the hybridization, as described in the *Eukaryotic Target Hybridization* chapter in Section 2.

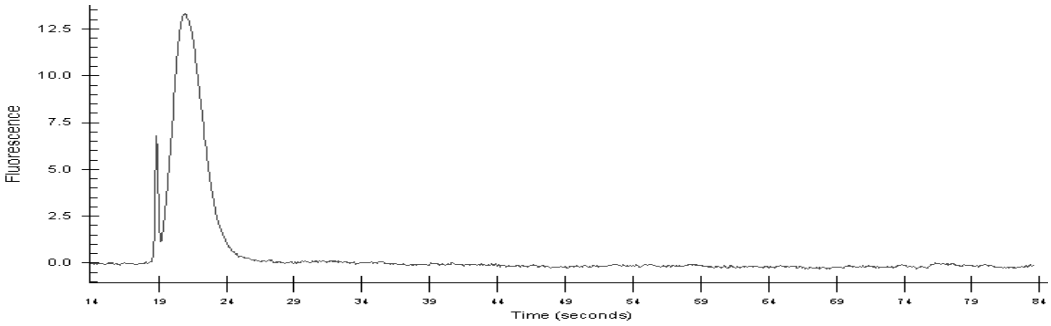


Figure 2.1.7
Fragmented cRNA. Bioanalyzer electropherogram for fragmented labeled cRNA from HeLa total RNA. This electropherogram displays the nucleotide size distribution for 150 ng of fragmented labeled cRNA resulting from one round of amplification. The average size is approximately 100 nt.

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

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), 200 U/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 0.1M 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	200U to 600U
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	10U
10 U/µL *E. coli* DNA Polymerase I	4 µL	40U
2 U/µL *E. coli* RNase H	1 µL	2U
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 [10U] 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 0.1M 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	200U to 400U
Total Volume		**20 µL**	

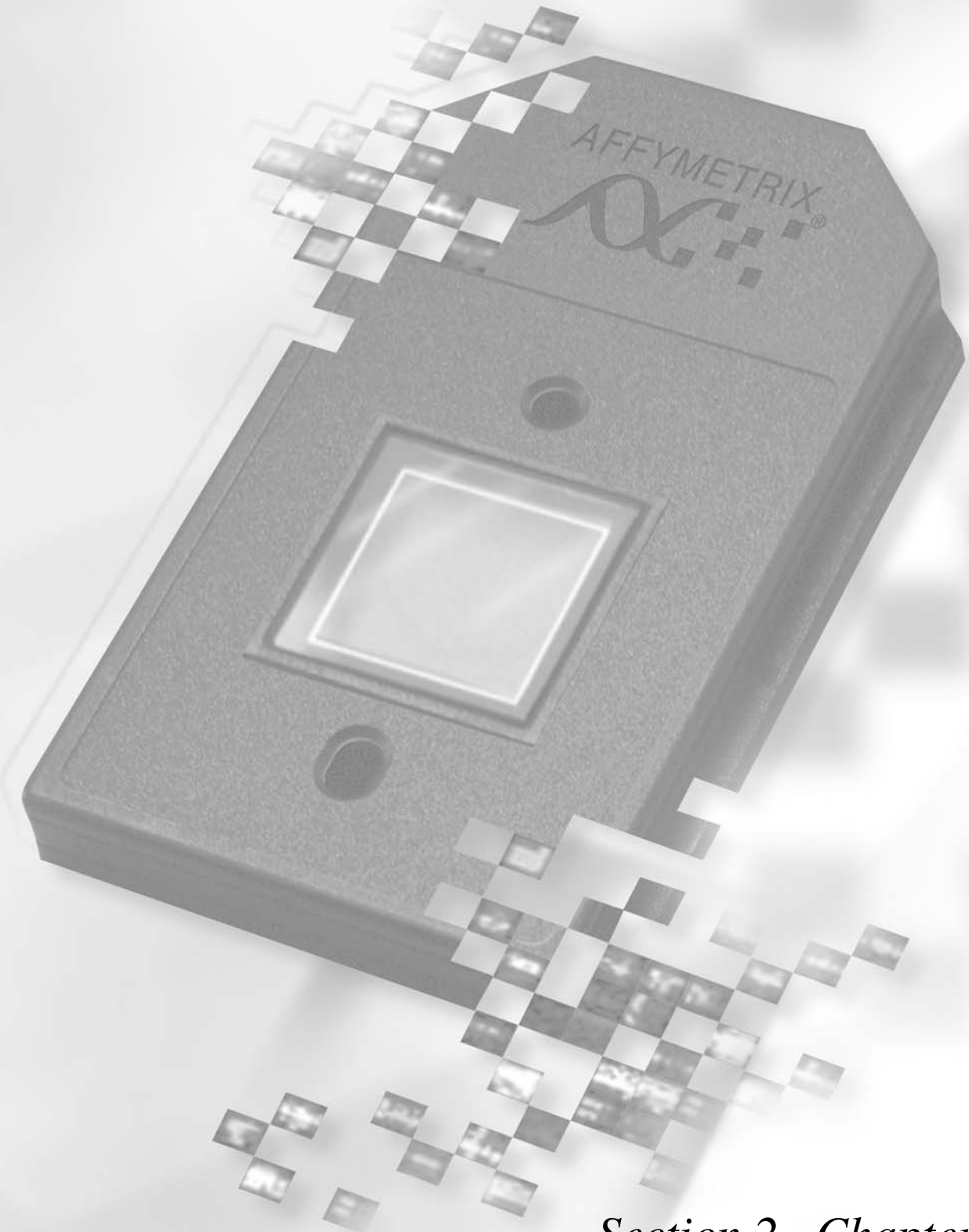
Step 2: Second-Strand cDNA Synthesis

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	10U
10 U/µL *E. coli* DNA Polymerase I	4 µL	40U
2 U/µL *E. coli* RNase H	1 µL	2U
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.



