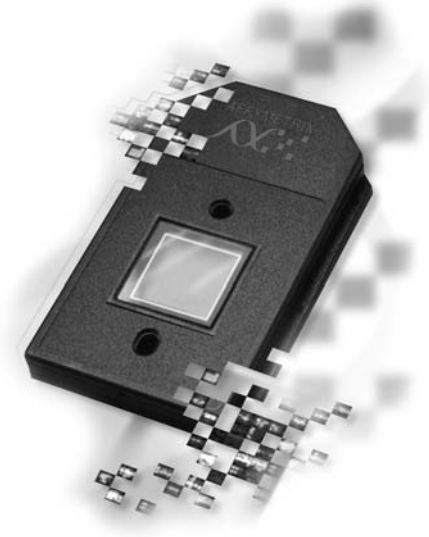
GENE EXPRESSION MONITORING

GeneChip® Expression Analysis



Technical Manual

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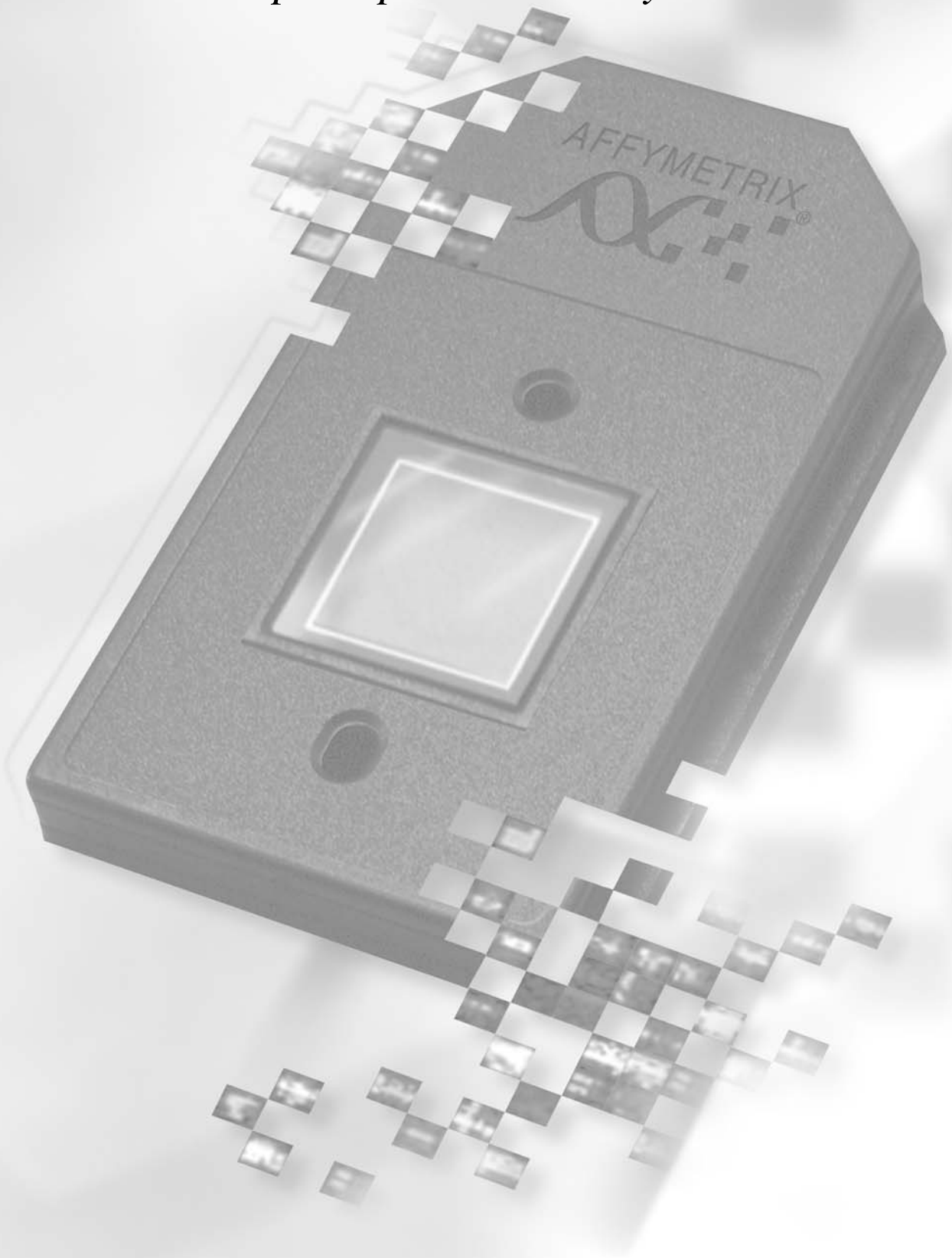
GeneChip® Expression Analysis Overview



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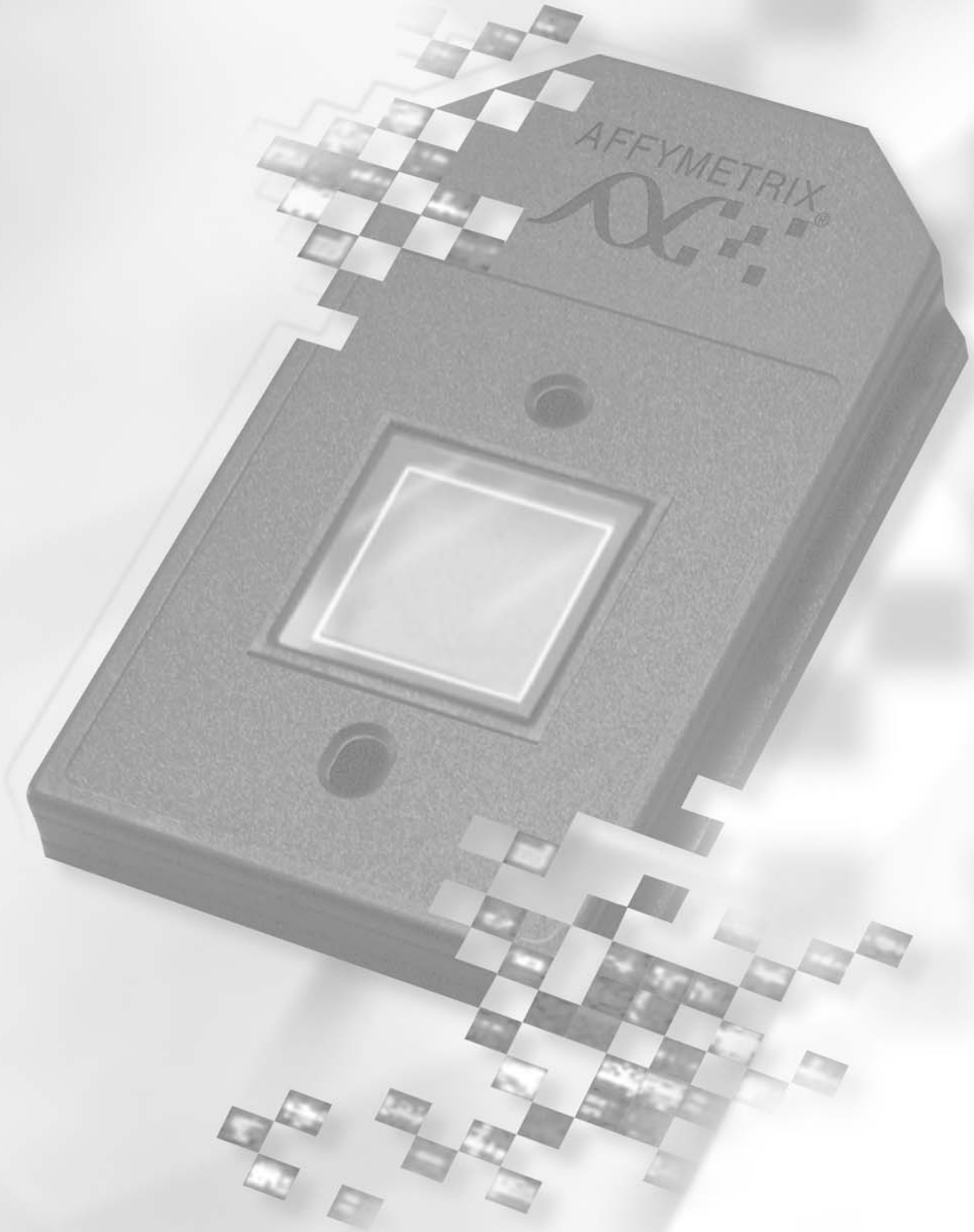

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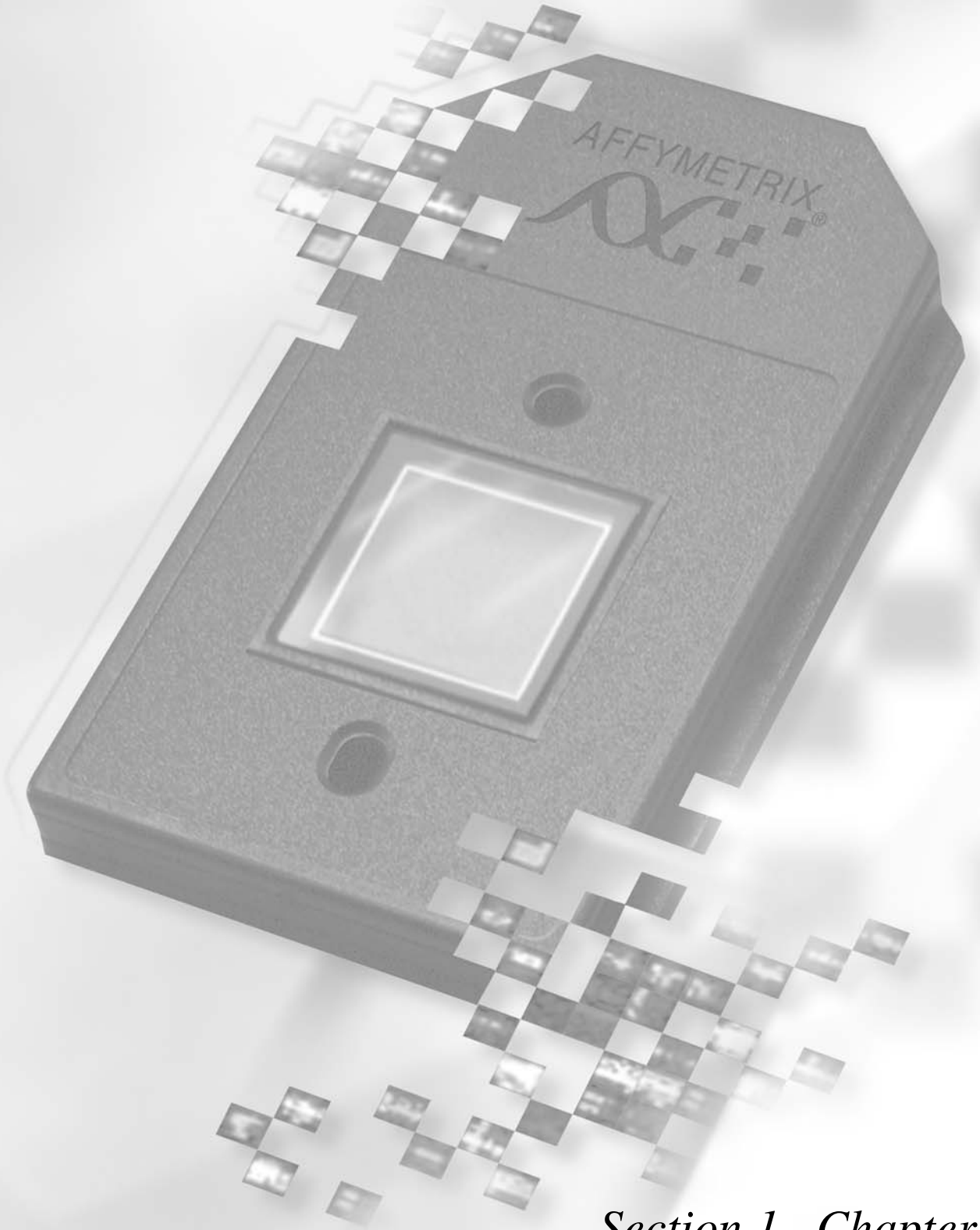