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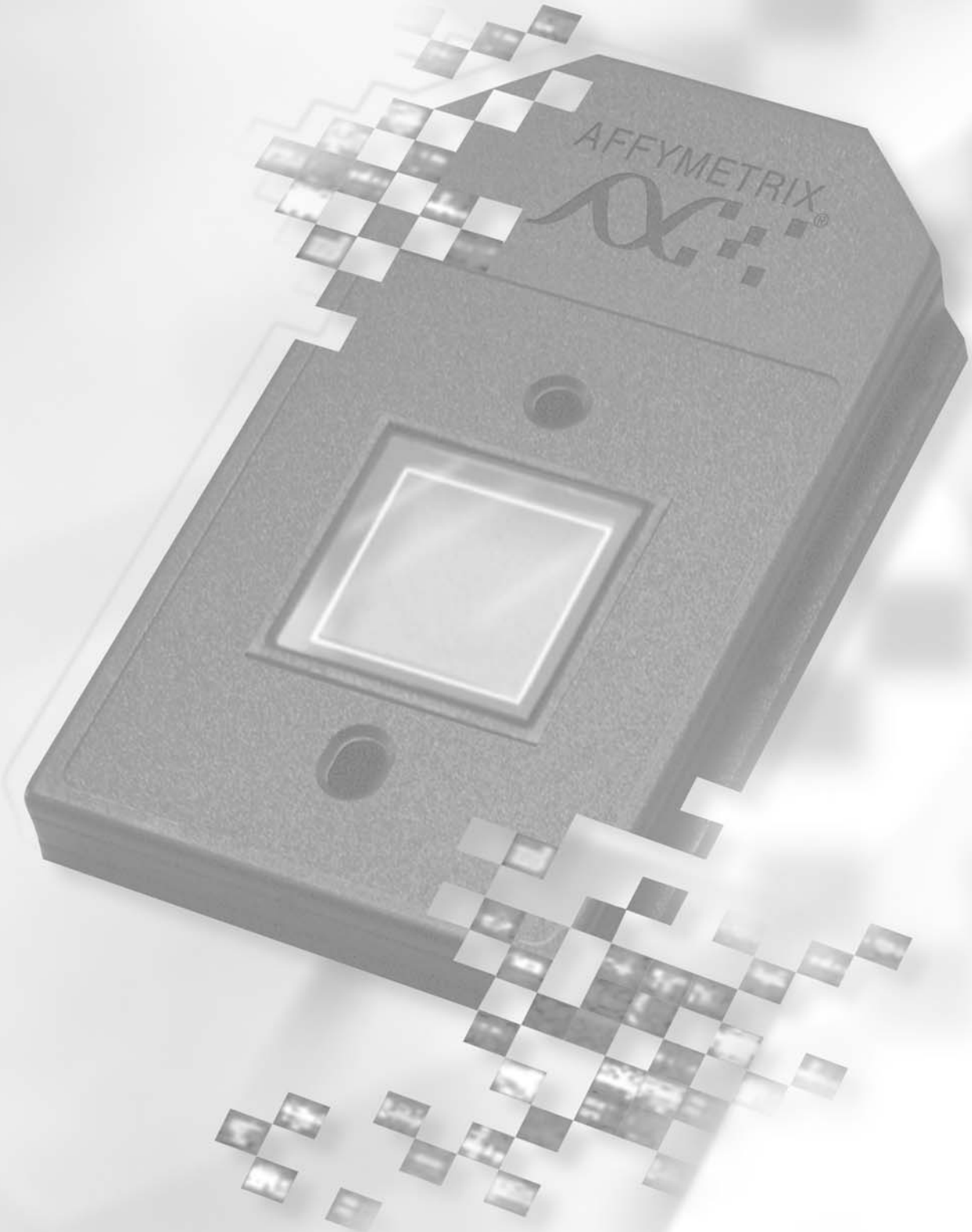


Eukaryotic Target Hybridization

This Chapter Contains:

- Detailed steps for preparing the eukaryotic hybridization mix containing labeled target and control cRNA.
- Instructions for hybridizing the target mix to a eukaryotic GeneChip® probe array.

After completing the procedures described in this chapter, the hybridized probe array is ready for washing, staining, and scanning, as detailed in Section 2, Chapter 3.

Reagents and Materials Required

The following reagents and materials are recommendations and have been tested and evaluated by Affymetrix scientists. Information and part numbers listed are based on U.S. catalog information. For supplier information, please refer to the Supplier Reference List in Appendix A of this manual.

- Water, Molecular Biology Grade, BioWhittaker Molecular Applications / Cambrex, P/N 51200
- Bovine Serum Albumin (BSA) solution (50 mg/mL), Invitrogen Life Technologies, P/N 15561-020
- Herring Sperm DNA, Promega Corporation, P/N D1811
- GeneChip Eukaryotic Hybridization Control Kit, Affymetrix, P/N 900454 (30 reactions) or P/N 900457 (150 reactions), contains Control cRNA and Control Oligo B2
- Control Oligo B2, 3 nM, Affymetrix, P/N 900301 (can be ordered separately)
- 5M NaCl, RNase-free, DNase-free, Ambion, P/N 9760G
- MES hydrate SigmaUltra, Sigma-Aldrich, P/N M5287
- MES Sodium Salt, Sigma-Aldrich, P/N M5057
- EDTA Disodium Salt, 0.5M solution (100 mL), Sigma-Aldrich, P/N E7889
- DMSO, Sigma-Aldrich, P/N D5879
- Surfact-Amps 20 (Tween-20), 10%, Pierce Chemical, P/N 28320

Miscellaneous Supplies

- Hybridization Oven 640, Affymetrix, P/N 800138 (110V) or 800139 (220V)
- 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 pipette tips and non-barrier pipette tips
- Heatblock

Reagent Preparation

12X MES Stock Buffer

(1.22M MES, 0.89M [Na^+])

For 1,000 mL:

64.61g of MES hydrate
193.3g of MES Sodium Salt
800 mL of Molecular Biology Grade water
Mix and adjust volume to 1,000 mL.
The pH should be between 6.5 and 6.7. Filter through a 0.2 µm filter.

IMPORTANT

Do not autoclave. Store at 2°C to 8°C, and shield from light.
Discard solution if yellow.

2X Hybridization Buffer

(Final 1X concentration is 100 mM MES, 1M [Na^+], 20 mM EDTA, 0.01% Tween-20)

For 50 mL:

8.3 mL of 12X MES Stock Buffer
17.7 mL of 5M NaCl
4.0 mL of 0.5M EDTA
0.1 mL of 10% Tween-20
19.9 mL of water
Store at 2°C to 8°C, and shield from light

Eukaryotic Target Hybridization

Please refer to the table below for the necessary amount of cRNA required for appropriate probe array format. These recipes take into account that it is necessary to make extra hybridization cocktail due to a small loss of volume (10-20 µL) during each hybridization.

1. Mix the following for each target, scaling up volumes for hybridization to multiple probe arrays.

IMPORTANT *If using the GeneChip IVT Labeling Kit to prepare the target, a final concentration of 10% DMSO needs to be added in the hybridization cocktail for optimal results.*

Table 2.2.1
Hybridization Cocktail for Single Probe Array*

Component	49 Format (Standard) / 64 Format Array	100 Format (Midi) Array	169 Format (Mini) Array / 400 Format (Micro) Array	Final Concentration
Fragmented cRNA **	15 µg	10 µg	5 µg	0.05 µg/µL
Control Oligonucleotide B2 (3 nM)	5 µL	3.3 µL	1.7 µL	50 pM
20X Eukaryotic Hybridization Controls (*bioB*, *bioC*, *bioD*, *cre*)	15 µL	10 µL	5 µL	1.5, 5, 25, and 100 pM respectively
Herring Sperm DNA (10 mg/mL)	3 µL	2 µL	1 µL	0.1 mg/mL
BSA (50 mg/mL)	3 µL	2 µL	1 µL	0.5 mg/mL
2X Hybridization Buffer	150 µL	100 µL	50 µL	1X
DMSO* **	30 µL	20 µL	10 µL	10%
H_2O	to final volume of 300 µL	to final volume of 200 µL	to final volume of 100 µL	
Final volume	**300 µL**	**200 µL**	**100 µL**	

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

**Please see Section 2, Chapter 1, for amount of adjusted fragmented cRNA to use when starting from total RNA.

*** Note that the addition of DMSO is different from previous recommendations. Follow this protocol for best results on arrays when using the GeneChip IVT Labeling Kit.

IMPORTANT *It is imperative that frozen stocks of 20X GeneChip Eukaryotic Hybridization Controls are heated to 65°C for 5 minutes to completely resuspend the cRNA before aliquotting.*

2. Equilibrate probe array to room temperature immediately before use.

Note *It is important to allow the arrays to equilibrate to room temperature completely. Specifically, if the rubber septa are not equilibrated to room temperature, they may be prone to cracking, which can lead to leaks.*

3. Heat the hybridization cocktail to 99°C for 5 minutes in a heat block.

4. Meanwhile, wet the array by filling it through one of the septa (see Figure 2.2.1 for location of the probe array septa) with appropriate volume of 1X Hybridization Buffer using a micropipettor and appropriate tips (Table 2.2.2).

✔ Note

It is necessary to use two pipette tips when filling the probe array cartridge: one for filling and the second to allow venting of air from the hybridization chamber.

5. Incubate the probe array filled with 1X Hybridization Buffer at 45°C for 10 minutes with rotation.

Table 2.2.2
Probe Array Cartridge Volumes

Array	Hybridization Volume	Total Fill Volume
49 Format (Standard)	200 µL	250 µL
64 Format	200 µL	250 µL
100 Format (Midi)	130 µL	160 µL
169 Format (Mini)	80 µL	100 µL
400 Format (Micro)	80 µL	100 µL

6. Transfer the hybridization cocktail that has been heated at 99°C, in step 3, to a 45°C heat block for 5 minutes.
7. Spin hybridization cocktail(s) at maximum speed in a microcentrifuge for 5 minutes to remove any insoluble material from the hybridization mixture.
8. Remove the buffer solution from the probe array cartridge and fill with appropriate volume (Table 2.2.2) of the clarified hybridization cocktail, avoiding any insoluble matter at the bottom of the tube.
9. Place probe array into the Hybridization Oven, set to 45°C.

   Avoid stress to the motor; load probe arrays in a balanced configuration around the axis. Rotate at 60 rpm.
10. Hybridize for 16 hours.

    During the latter part of the 16-hour hybridization, proceed to Section 2, Chapter 3 to prepare reagents required immediately after completion of hybridization.

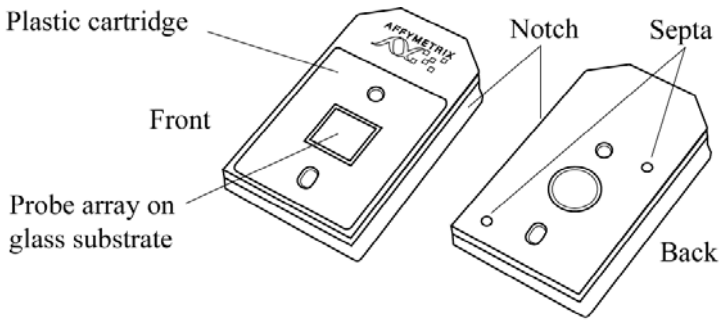
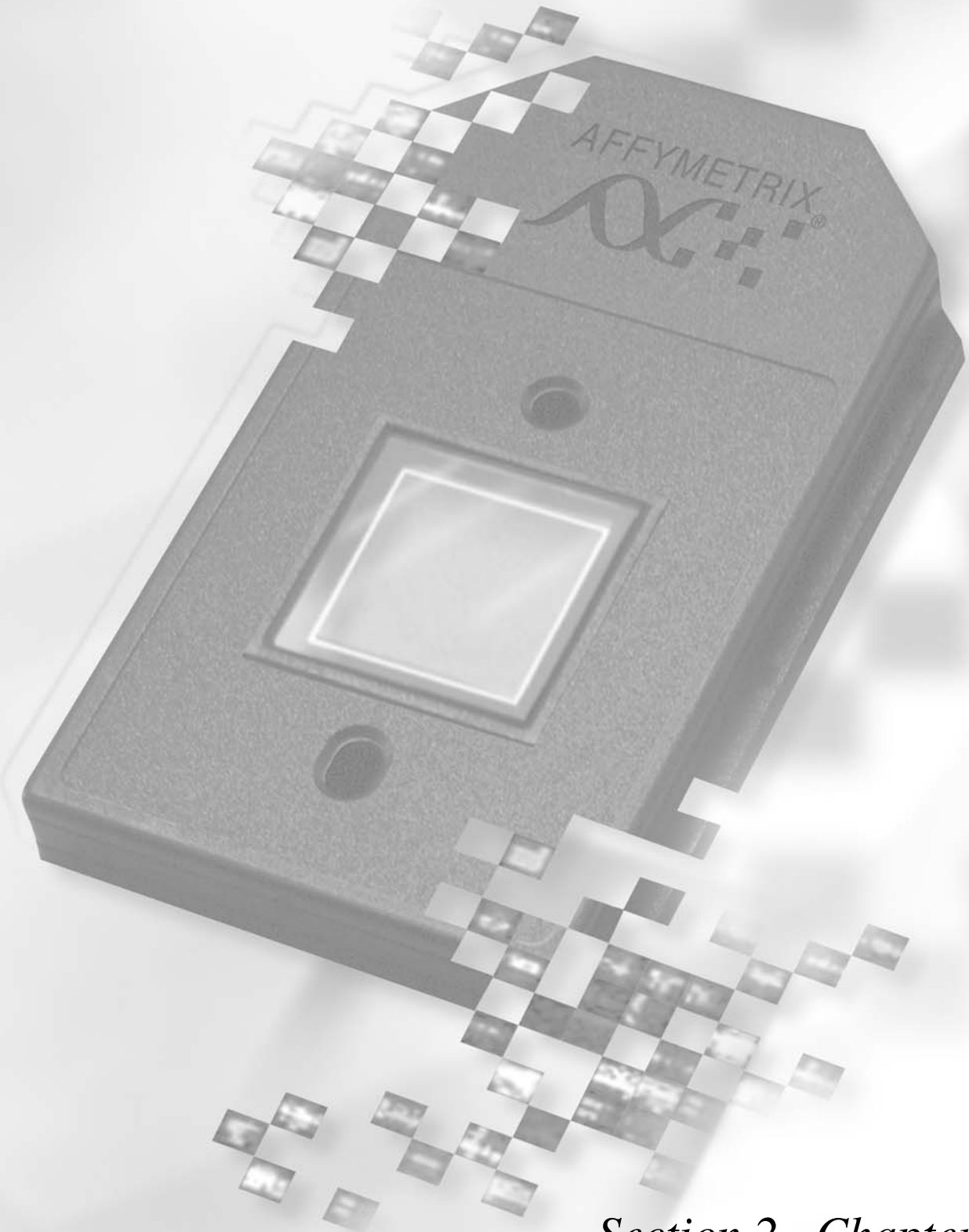