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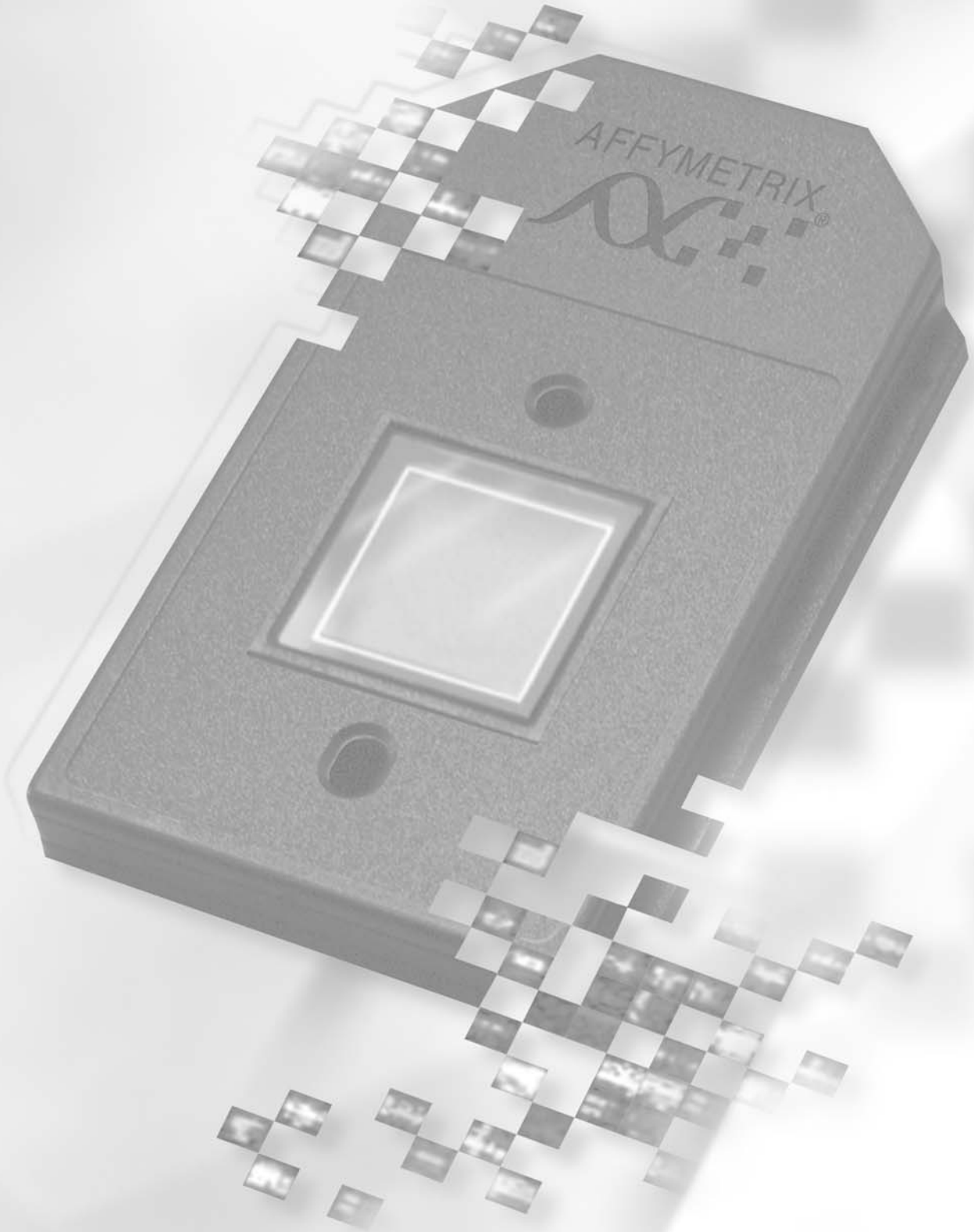

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This Chapter Contains:

- An overview of GeneChip® Expression Analysis.
- A summary of the procedures covered in the remainder of the manual.

Introduction and Objectives

Welcome to the *Affymetrix GeneChip® Expression Analysis Technical Manual*. This manual is a technical guide for using GeneChip expression analysis probe arrays. All protocols included in this manual have been used successfully by scientists at Affymetrix, or have been recommended by our collaborators during the development of particular products. The field of mRNA gene expression monitoring is rapidly evolving and periodic technical updates to this manual will reflect the newest protocols and information for using GeneChip probe arrays. This manual applies to all GeneChip expression products.

As an Affymetrix GeneChip user, your feedback is welcome. Please contact our technical support team with any input on how we can improve this resource.

Explanation of GeneChip® Probe Arrays

GeneChip probe arrays are manufactured using technology that combines photolithography and combinatorial chemistry.[1,2] Up to 1.3 million different oligonucleotide probes are synthesized on each array. Each oligonucleotide is located in a specific area on the array called a probe cell. Each probe cell contains hundreds of thousands to millions of copies of a given oligonucleotide.

Probe arrays are manufactured in a series of cycles. Initially, a glass substrate is coated with linkers containing photolabile protecting groups. Then, a mask is applied that exposes selected portions of the probe array to ultraviolet light. Illumination removes the photolabile protecting groups enabling selective nucleoside phosphoramidite addition only at the previously exposed sites. Next, a different mask is applied and the cycle of illumination and chemical coupling is performed again. By repeating this cycle, a specific set of oligonucleotide probes is synthesized with each probe type in a known location. The completed probe arrays are packaged into cartridges.

During the laboratory procedure described in this manual, biotin-labeled RNA or DNA fragments referred to as the “target” are hybridized to the probe array. The hybridized probe array is stained with streptavidin phycoerythrin conjugate and scanned by the GeneChip® Scanner 3000, or the GeneArray® Scanner. The amount of light emitted at 570 nm is proportional to the bound target at each location on the probe array.

GeneChip® Expression Analysis Overview

The following major steps outline GeneChip expression analysis:

1. Target Preparation
2. Target Hybridization
3. Fluidics Station Setup
4. Probe Array Washing and Staining
5. Probe Array Scan
6. Data Analysis

Due to the differences in the RNA species between eukaryotic and prokaryotic organisms, different target labeling protocols have been optimized. Sections 2 and 3 provide detailed protocols for target preparation, hybridization, array washing, and staining for eukaryotic and prokaryotic arrays, respectively. Please refer to the sections in this manual for detailed protocols appropriate for your arrays.

Step 1: Target Preparation

This manual describes procedures for preparing biotinylated target from purified eukaryotic and prokaryotic RNA samples suitable for hybridization to GeneChip expression probe arrays. These procedures are recommendations only. For more information on these procedures, please contact Affymetrix Technical Support at 1-888-DNA-CHIP, +44 (0)1628 552550 in Europe, or +81-(0)3-5730-8200 in Japan.

For eukaryotic samples, using protocols in this manual Section 2, double-stranded cDNA is synthesized from total RNA or purified poly-A messenger RNA isolated from tissue or cells. An *in vitro* transcription (IVT) reaction is then done to produce biotin-labeled cRNA from the cDNA. The cRNA is fragmented before hybridization.

For prokaryotic samples, Section 3 describes a detailed protocol to isolate total RNA followed by reverse transcription with random hexamers to produce cDNA. After fragmentation by DNase I, the cDNA is end-labeled with biotin by terminal transferase.

Step 2: Target Hybridization

A hybridization cocktail is prepared, including the fragmented target, probe array controls, BSA, and herring sperm DNA. It is then hybridized to the probe array during a 16-hour incubation. The hybridization process is described in the respective sections for the different probe array types.

Step 3: Fluidics Station Setup

Specific experimental information is defined using Affymetrix® Microarray Suite or GeneChip Operating Software (GCOS) on a PC-compatible workstation. The probe array type, sample description, and comments are entered and saved with a unique experiment name. The fluidics station is then prepared for use by priming with the appropriate buffers. For more information on the fluidics station, refer to the *Fluidics Station User's Guide*.

Step 4: Probe Array Washing and Staining

Immediately following hybridization, the probe array undergoes an automated washing and staining protocol on the fluidics station.

Step 5: Probe Array Scan

Once the probe array has been hybridized, washed, and stained, it is scanned. Each workstation running Affymetrix Microarray Suite or GCOS can control one scanner. The software defines the probe cells and computes an intensity for each cell.

Each complete probe array image is stored in a separate data file identified by the experiment name and is saved with a data image file (.dat) extension.

Review the scanner user's manual for safety precautions and for more information on using the scanner.

Step 6: Data Analysis

The .dat image is analyzed for probe intensities; results are reported in tabular and graphical formats. Information on data analysis is provided in the enclosed *GeneChip® Expression Analysis: Data Analysis Fundamentals* booklet (P/N 701190).

Precautions

1. FOR RESEARCH USE ONLY; NOT FOR USE IN DIAGNOSTIC PROCEDURES.
2. Avoid microbial contamination, which may cause erroneous results.

▲ WARNING *All biological specimens and materials with which they come into contact should be handled as if capable of transmitting infection and disposed of with proper precautions in accordance with federal, state, and local regulations. This includes adherence to the OSHA Bloodborne Pathogens Standard (29 CFR 1910.1030) for blood-derived and other samples governed by this act. Never pipet by mouth. Avoid specimen contact with skin and mucous membranes.*

3. Exercise standard precautions when obtaining, handling, and disposing of potentially carcinogenic reagents.
4. Exercise care to avoid cross contamination of samples during all steps of this procedure, as this may lead to erroneous results.
5. Use powder-free gloves whenever possible to minimize introduction of powder particles into sample or probe array cartridges.

Terminology

Probes	The oligonucleotides on the surface of the probe arrays are called probes because they probe, or interrogate, the sample.
Target	The target is the labeled nucleic acid that is being interrogated. It is hybridized to the probes on the array.
Probe Cell	Specific areas on the probe array that contain oligonucleotides of a specific sequence.

Interfering Conditions

! CAUTION *Wear powder-free gloves throughout procedure. Take steps to minimize the introduction of exogenous nucleases. Water used in the protocols below is molecular biology grade (nuclease free).*

Proper storage and handling of reagents and samples is essential for robust performance.

All laboratory equipment used to prepare the target during this procedure should be calibrated and carefully maintained to ensure accuracy, as incorrect measurement of reagents may affect the outcome of the procedure.

Instruments

The Affymetrix GeneChip Expression Analysis Technical Manual is designed for use in a system consisting of a Fluidics Station, a Hybridization Oven 640, and a Scanner.

References

1. Sambrook, J., Fritsch, E.F., Maniatis, T. *Molecular Cloning: A Laboratory Manual, v.1* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY p 21-52 (1989).
2. See www.affymetrix.com for current GeneChip technology references.

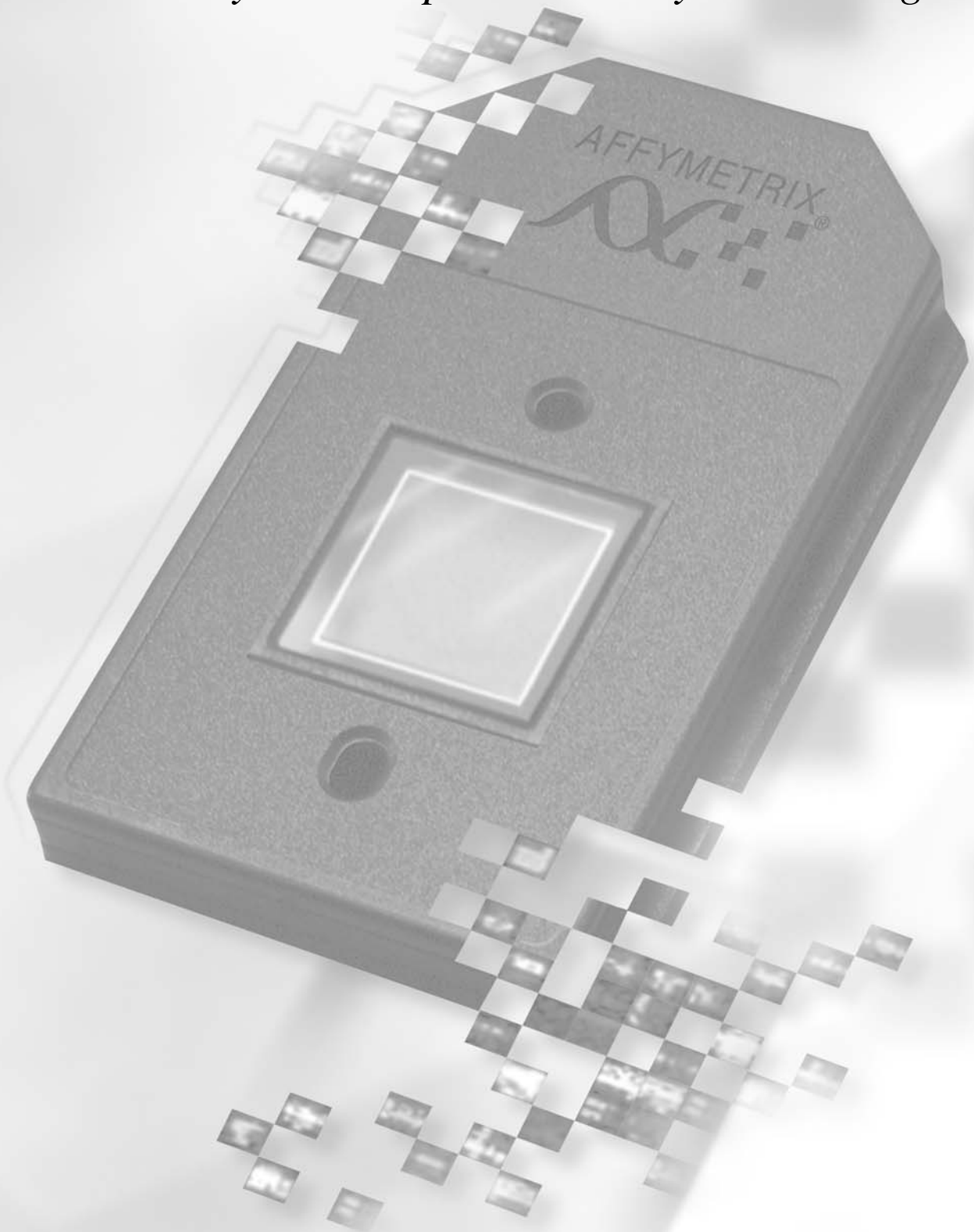
Limitations

- The results of the assay are dependent upon the quality of the input RNA, subsequent proper handling of nucleic acids and other reagents.
- The results should be evaluated by a qualified individual.

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

Section 2:

Eukaryotic Sample and Array Processing



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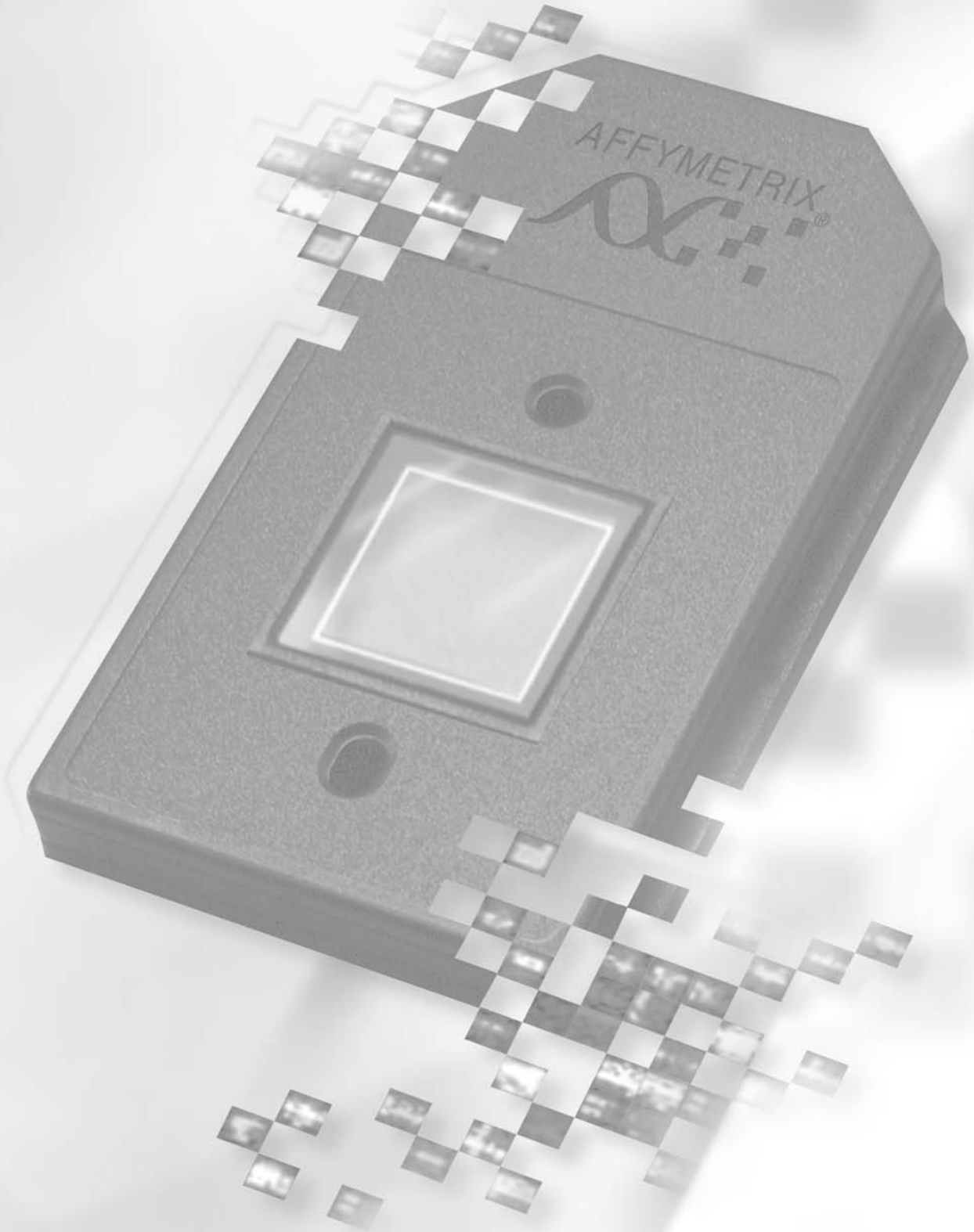

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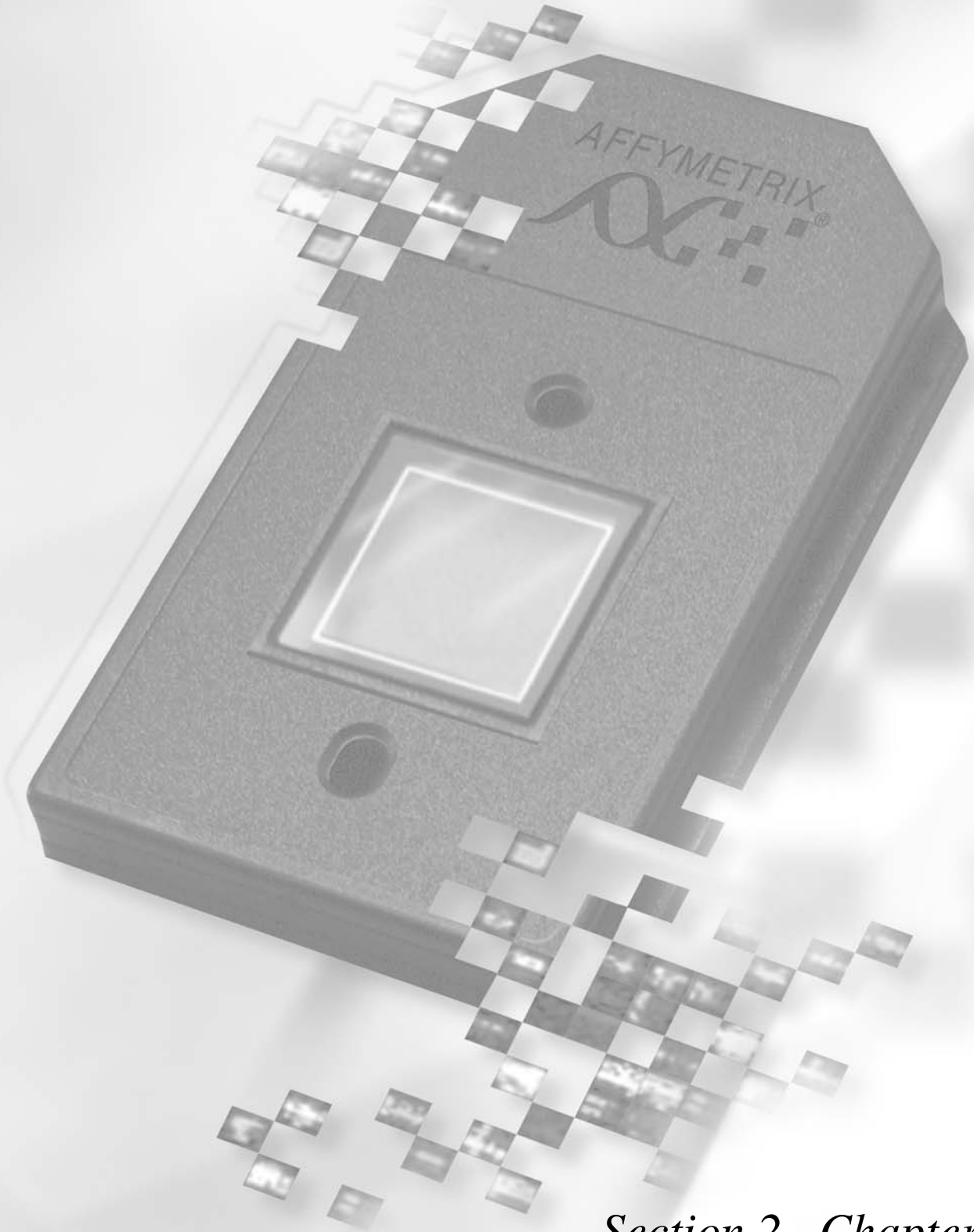

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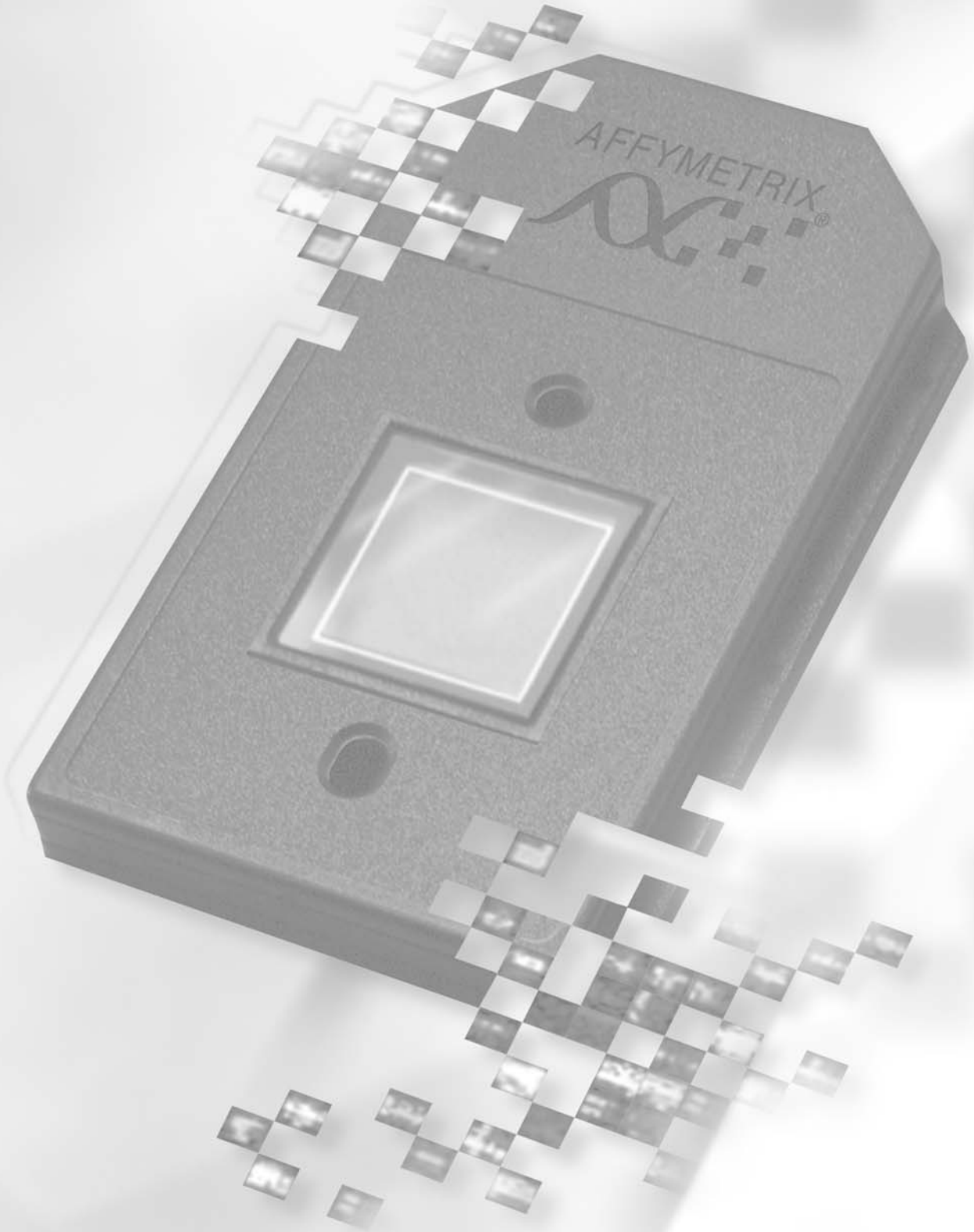

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This Chapter Contains:

- 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.

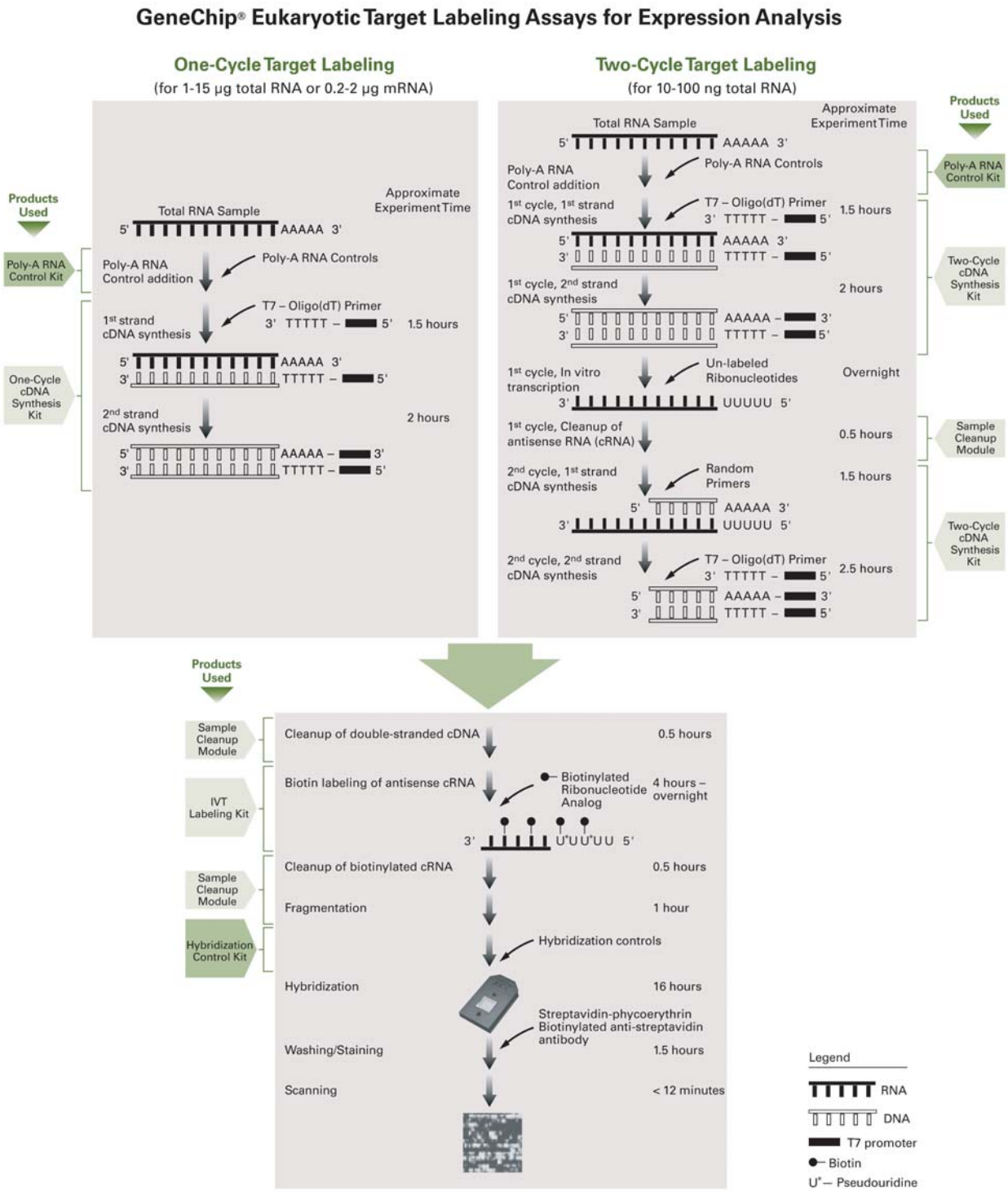

Figure 2.1.1
GeneChip Eukaryotic Labeling Assays for Expression Analysis

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

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.