Figure 2.2.1
GeneChip® Probe Array



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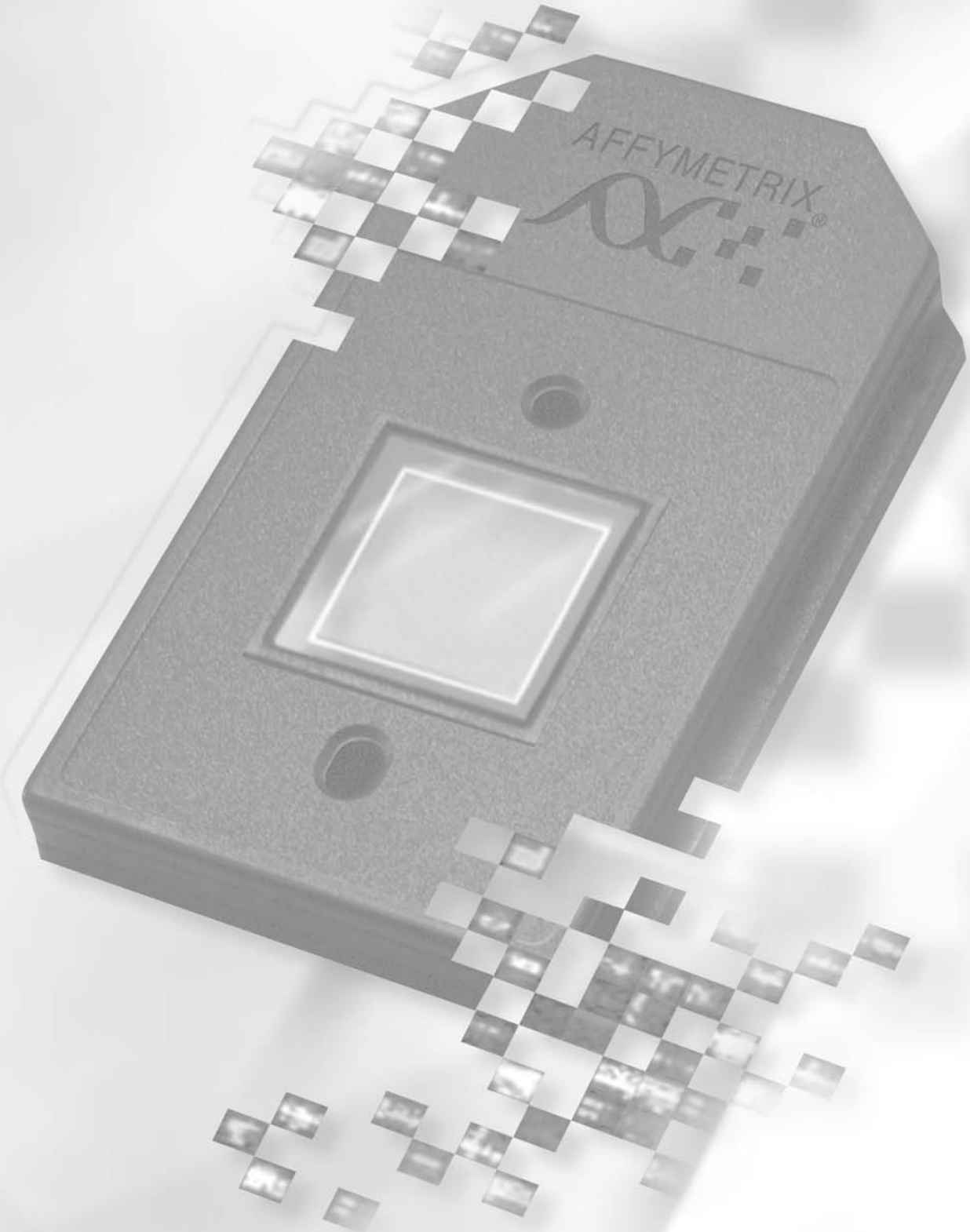


Eukaryotic Arrays: Washing, Staining, and Scanning

This Chapter Contains:

- Instructions for using the Fluidics Station 400 and 450/250 to automate the washing and staining of eukaryotic GeneChip® expression probe arrays.
- Instructions for scanning probe arrays using the GeneArray® Scanner or the GeneChip® Scanner 3000.

After completing the procedures described in this chapter, the scanned probe array image (.dat file) is ready for analysis, as explained in the enclosed *GeneChip Expression Analysis: Data Analysis Fundamentals* booklet (P/N 701190).

Reagents and Materials Required

The following reagents and materials are recommendations and have been tested and evaluated by Affymetrix scientists. Information and part numbers listed are based on U.S. catalog information. For supplier information, please refer to the Supplier Reference List in Appendix A of this manual.