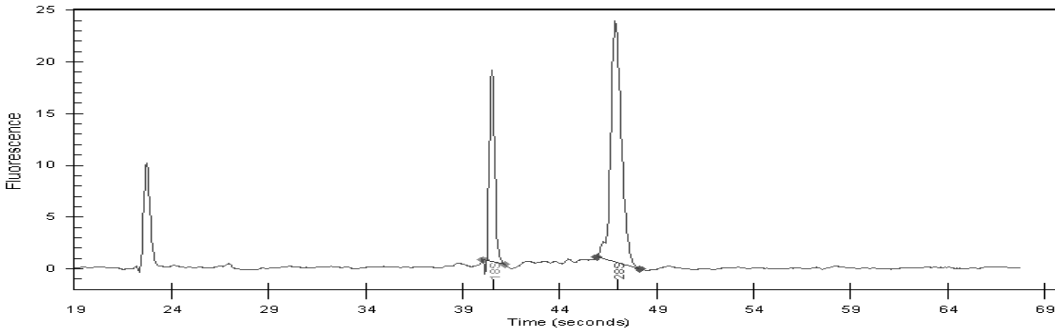

Figure 2.1.2
Electropherogram (from the Agilent 2100 Bioanalyzer) for HeLa Total RNA. For a high-quality total RNA sample, two well-defined peaks corresponding to the 18S and 28S ribosomal RNAs should be observed, similar to a denaturing agarose gel, with ratios approaching 2:1 for the 28S to 18S bands.

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.

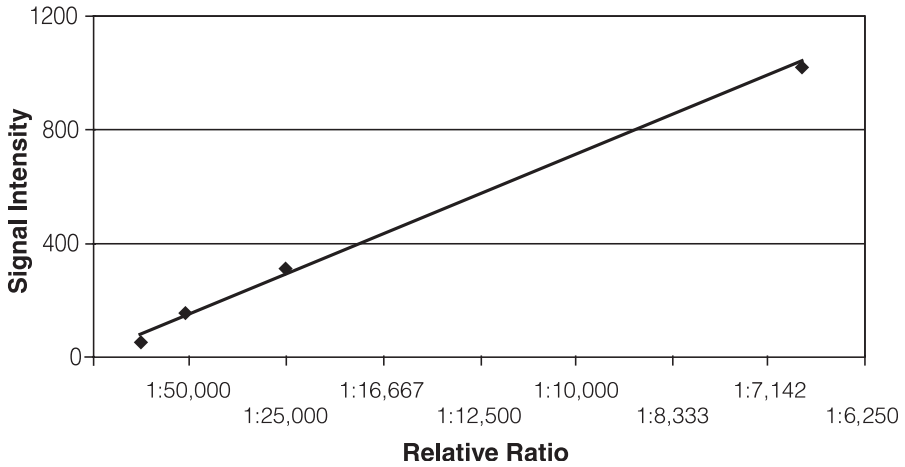

Figure 2.1.3
Poly-A RNA spikes amplified using 5 µg of a complex human HeLa total RNA sample.
Labeled target was hybridized onto a GeneChip® Human Genome U133A 2.0 Array. Signal intensities of the 3' AFFX-r2-Bs probe set of the control transcripts were obtained using GeneChip Operating Software version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

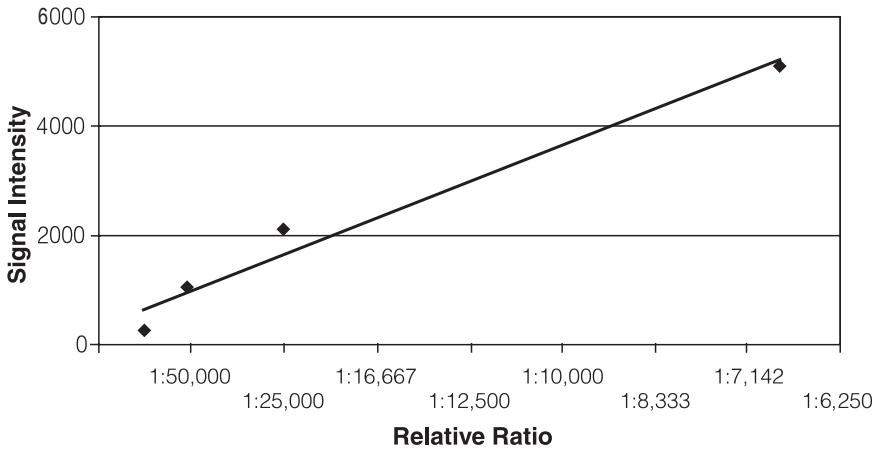

Figure 2.1.4
Poly-A RNA spikes amplified using 10 ng of a complex human brain total RNA sample.
Labeled target was hybridized onto a GeneChip® Human Genome U133 Plus 2.0 Array. Signal intensities of the 3' AFFX-r2-Bs probe set of the control transcripts were obtained using GeneChip Operating Software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

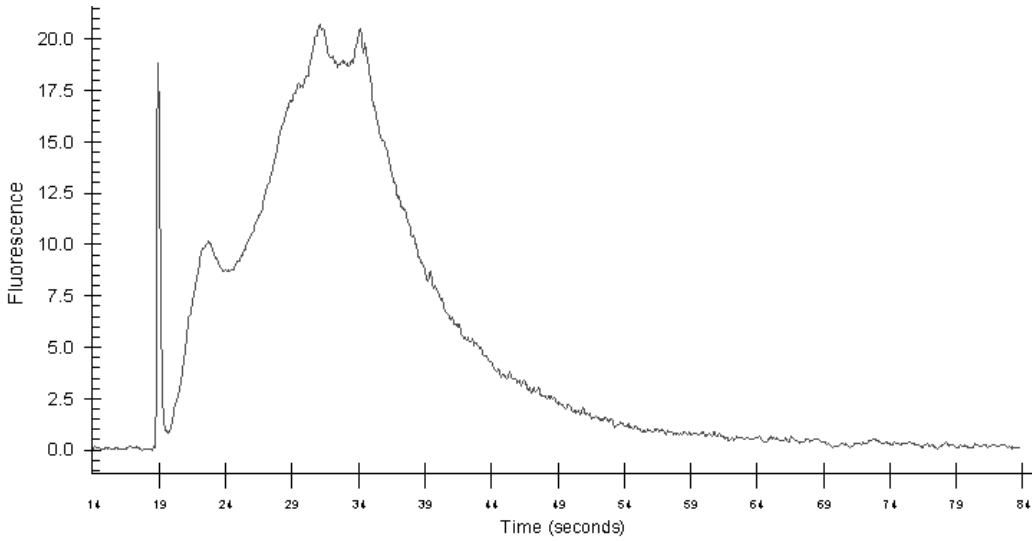

Figure 2.1.5
Biotin-labeled cRNA from One-Cycle cDNA Synthesis Kit. Bioanalyzer electropherogram for labeled cRNA from HeLa total RNA using the One-Cycle Kit. This electropherogram displays the nucleotide size distribution for 400 ng of labeled cRNA resulting from one round of amplification. The average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

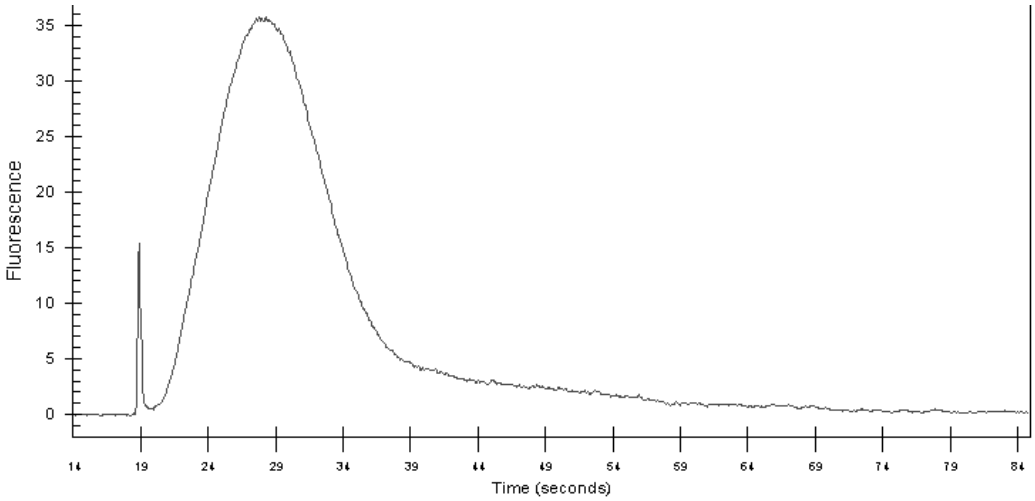

Figure 2.1.6
Biotin-labeled cRNA from Two-Cycle cDNA Synthesis Kit. Bioanalyzer electropherogram for labeled cRNA from HeLa total RNA using the Two-Cycle Kit. This electropherogram displays the nucleotide size distribution for 400 ng of labeled cRNA resulting from two rounds of amplification. The average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

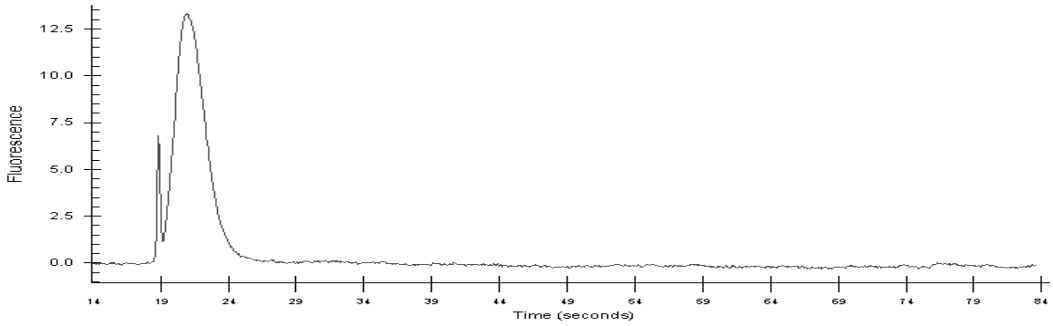

Figure 2.1.7
Fragmented cRNA. Bioanalyzer electropherogram for fragmented labeled cRNA from HeLa total RNA. This electropherogram displays the nucleotide size distribution for 150 ng of fragmented labeled cRNA resulting from one round of amplification. The average size is approximately 100 nt.