- Water, Molecular Biology Grade, BioWhittaker Molecular Applications / Cambrex, P/N 51200
- Distilled water, Invitrogen Life Technologies, P/N 15230-147
- Bovine Serum Albumin (BSA) solution (50 mg/mL), Invitrogen Life Technologies, P/N 15561-020
- R-Phycoerythrin Streptavidin, Molecular Probes, P/N S-866
- 5M NaCl, RNase-free, DNase-free, Ambion, P/N 9760G
- PBS, pH 7.2, Invitrogen Life Technologies, P/N 20012-027
- 20X SSPE (3M NaCl, 0.2M NaH_2PO_4, 0.02M EDTA), BioWhittaker Molecular Applications / Cambrex, P/N 51214
- Goat IgG, Reagent Grade, Sigma-Aldrich, P/N I 5256
- Anti-streptavidin antibody (goat), biotinylated, Vector Laboratories, P/N BA-0500
- Surfact-Amps 20 (Tween-20), 10%, Pierce Chemical, P/N 28320

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 pipette tips and non-barrier pipette tips
- Tygon Tubing, 0.04″ inner diameter, Cole-Parmer, P/N H-06418-04
- Tough-Spots, Label Dots, USA Scientific, P/N 9185-0000

Reagent Preparation

Wash Buffer A: Non-Stringent Wash Buffer

(6X SSPE, 0.01% Tween-20)

For 1,000 mL:
300 mL of 20X SSPE
1.0 mL of 10% Tween-20
699 mL of water
Filter through a 0.2 µm filter

Wash Buffer B: Stringent Wash Buffer

(100 mM MES, 0.1M [Na^+], 0.01% Tween-20)

For 1,000 mL:
83.3 mL of 12X MES Stock Buffer (see Section 2, Chapter 2 for reagent preparation)
5.2 mL of 5M NaCl
1.0 mL of 10% Tween-20
910.5 mL of water
Filter through a 0.2 µm filter
Store at 2°C to 8°C and shield from light

2X Stain Buffer

(Final 1X concentration: 100 mM MES, 1M [Na^+], 0.05% Tween-20)

For 250 mL:
41.7 mL of 12X MES Stock Buffer (see Section 2, Chapter 2 for reagent preparation)
92.5 mL of 5M NaCl
2.5 mL of 10% Tween-20
113.3 mL of water
Filter through a 0.2 µm filter
Store at 2°C to 8°C and shield from light

10 mg/mL Goat IgG Stock

Resuspend 50 mg in 5 mL of 150 mM NaCl
Store at 4°C

✔ Note

If a larger volume of the 10 mg/mL IgG stock is prepared, aliquot and store at -20°C until use. After the solution has been thawed it should be stored at 4°C. Avoid additional freezing and thawing.

Experiment and Fluidics Station Setup

Step 1: Defining File Locations

Before working with Affymetrix® Microarray Suite, it is important to define where the program stores and looks for files.

✔ Note

For GeneChip® Operating Software (GCOS), this step is not necessary. Proceed directly to Step 2: Entering Experiment Information.

1. Launch Microarray Suite from the workstation and select **Tools → Defaults → File Locations** from the menu bar.
2. The File Locations window displays the locations of the following files:
   - Probe Information (library files, mask files)
   - Fluidics Protocols (fluidics station scripts)
   - Experiment Data (.exp, .dat, .cel, and .chp files are all saved to location selected here)
3. Verify that all three file locations are set correctly and click **OK**.

   Contact Affymetrix Technical Support if you have any questions regarding this procedure.

Step 2: Entering Experiment Information

To wash, stain, and scan a probe array, an experiment must first be registered in GCOS or Microarray Suite. Please follow the instructions detailed in the "*Setting Up an Experiment*" section of the appropriate GCOS or Microarray Suite User's Guide.

The fields of information required for registering experiments in Microarray Suite are:

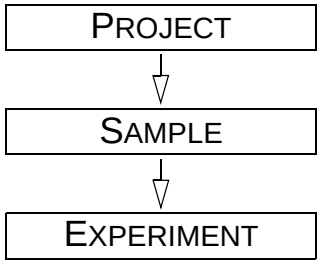
- Experiment Name
- Probe Array Type

In GCOS, three additional fields are required:

- Sample Name
- Sample Type
- Project

Sample templates, Experiment templates, and array barcodes can also be employed in GCOS to standardize and simplify the registration process. Please see the GCOS User's Guide for more information.

The Project, Sample Name, and Experiment Name fields establish a sample hierarchy that organizes GeneChip gene expression data in GCOS. In terms of the organizational structure, the Project is at the top of the hierarchy, followed by Sample Name, and then Experiment Name.



Step 3: Preparing the Fluidics Station

The Fluidics Station 400, or 450/250 is used to wash and stain the probe arrays. It is operated using GCOS/Microarray Suite.

Setting Up the Fluidics Station

1. Turn on the Fluidics Station using the toggle switch on the lower left side of the machine.
2. Select **Run** → **Fluidics** from the menu bar.
   ⇒ The Fluidics Station dialog box appears with a drop-down list for selecting the experiment name for each of the fluidics station modules. A second drop-down list is accessed for choosing the Protocol for each of the fluidics station modules.

✔ Note

Refer to the Fluidics Station User's Guide for instructions on connecting and addressing multiple fluidics stations.

Priming the Fluidics Station

Priming ensures that the lines of the fluidics station are filled with the appropriate buffers and the fluidics station is ready for running fluidics station protocols.

Priming should be done:

- when the fluidics station is first started.
- when wash solutions are changed.
- before washing, if a shutdown has been performed.
- if the LCD window instructs the user to prime.

1. To prime the fluidics station, select **Protocol** in the Fluidics Station dialog box.
2. Choose **Prime** or **Prime_450** for the respective modules in the Protocol drop-down list.
3. Change the intake buffer reservoir A to **Non-Stringent Wash Buffer**, and intake buffer reservoir B to **Stringent Wash Buffer**.
4. For MAS, click **Run** for each module to begin priming. In GCOS, select the **All Modules** check box, then click **Run**.

Probe Array Wash and Stain

After 16 hours of hybridization, remove the hybridization cocktail from the probe array and fill the probe array completely with the appropriate volume of Non-Stringent Wash Buffer (Wash Buffer A), as given in Table 2.2.2 on page 2.2.8.

✔ Note

If necessary, at this point, the probe array can be stored at 4°C for up to 3 hours before proceeding with washing and staining. Equilibrate the probe array to room temperature before washing and staining.

This protocol is recommended for use with probe arrays with probe cells of 24 µm or smaller. This procedure takes approximately 90 minutes to complete.

Preparing the Staining Reagents

Prepare the following reagents. Volumes given are sufficient for one probe array.

SAPE Stain Solution

Streptavidin Phycoerythrin (SAPE) should be stored in the dark at 4°C, either foil-wrapped or kept in an amber tube. Remove SAPE from the refrigerator and tap the tube to mix well before preparing stain solution. Do not freeze SAPE. Always prepare the SAPE stain solution fresh, on the day of use.

Table 2.3.1
SAPE Solution Mix

Components	Volume	Final Concentration
2X Stain Buffer	600.0 µL	1X
50 mg/mL BSA	48.0 µL	2 mg/mL
1 mg/mL Streptavidin Phycoerythrin (SAPE)	12.0 µL	10 µg/mL
DI H_20	540.0 µL	—
Total Volume	**1200 µL**	

Mix well and divide into two aliquots of 600 µL each to be used for stains 1 and 3.

Antibody Solution

Table 2.3.2
Antibody Solution Mix

Components	Volume	Final Concentration
2X Stain Buffer	300.0 µL	1X
50 mg/mL BSA	24.0 µL	2 mg/mL
10 mg/mL Goat IgG Stock	6.0 µL	0.1 mg/mL
0.5 mg/mL biotinylated antibody	3.6 µL	3 µg/mL
DI H_20	266.4 µL	—
Total Volume	**600 µL**	

Table 2.3.3
Fluidics Scripts for 11 µm Feature Size Eukaryotic Arrays*

Format	49	64	100	169	400
Using GeneChip® IVT Labeling Kit	EukGE-WS2v5	EukGE-WS2v5	Midi_euk2v3	Mini_euk2v3	Micro_1v1
Using all other labeling kits	EukGE-WS2v4	EukGE-WS2v4	Midi_euk2v3	Mini_euk2v3	Micro_1v1

* When using the Fluidics Station 450 or 250, add _450 at the end of the fluidics script's name.

Table 2.3.4
Fluidics Scripts for ≥ 18 µm Feature Size Eukaryotic Arrays*

Format	49	64	100	169	400
Using GeneChip® IVT Labeling Kit	EukGE-WS2v4	EukGE-WS2v4	Midi_euk2v3	Mini_euk2v3	Micro_1v1
Using all other labeling kits	EukGE-WS2v4	EukGE-WS2v4	Midi_euk2v3	Mini_euk2v3	Micro_1v1

* When using the Fluidics Station 450 or 250, add _450 at the end of the fluidics script's name.

Table 2.3.5
Fluidics Protocols - Antibody Amplification for Eukaryotic Targets
(protocols for the Fluidics Station 450/250 will have _450 as a suffix).

	EukGE-WS2v4*	EukGE-WS2v5* Midi_euk2*	Micro_1* Mini_euk2*
Post Hyb Wash #1	10 cycles of 2 mixes/cycle with Wash Buffer A at 25°C	10 cycles of 2 mixes/cycle with Wash Buffer A at 30°C	10 cycles of 2 mixes/cycle with Wash Buffer A at 25°C
Post Hyb Wash #2	4 cycles of 15 mixes/cycle with Wash Buffer B at 50°C	6 cycles of 15 mixes/cycle with Wash Buffer B at 50°C	8 cycles of 15 mixes/cycle with Wash Buffer B at 50°C
Stain	Stain the probe array for 10 minutes in SAPE solution at 25°C	Stain the probe array for 5 minutes in SAPE solution at 35°C	Stain the probe array for 10 minutes in SAPE solution at 25°C
Post Stain Wash	10 cycles of 4 mixes/cycle with Wash Buffer A at 25°C	10 cycles of 4 mixes/cycle with Wash Buffer A at 30°C	10 cycles of 4 mixes/cycle with Wash Buffer A at 30°C
2nd Stain	Stain the probe array for 10 minutes in antibody solution at 25°C	Stain the probe array for 5 minutes in antibody solution at 35°C	Stain the probe array for 10 minutes in antibody solution at 25°C
3rd Stain	Stain the probe array for 10 minutes in SAPE solution at 25°C	Stain the probe array for 5 minutes in SAPE solution at 35°C	Stain the probe array for 10 minutes in SAPE solution at 25°C
Final Wash	15 cycles of 4 mixes/cycle with Wash Buffer A at 30°C. The holding temperature is 25°C	15 cycles of 4 mixes/cycle with Wash Buffer A at 35°C. The holding temperature is 25°C	15 cycles of 4 mixes/cycle with Wash Buffer A at 35°C. The holding temperature is 25°C

- Wash Buffer A = non-stringent wash buffer
- Wash Buffer B = stringent wash buffer

* When using the Fluidics Station 450 or 250 add _450 at the end of the fluidics script's name.

FS-450

If you are using the Fluidics Station 450/250:

Washing and Staining the Probe Array

1. In the Fluidics Station dialog box on the workstation, select the correct experiment name from the drop-down **Experiment** list.

   ⇒ The **Probe Array Type** appears automatically.

2. In the **Protocol** drop-down list, select the appropriate antibody amplification protocol to control the washing and staining of the probe array format being used: Table 2.3.3 and Table 2.3.4.

3. Choose **Run** in the Fluidics Station dialog box to begin the washing and staining. Follow the instructions in the LCD window on the fluidics station.

   If you are unfamiliar with inserting and removing probe arrays from the fluidics station modules, please refer to the appropriate *Fluidics Station User's Guide* or *Quick Reference Card* (P/N 08-0093 for the FS-450/250 fluidics stations).

4. Insert the appropriate probe array into the designated module of the fluidics station while the cartridge lever is in the down, or eject, position. When finished, verify that the cartridge lever is returned to the up, or engaged, position.

5. Remove any microcentrifuge vial remaining in the sample holder of the fluidics station module(s) being used.

6. If prompted to "Load Vials 1-2-3," place the three experiment sample vials (the microcentrifuge vials) into the sample holders 1, 2, and 3 on the fluidics station.
   - Place one vial containing 600 µL of streptavidin phycoerythrin (SAPE) solution in sample holder 1.
   - Place one vial containing 600 µL of anti-streptavidin biotinylated antibody solution in sample holder 2.
   - Place one vial containing 600 µL of streptavidin phycoerythrin (SAPE) solution in sample holder 3.
   - Press down on the needle lever to snap needles into position and to start the run.

   The run begins. The Fluidics Station dialog box at the workstation terminal and the LCD window display the status of the washing and staining as they progress.

7. At the end of the run, or at the appropriate prompt, remove the microcentrifuge vials and replace with three empty microcentrifuge vials.

8. Remove the probe arrays from the fluidics station modules by first pressing down the cartridge lever to the eject position.

9. Check the probe array window for large bubbles or air pockets.
   - If bubbles are present, proceed to Table 2.3.6.
   - If the probe array has no large bubbles, it is ready to scan on the GeneArray® Scanner or the GeneChip® Scanner 3000. Pull up on the cartridge lever to engage washblock and proceed to *Probe Array Scan* on page 2.3.15.

   If you do not scan the arrays right away, keep the probe arrays at 4°C and in the dark until ready for scanning.

If there are no more samples to hybridize, shut down the fluidics station following the procedure outlined in the section, *Shutting Down the Fluidics Station* on page 2.3.17.