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**	

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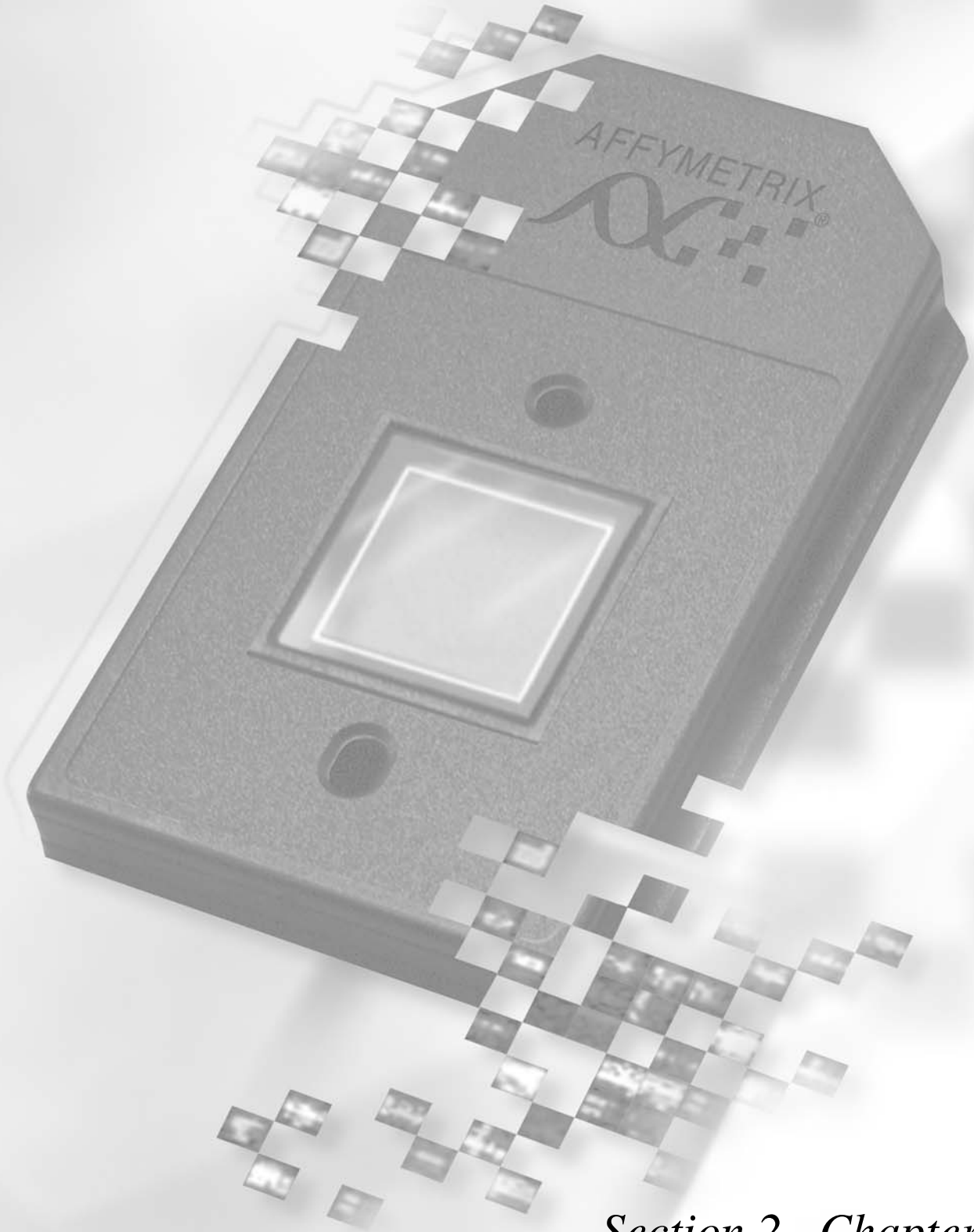
Step 2: Second-Strand cDNA Synthesis

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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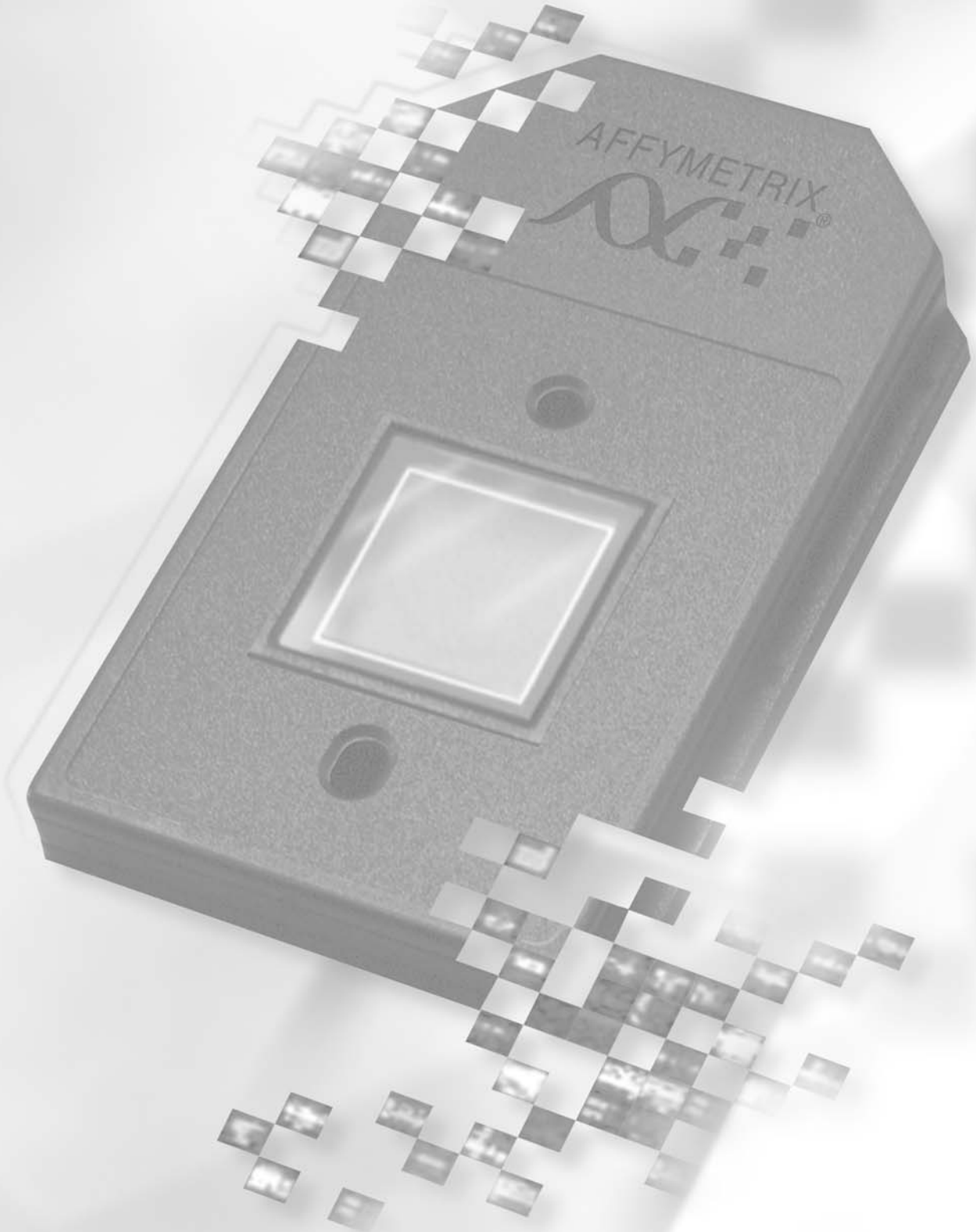


Eukaryotic Target Hybridization

Reagents and Materials Required 2.2.5
Reagent Preparation 2.2.6
Eukaryotic Target Hybridization 2.2.7

Eukaryotic

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.

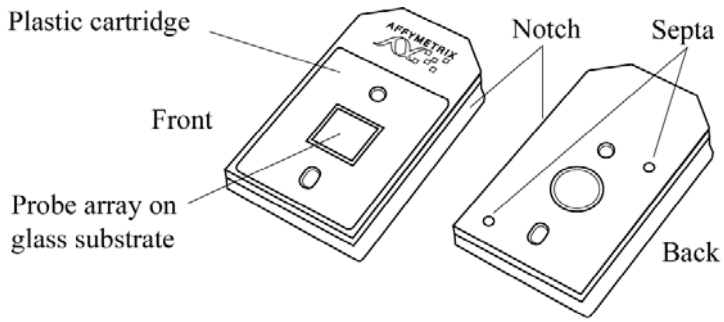
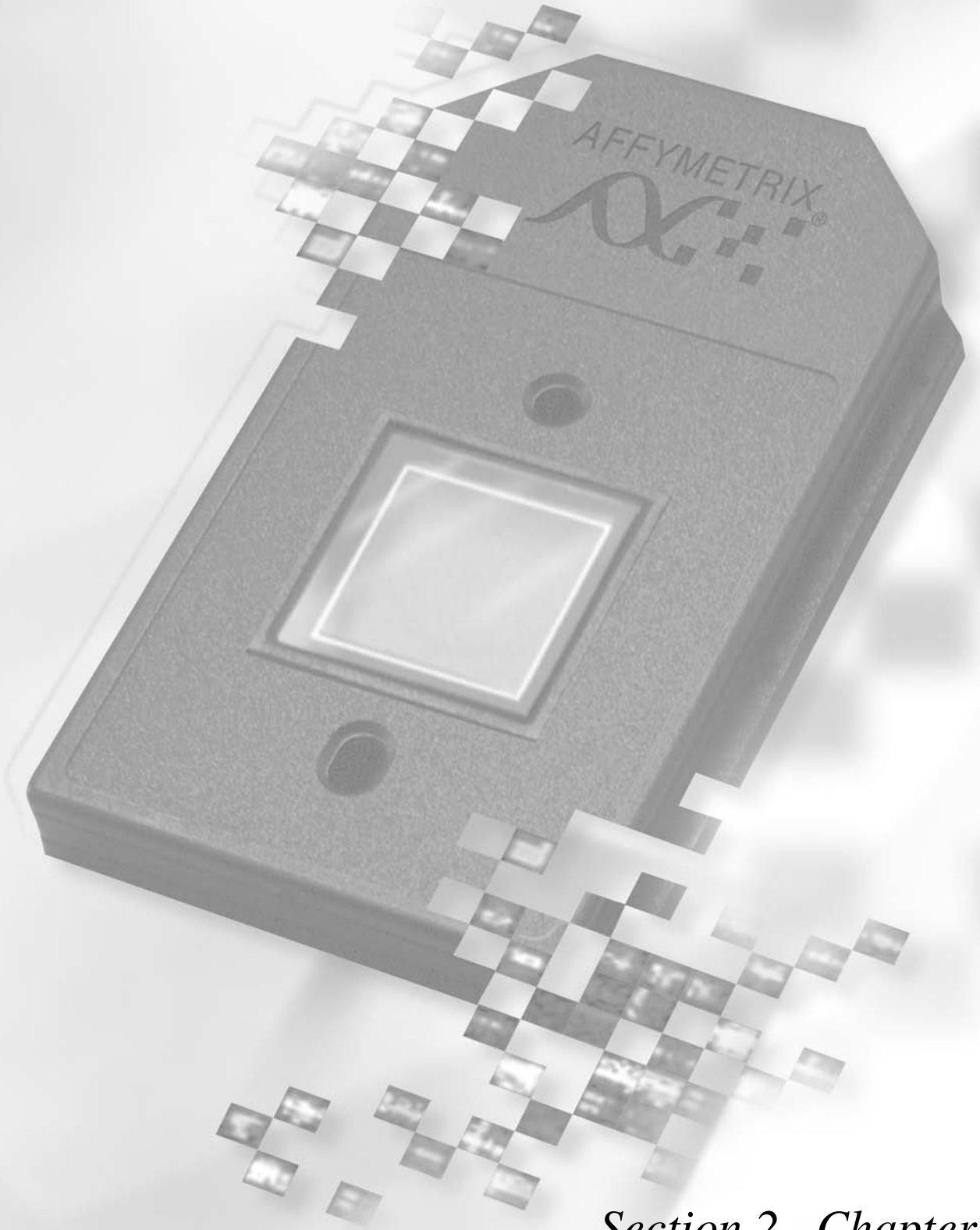


Figure 2.2.1
GeneChip® Probe Array



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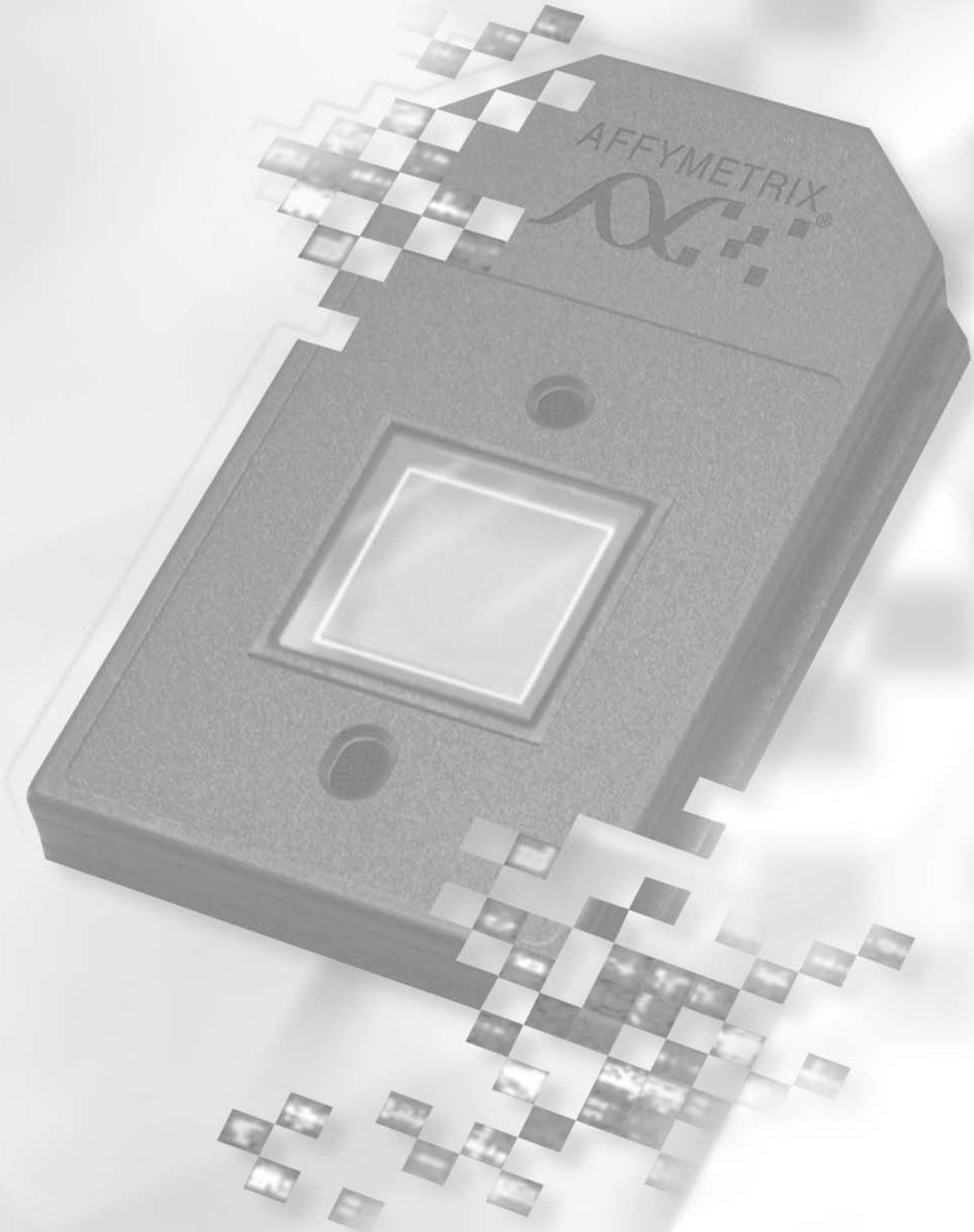


Eukaryotic Arrays: Washing, Staining, and Scanning

Reagents and Materials Required 2.3.5
Reagent Preparation 2.3.6
Experiment and Fluidics Station Setup 2.3.7
Step 1: Defining File Locations 2.3.7
Step 2: Entering Experiment Information. 2.3.7
Step 3: Preparing the Fluidics Station. 2.3.8
Probe Array Wash and Stain 2.3.9
Probe Array Scan 2.3.15
Handling the GeneChip® Probe Array 2.3.15
Scanning the Probe Array 2.3.16
Shutting Down the Fluidics Station 2.3.17
Customizing the Protocol 2.3.18

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:

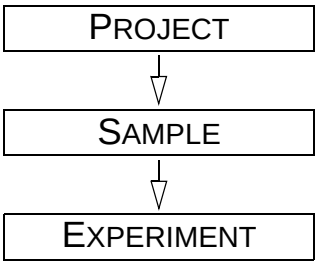
- Experiment Name
- Probe Array Type

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_2$0	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.