✔ Note

For proper cleaning and maintenance of the fluidics station, including the bleach protocol, refer to Section 4, Fluidics Station Maintenance Procedures.

Table 2.3.6
If Bubbles are Present

Return the probe array to the probe array holder. Engage the washblock by gently pushing up on the cartridge lever to the engaged, or closed, position. The fluidics station will drain the probe array and then fill it with a fresh volume of the last wash buffer used. When it is finished, the LCD window will display **EJECT CARTRIDGE.** Again, remove the probe array and inspect it for bubbles. If no bubbles are present, it is ready to scan. Proceed to *Probe Array Scan* on page 2.3.15. If several attempts to fill the probe array without bubbles are unsuccessful, the array should be filled with **Wash Buffer A (non-stringent buffer)** manually, using a micropipette. Excessive washing will result in a loss of signal intensity.

FS-400

If you are using the Fluidics Station 400:

Washing and Staining the Probe Array

1. In the Fluidics Station dialog box on the workstation, select the correct experiment name in the drop-down **Experiment** list. The probe array type will appear automatically.
2. In the **Protocol** drop-down list, select the appropriate antibody amplification protocol to control the washing and staining of the probe array format being used: Table 2.3.3 and Table 2.3.4.
3. Choose **Run** in the Fluidics Station dialog box to begin the washing and staining. Follow the instructions on the LCD window on the fluidics station.
4. If you are unfamiliar with inserting and removing probe arrays from the fluidics station modules, please refer to the *Fluidics Station 400 User's Guide*, *Fluidics Station 400 Video In-Service CD* (P/N 900374), or *Quick Reference Card* (P/N 08-0072).
5. Insert the appropriate probe array into the designated module of the fluidics station while the cartridge lever is in the **EJECT** position. When finished, verify that the cartridge lever is returned to the **ENGAGE** position.
6. Remove any microcentrifuge vials remaining in the sample holder of the fluidics station module(s) being used.
7. When the LCD window indicates, place the microcentrifuge vial containing 600 µL of streptavidin phycoerythrin (SAPE) stain solution into the sample holder. Verify that the metal sampling needle is in the vial with its tip near the bottom.
8. When the LCD window indicates, replace the microcentrifuge vial containing the streptavidin phycoerythrin (SAPE) stain solution with a microcentrifuge vial containing antibody stain solution into the sample holder, making sure that the metal sampling needle is in the vial with its tip near the bottom.

9. When the LCD window indicates, replace the microcentrifuge vial containing the antibody stain solution with a microcentrifuge vial containing 600 µL of streptavidin phycoerythrin (SAPE) stain solution into the sample holder. Verify that the metal sampling needle is in the vial with its tip near the bottom.

   ⇒ The Fluidics Station dialog box and the LCD window display the status of the washing and staining as they progress. When the wash is complete, the LCD window displays the message **EJECT CARTRIDGE**.

10. At the end of the run, or at the appropriate prompt, remove microcentrifuge vial containing stain and replace with an empty microcentrifuge vial.

11. Remove the probe arrays from the fluidics station modules by first moving the probe array holder lever to the **EJECT** position.

12. Check the probe array window for large bubbles or air pockets.

    - If bubbles are present, proceed to Table 2.3.7.
    - If the probe array has no large bubbles, it is ready to scan on the GeneChip® Scanner 3000 or GeneArray® Scanner. **ENGAGE** wash block and proceed to *Probe Array Scan* on page 2.3.15.

    If you do not scan the arrays right away, keep the probe arrays at 4°C and in the dark until ready for scanning.

    If there are no more samples to hybridize, shut down the fluidics station following the procedure outlined in the section, *Shutting Down the Fluidics Station* on page 2.3.17.

✔ Note

For proper cleaning and maintenance of the fluidics station including the bleach protocol, refer to Section 4, Fluidics Station Maintenance Procedures.

Table 2.3.7
If Bubbles are Present

Return the probe array to the probe array holder. Latch the probe array holder by gently pushing it up until a light click is heard. Engage the wash block by firmly pushing up on the cartridge lever to the **ENGAGE** position. The fluidics station will drain the probe array and then fill it with a fresh volume of the last wash buffer used. When it is finished, the LCD window displays **EJECT CARTRIDGE** again, remove the probe array and inspect it again for bubbles. If no bubbles are present, it is ready to scan. Proceed to *Probe Array Scan* on page 2.3.15. If several attempts to fill the probe array without bubbles are unsuccessful, the array should be filled with **Wash Buffer A (non-stringent buffer)** manually, using a micropipette. Excessive washing will result in a loss of signal intensity.

Probe Array Scan

The scanner is also controlled by Affymetrix® Microarray Suite or GCOS. The probe array is scanned after the wash protocols are complete. Make sure the laser is warmed up prior to scanning by turning it on at least 15 minutes before use if you are using the Agilent GeneArray® Scanner, or 10 minutes if you are using the Affymetrix® GeneChip® Scanner 3000. If probe array was stored at 4°C, warm to room temperature before scanning. Refer to the Microarray Suite or GCOS online help and the appropriate scanner user's manual for more information on scanning.

✔ Note

The scanner uses a laser and is equipped with a safety interlock system. Defeating the interlock system may result in exposure to hazardous laser light.

You must have read, and be familiar with, the operation of the scanner before attempting to scan a probe array. Please refer to the Microarray Suite User's Guide (P/N 08-0081) or to the GeneChip® Scanner 3000 quick reference card (P/N 08-0075).

Handling the GeneChip® Probe Array

Before you scan the probe array, follow the directions in this section on handling the probe array. If necessary, clean the glass surface of the probe array with a non-abrasive towel or tissue before scanning. Do not use alcohol to clean glass.

Before scanning the probe array cartridge, apply Tough-Spots™ to each of the two septa on the probe array cartridge to prevent the leaking of fluids from the cartridge during scanning.

IMPORTANT *Apply the spots just before scanning. Do not use them in the hyb process.*

1. On the back of the probe array cartridge, clean excess fluid from around septa.
2. Carefully apply one Tough-Spots to each of the two septa. Press to ensure that the spots remain flat. If the Tough-Spots do not apply smoothly, that is, if you observe bumps, bubbles, tears, or curled edges, do not attempt to smooth out the spot. Remove the spot and apply a new spot. See Figure 2.3.1.

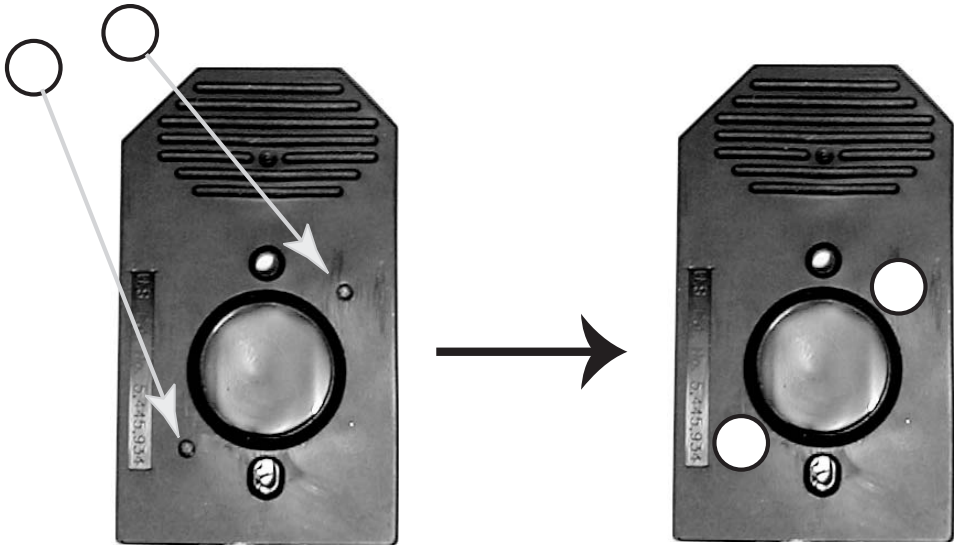


Figure 2.3.1
Applying Tough-Spots™ to the probe array cartridge

3. Insert the cartridge into the scanner and test the autofocus to ensure that the Tough-Spots do not interfere with the focus. If you observe a focus error message, remove the spot and apply a new spot. Ensure that the spots lie flat.

Scanning the Probe Array

1. Select **Run** → **Scanner** from the menu bar. Alternatively, click the **Start Scan** icon in the tool bar.

   ⇒ The Scanner dialog box appears with a drop-down list of experiments that have not been run.

2. Select the experiment name that corresponds to the probe array to be scanned.

   A previously run experiment can also be selected by using the **Include Scanned Experiments** option box. After selecting this option, previously scanned experiments appear in the drop-down list.

3. By default, for the GeneArray® Scanner only, after selecting the experiment the number [**2**] is displayed in the **Number of Scans** box to perform the recommended 2X image scan. For the GeneChip® Scanner 3000, only one scan is required.

4. Once the experiment has been selected, click the **Start** button.

   ⇒ A dialog box prompts you to load an array into the scanner.

5. If you are using the GeneArray® Scanner, click the **Options** button to check for the correct pixel value and wavelength of the laser beam.

   - Pixel value = 3 µm
   - Wavelength = 570 nm

   If you are using the GeneChip Scanner 3000, pixel resolution and wavelength are preset and cannot be changed.

6. Open the sample door on the scanner and insert the probe array into the holder. Do not force the probe array into the holder. Close the sample door of the scanner.
7. Click **OK** in the Start Scanner dialog box.
   ⇒ The scanner begins scanning the probe array and acquiring data. When **Scan in Progress** is selected from the **View** menu, the probe array image appears on the screen as the scan progresses.

Shutting Down the Fluidics Station

1. After removing a probe array from the probe array holder, the LCD window displays the message **ENGAGE WASHBLOCK**.
2. If you are using the FS-400, latch the probe array holder by gently pushing up until a light click is heard. Engage the washblock by firmly pushing up on the cartridge lever to the **ENGAGE** position.

   If you are using the FS-450, gently lift up the cartridge lever to engage, or close, the washblock.
   ⇒ The fluidics station automatically performs a Cleanout procedure. The LCD window indicates the progress of the Cleanout procedure**.**
3. When the fluidics station LCD window indicates **REMOVE VIALS**, the Cleanout procedure is complete.
4. Remove the sample microcentrifuge vial(s) from the sample holder(s).
5. If no other hybridizations are to be performed, place wash lines into a bottle filled with deionized water.
6. Choose **Shutdown** or **Shutdown_450** for all modules from the drop-down **Protocol** list in the Fluidics Station dialog box. Click the **Run** button for all modules.

   The Shutdown protocol is critical to instrument reliability. Refer to the appropriate *Fluidics Station User's Guide* for more information.
7. After Shutdown protocol is complete, flip the ON/OFF switch of the fluidics station to the OFF position.

IMPORTANT

To maintain the cleanliness of the fluidics station and obtain the highest quality image and data possible, a weekly bleach protocol and a monthly decontamination protocol are highly recommended. Please refer to Section 4, Fluidics Station Maintenance Procedures for further detail.

Customizing the Protocol

There may be times when the fluidics protocols need to be modified. Modification of protocols must be done before downloading the protocol to the fluidics station. Protocol changes will not affect runs in progress. For more specific instructions, refer to the Microarray Suite/GCOS online help.

1. Select **Tools → Edit Protocol** from the menu bar.
   ⇒ The Edit Protocol dialog box appears.
2. Select the protocol to be changed from the **Protocol Name** drop-down list.
   ⇒ The name of the protocol is displayed in the Protocol Name box. The conditions for that protocol are displayed on the right side of the Edit Protocol dialog box.
3. Select the items to be changed and input the new parameters as needed, keeping the parameters within the ranges shown below in Table 2.3.8.

Table 2.3.8
Valid Ranges for Wash/Stain Parameters

Parameter	Valid Range
Wash Temperature for A1, B, A2, or A3 (°C)	15 to 50
Number of Wash Cycles for A1, B, A2, or A3	0 to 99
Mixes / Wash cycle for A1, B, A2, or A3	1 to 99
Stain Time (seconds)	0 to 86399
Stain Temperature (°C)	15 to 50
Holding Temperature (°C)	15 to 50

- Wash A1 corresponds to Post Hyb wash #1 in Table 2.3.5.
- Wash B corresponds to Post Hyb wash #2 in Table 2.3.5.
- Wash A2 corresponds to Post Stain Wash in Table 2.3.5.
- Wash A3 corresponds to Final Wash in Table 2.3.5.

4. To return to the default values for the protocol selected, click the **Defaults** button.
5. After all the protocol conditions are modified as desired, change the name of the edited protocol in the **Protocol Name** box.

! CAUTION *If the protocol is saved without entering a new **Protocol Name**, the original protocol parameters will be overwritten.*

6. Click **Save**, then close the dialog box.

   Enter **0** (zero) for hybridization time if hybridization step is not required. Likewise, enter **0** (zero) for the stain time if staining is not required. Enter **0** (zero) for the number of wash cycles if a wash solution is not required.