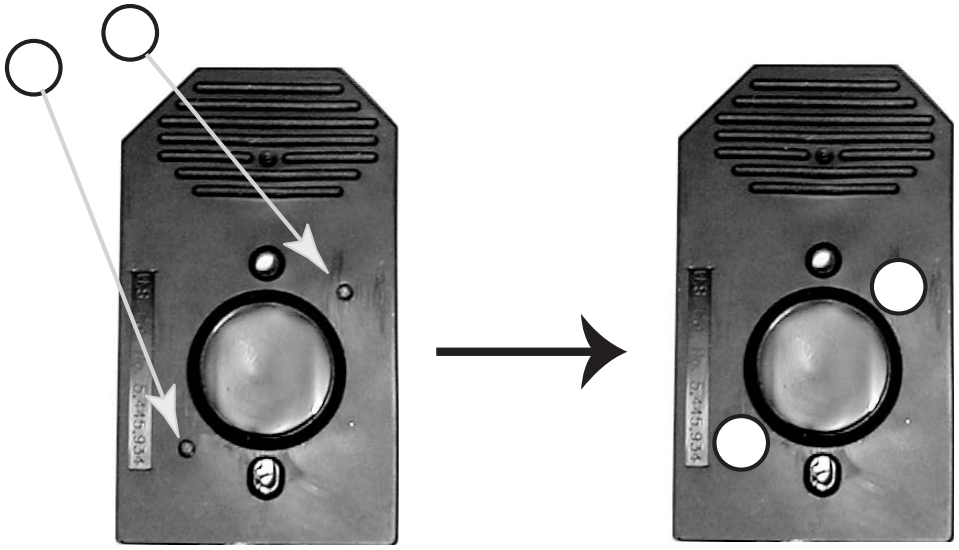


Figure 2.3.1
Applying Tough-Spots™ to the probe array cartridge

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.

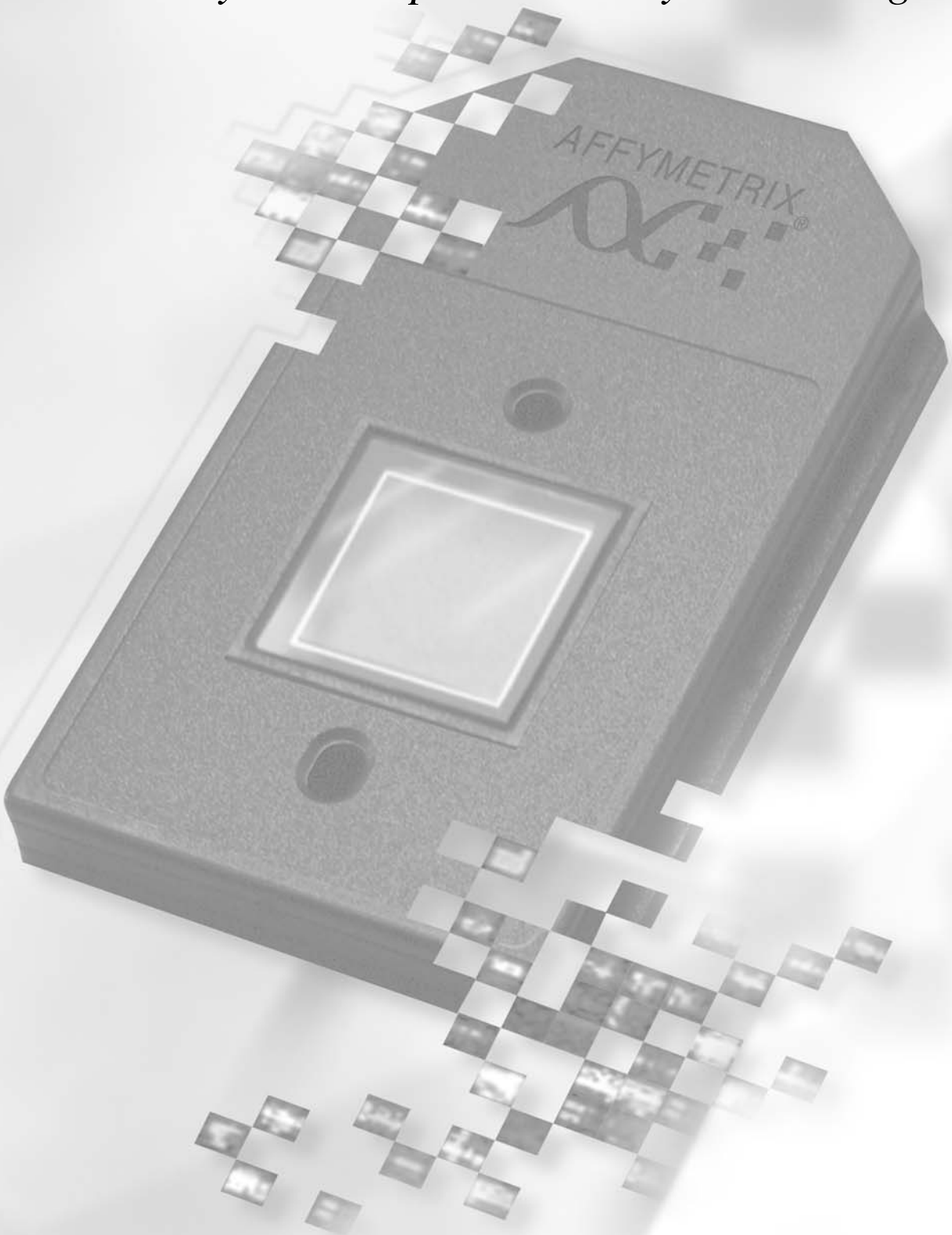
4. To return to the default values for the protocol selected, click the **Defaults** button.
5. After all the protocol conditions are modified as desired, change the name of the edited protocol in the **Protocol Name** box.

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

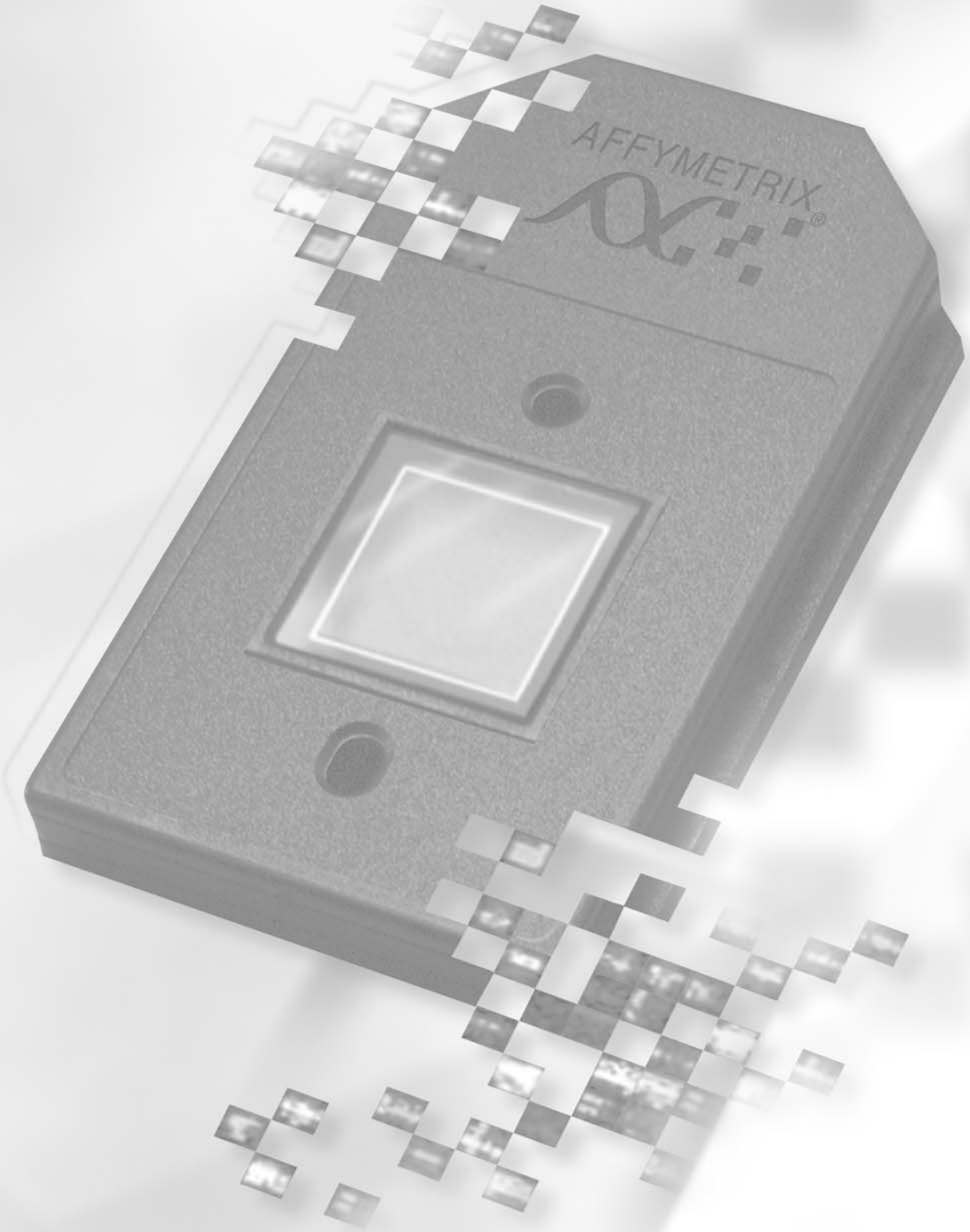
6. Click **Save**, then close the dialog box.
   Enter **0** (zero) for hybridization time if hybridization step is not required. Likewise, enter **0** (zero) for the stain time if staining is not required. Enter **0** (zero) for the number of wash cycles if a wash solution is not required.

Section 3:

Prokaryotic Sample and Array Processing



Section 3



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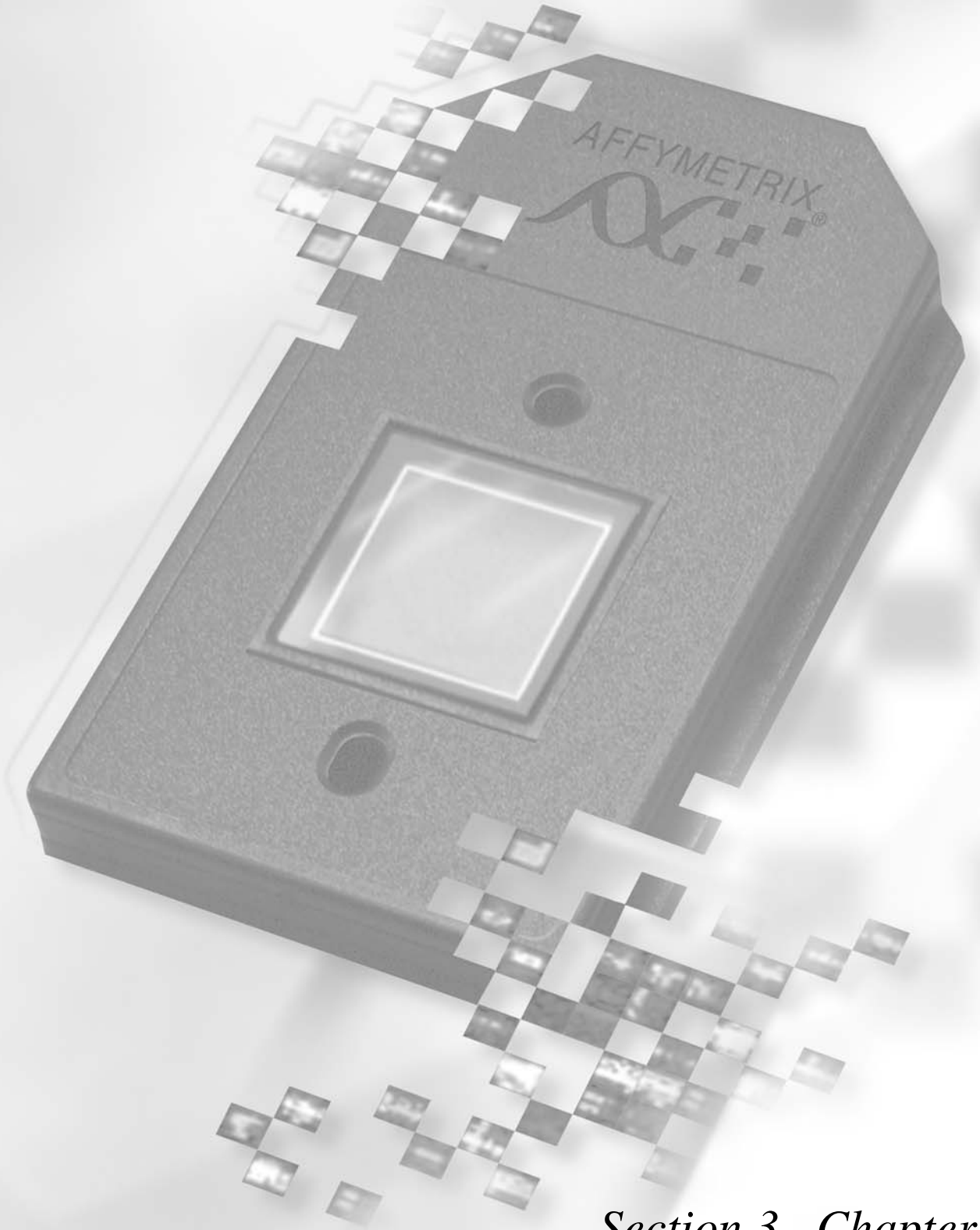

Prokaryotic

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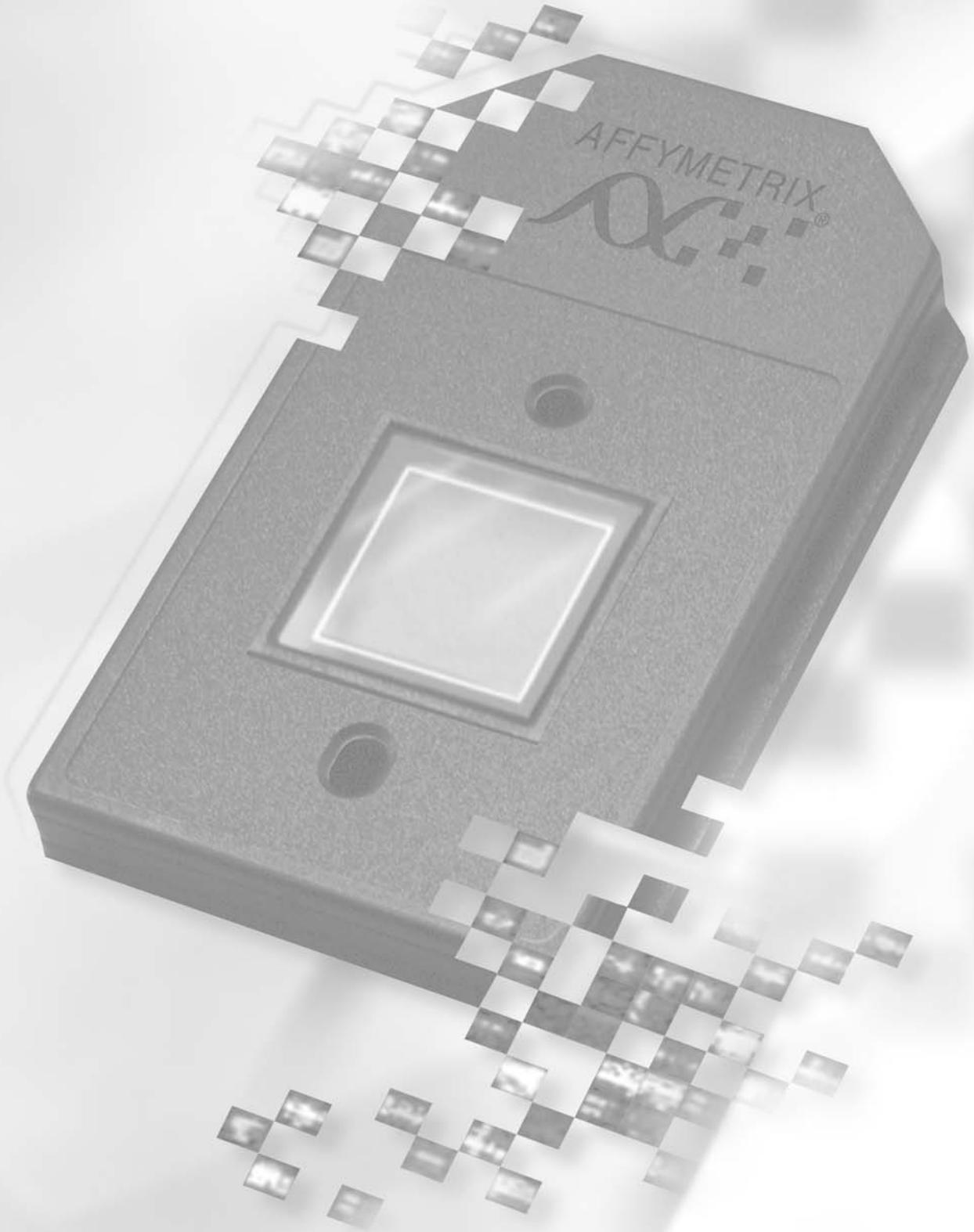

Prokaryotic Target Preparation

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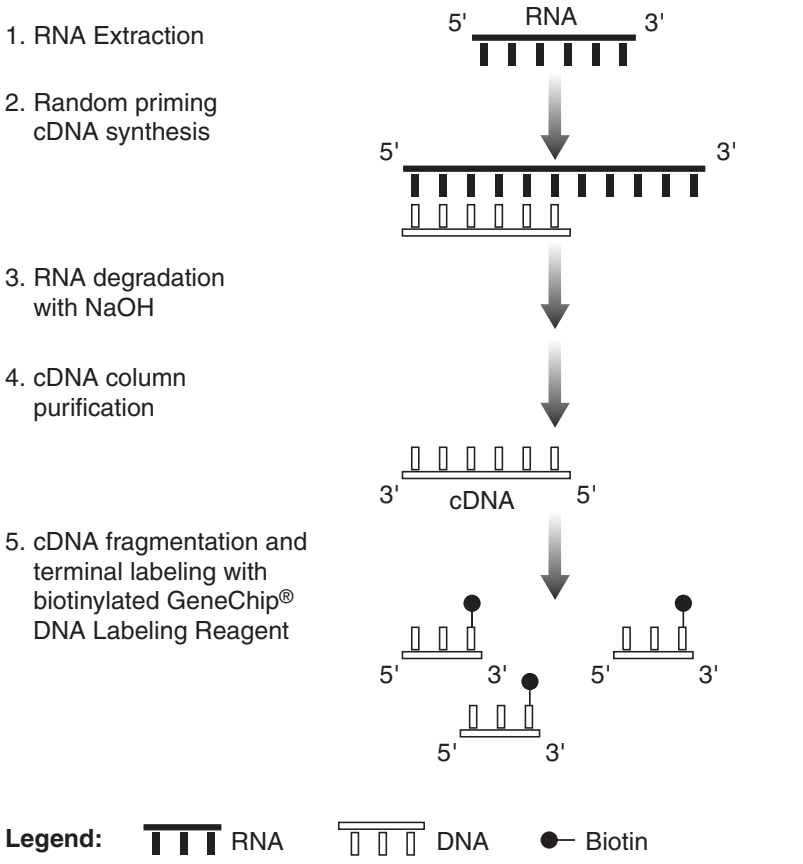
This Chapter Contains:

This chapter describes the assay procedures recommended for use with the GeneChip® *P. aeruginosa* Genome Array and the GeneChip® *E. coli* Antisense Genome Array. The assay utilizes reverse transcriptase and random hexamer primers to produce DNA complementary to the RNA. The cDNA products are then fragmented by DNase I and labeled with terminal transferase and biotinylated GeneChip® DNA Labeling Reagent at the 3' termini.

This protocol is presented as a recommendation only, and has not been validated by Affymetrix.

Target Labeling for Prokaryotic GeneChip® Antisense Arrays



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.

Labeling

- dNTP, Invitrogen Life Technologies, P/N 18427-013
- Random Primers, 3 µg/µL, Invitrogen Life Technologies, P/N 48190-011
- GeneChip® Eukaryotic Poly-A RNA Control Kit, Affymetrix, P/N 900433
- SuperScript II™ Reverse Transcriptase, Invitrogen Life Technologies, P/N 18064-071
- SUPERase•In™, Ambion, P/N 2696
- Nuclease-free Water, Ambion, P/N 9930
- NaOH, 1N solution, VWR Scientific Products, P/N MK469360
- HCl, 1N solution, VWR Scientific Products, P/N MK638860
- MinElute PCR Purification Kit, QIAGEN, P/N 28004
- 10X One-Phor-All Buffer, Amersham Biosciences, P/N 27-0901-02
- Deoxyribonuclease I (DNase I), Amersham Biosciences, P/N 27-0514-01
- GeneChip® DNA Labeling Reagent, Affymetrix, P/N 900542
- Terminal Deoxynucleotidyl Transferase, Promega, P/N M1875
- EDTA, 0.5M, pH 8.0, Invitrogen Life Technologies, P/N 15575-020
- Non-stick RNase-free microfuge tubes, 0.5 mL and 1.5 mL, Ambion, P/N 12350 and P/N 12450, respectively

Gel-Shift Assay

- Novex XCell SureLock™ Mini-Cell, Invitrogen Life Technologies, P/N EI0001
- 4-20% TBE Gel, 1.0 mm, 12 well, Invitrogen Life Technologies, P/N EC62252
- Sucrose Gel Loading Dye, 5X, Amresco, P/N E-274
- 10X TBE Running Buffer
- SYBR Gold, Molecular Probes, P/N S-11494
- 10 bp and 100 bp DNA ladder, Invitrogen Life Technologies, P/N 10821-015 and 15628-019, respectively
- ImmunoPure NeutrAvidin, Pierce Chemical, P/N 31000
- 1M Tris, pH 7.0, Ambion, P/N 9850G
- PBS, pH 7.2, Invitrogen Life Technologies, P/N 20012-027

Reagent Preparation

75 ng/µL Random Primers

For 1000 µL:

25 µL of 3 µg/µL Random Primers

975 µL of Nuclease-free H_2O

Store at -20°C in a non-frost-free freezer.

2 mg/mL NeutrAvidin

Resuspend 10 mg NeutrAvidin in 5 mL PBS solution. Store at 4°C.

Total RNA Isolation

As starting material for the cDNA synthesis procedure, total RNA can be isolated by using standard procedures for bacterial RNA isolation or various commercial RNA isolation kits.

For *Pseudomonas aeruginosa* and *E. coli*, we have successfully used the QIAGEN® RNeasy Mini Purification Kit. Caution should be used to minimize chromosomal DNA contamination during the isolation, due to the high sensitivity of the assay. It is suggested that no more than 1 X 10^9 cells are applied to a single purification column. Also, use the lysozyme at a concentration of 1 mg/mL, and not the recommended 400 µg/mL. Additional DNase I treatment may be required to eliminate DNA contamination when the bacterial culture is grown at high density.

After purification, RNA concentration is determined by absorbance at 260 nm on a spectrophotometer (1 absorbance unit = 40 µg/mL RNA). The A_{260}/A_{280} ratio should be approximately 2.0, with ranges between 1.8 to 2.1 considered acceptable. We recommend checking the quality of RNA by running it on an agarose gel prior to starting the assay. The 23S and 16S rRNA bands should be clear without any obvious smears. Any indication of the presence of chromosomal DNA contamination (high molecular weight bands or smears on the gel) would require additional DNase treatment before proceeding to cDNA synthesis.

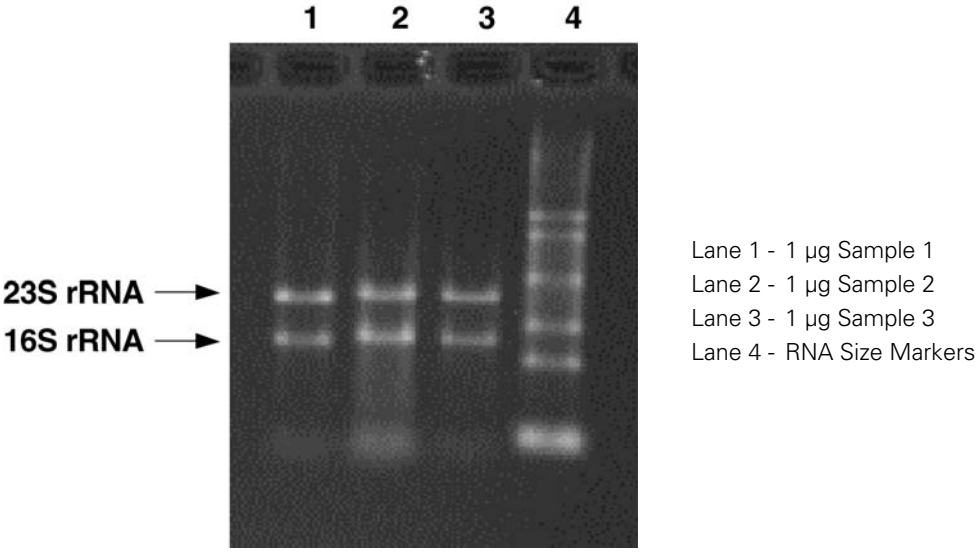


Figure 3.1.1
Typical RNA preparation from *E. coli*

cDNA Synthesis

The following protocol starts with 10 µg of total RNA. Incubations are performed in a thermal cycler.

✔ Note *The integrity of total RNA is essential for the success of the assay. Exercise precautions and follow standard laboratory procedures when handling RNA samples.*

Step 1: Preparation of Poly-A RNA Controls

The **Poly-A RNA Control Stock** and **Poly-A Control Dil Buffer** are provided with the Poly-A RNA Control Kit (P/N 900433) to prepare the appropriate serial dilutions based on the following recommendation:

Table 3.1.1
Serial Dilutions of Poly-A RNA Control Stock

Array Format*	Serial Dilutions		Spike-in Volume
	First	Second	
169 Format (Mini)	1:20	1:16	2 µL
100 Format (Midi)	1:20	1:20	2 µL
49 Format (Standard)	1:20	1:13	2 µL

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

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

The **Poly-A RNA Control Stock** contains *in vitro* synthesized, polyadenylated transcripts for *B. subtilis* genes that are pre-mixed at staggered concentrations. The concentrations of the spikes in the stock solution are: *lys* 7.6 nM, *phe* 15.2 nM, *thr* 30.4 nM, and *dap* 114.0 nM. Following the recommended dilutions as shown above, the final concentrations of the spikes in the hybridization cocktail (Table 3.2.1) are *lys* 0.256 pM, *phe* 0.511 pM, *thr* 1.022 pM, and *dap* 3.833 pM.

✔ Note *We strongly recommend using control transcripts to monitor the assay sensitivity and performance. Probe sets for these control genes from B. subtilis have been tiled on the GeneChip® P. aeruginosa Genome Array and E. coli Antisense Genome Array.*

For example, to prepare the poly-A RNA dilutions for a 100 format array:

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

a. Add 2 µL of the **Poly-A RNA Control Stock** to 38 µL of **Poly-A Control Dil Buffer** for the First Dilution (1:20).

b. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.

c. Add 2 µL of the First Dilution to 38 µL of **Poly-A Control Dil Buffer** to prepare the Second Dilution (1:20).

d. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.

e. Add 2 µL of this Second Dilution to the total RNA as indicated in Table 3.1.2.

✔ 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: cDNA Synthesis

1. Prepare the following mixture for primer annealing:

Table 3.1.2
Primer Hybridization Mix

Components	Volume	Final Concentration
Total RNA	10 µg	0.33 µg/µL
75 ng/µL Random Primers	10 µL	25 ng/µL
Diluted poly-A RNA controls	2 µL	Variable
Nuclease-free H_2O	Up to 30.0 µL	—
Total Volume	**30 µL**	

✔ Note

The random primers supplied by Invitrogen Life Technologies are oligodeoxynucleotides composed mainly of hexamers. Random primers of different length or GC content have been successfully applied to the procedure.

2. Incubate the RNA/Primer mix at the following temperatures:
   - 70°C for 10 minutes
   - 25°C for 10 minutes
   - Chill to 4°C
3. Prepare the reaction mix for cDNA synthesis. Briefly centrifuge the reaction tube to collect sample at the bottom and add the cDNA synthesis mix from Table 3.1.3 to the RNA/primer hybridization mix.

Table 3.1.3
cDNA Synthesis Components

Components	Volume	Final Concentration
RNA/Primer hybridization mix (from previous step)	30 µL	
5X 1st Strand Buffer	12 µL	1X
100 mM DTT	6 µL	10 mM
10 mM dNTPs	3 µL	0.5 mM
SUPERase•In (20 U/µL)	1.5 µL	0.5 U/µL
SuperScript II (200 U/µL)	7.5 µL	25 U/µL
Total Volume	**60 µL**	

4. Incubate the reaction at the following temperatures:
   - 25°C for 10 minutes
   - 37°C for 60 minutes
   - 42°C for 60 minutes
   - Inactivate SuperScript II at 70°C for 10 minutes
   - Chill to 4°C

Step 3: Removal of RNA

1. Add 20 µL of 1N NaOH and incubate at 65°C for 30 minutes.
2. Add 20 µL of 1N HCl to neutralize.

Step 4: Purification and Quantitation of cDNA

1. Use MinElute PCR Purification Columns to clean up the cDNA synthesis product (for detailed protocol, see MinElute PCR Purification Kit Protocols provided by the supplier). Elute the product with 12 µL of EB Buffer (supplied with the kit). The average volume of eluate is 11 µL from 12 µL of EB Buffer.
2. Quantify the purified cDNA product by 260 nm absorbance (1.0 A_{260} unit = 33 µg/mL of single-stranded DNA).

✔ Note

Typical yields of cDNA are 3 to 7 µg. A minimum of 1.5 µg of cDNA is required for subsequent procedures to obtain sufficient material to hybridize onto the array and to perform necessary quality control experiments.

cDNA Fragmentation

1. Prepare the following reaction mix:

Table 3.1.4
Fragmentation Reaction

Components	Volume	Concentration
10X One-Phor-All Buffer	2 µL	1X
cDNA	10 µL	-
DNase I (see note below)	X µL	0.6 U/µg of cDNA
Nuclease-free H_2O	Up to 20 µL	-
Total Volume	**20 µL**	

✔ Note

Use all remaining cDNA purified from the previous step in this reaction. Do not proceed if the yield is lower than 1.5 µg. Dilute DNase I to 0.6 U/µL in 1X One-Phor-All Buffer. Prepare fresh dilution each time immediately before use.

IMPORTANT

It is anticipated that DNase I enzyme activity may vary from lot to lot. A titration assay is strongly recommended for each new lot of enzyme to determine the dosage of the DNase I (unit of DNase I per µg of cDNA) to be used in the fragmentation reaction. 0.6U for each µg of cDNA can be used as a starting point for the titration.

2. Incubate the reaction at 37°C for 10 minutes.
3. Inactivate DNase I at 98°C for 10 minutes.
4. The fragmented cDNA is applied directly to the terminal labeling reaction. Alternatively, the material can be stored at -20°C for later use.

✔ Note

To examine the fragmentation result, load ~200 ng of the product on a 4% to 20% acrylamide gel and stain with SYBR Gold. The majority of the fragmented cDNA should be in the 50 to 200 base-pairs range.

Terminal Labeling

Use GeneChip® DNA Labeling Reagent (Affymetrix, P/N 900542) to label the 3' termini of the fragmentation products.

1. Prepare the following reaction mix:

Table 3.1.5
Terminal Label Reaction

Components	Volume
5X Reaction Buffer	10 µL
GeneChip DNA Labeling Reagent, 7.5 mM	2 µL
Terminal Deoxynucleotidyl Transferase	2 µL
Fragmentation cDNA Product	Up to 20 µL
H_2O	16 µL
Total Volume	**50 µL**

2. Incubate the reaction at 37°C for 60 minutes.
3. Stop the reaction by adding 2 µL of 0.5M EDTA.
4. The target is ready to be hybridized onto probe arrays, as described in Section 3, Chapter 2, *Prokaryotic Target Hybridization*. Alternatively, it may be stored at -20°C for later use.

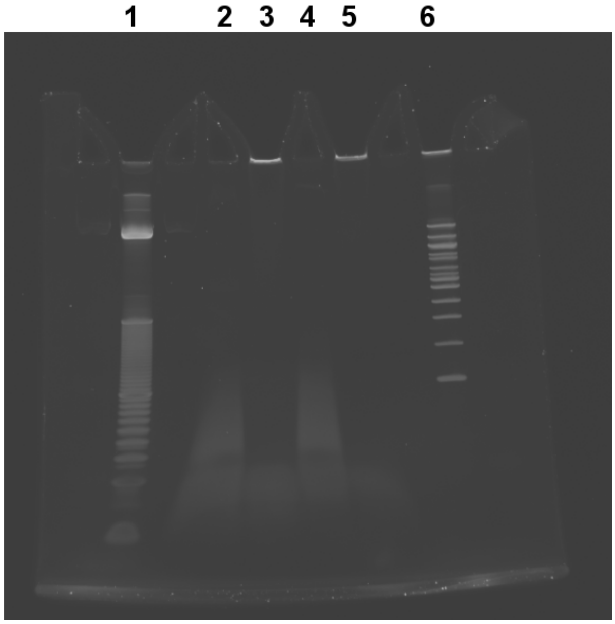
To estimate the labeling efficiency, a gel-shift assay can be performed (see below). In general, greater than 90% of the fragments should be labeled and, therefore, shifted.

Gel-Shift Assay

The efficiency of the labeling procedure can be assessed using the following procedure. This quality control protocol prevents hybridizing poorly labeled target onto the probe array. The addition of biotin residues is monitored in a gel-shift assay, where the fragments are incubated with avidin prior to electrophoresis. The nucleic acids are then detected by staining, as shown in the gel photograph (Figure 3.1.2). The procedure takes approximately 90 minutes to complete.

✔ Note

The absence of a shift pattern indicates poor biotin labeling. The problem should be addressed before proceeding to the hybridization step.



Lane 1	10 bp Ladder
Lane 2 & 4	Fragmented and labeled cDNA from *P. aeruginosa*
Lane 3 & 5	Fragmented and labeled cDNA from *P. aeruginosa* with avidin
Lane 6	100 bp Ladder

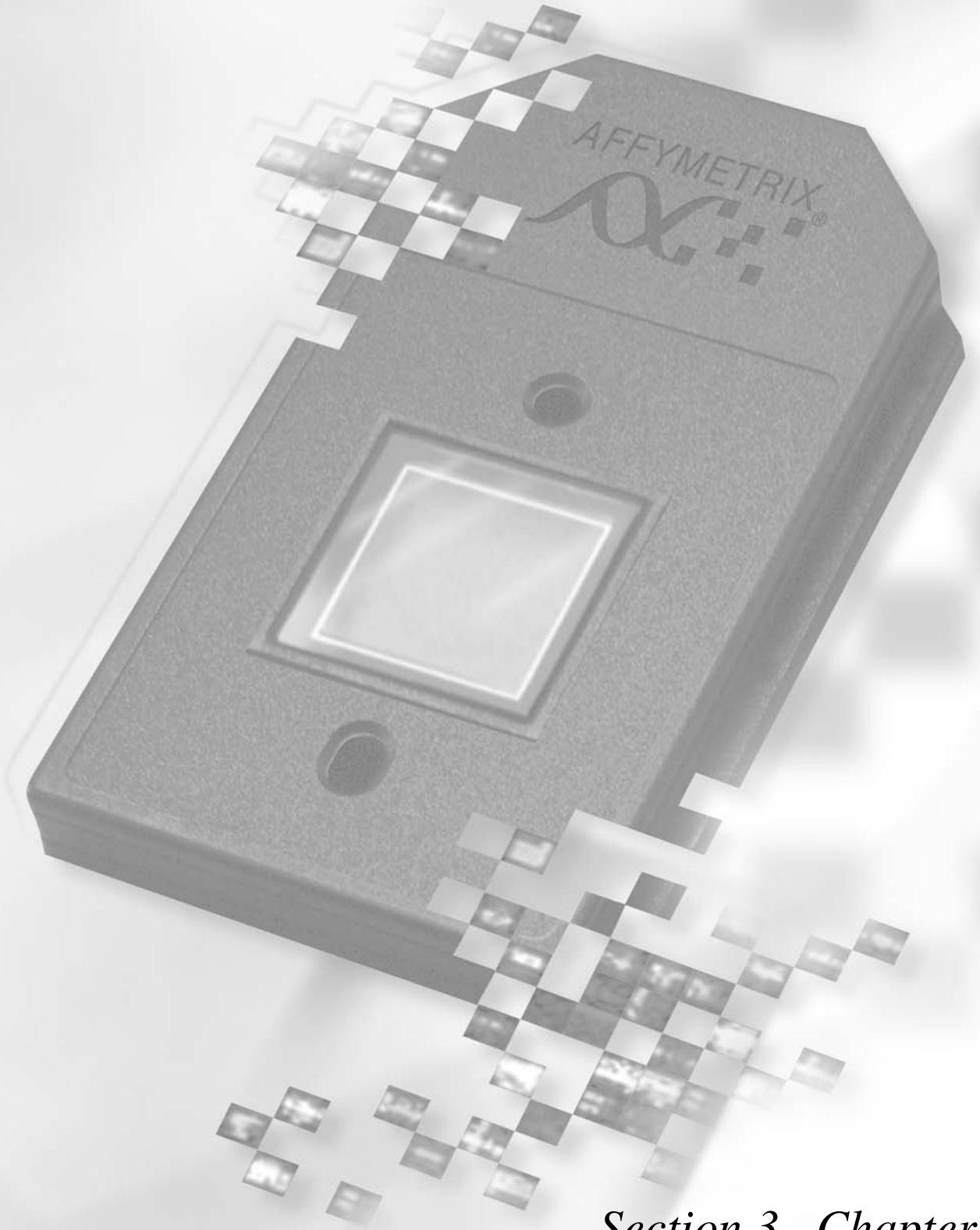
Figure 3.1.2
Gel-shift for monitoring *P. aeruginosa* target labeling efficiency. Notice that the majority of the avidin-conjugated product, in both lanes 3 and 5, is highlighted as a bright band towards the top of the gel.

1. Prepare a NeutrAvidin solution of 2 mg/mL in PBS.
2. Place a 4% to 20% TBE gel into the gel holder and load system with 1X TBE Buffer.
3. For each sample to be tested, remove two 150 to 200 ng aliquots of fragmented and biotinylated sample to fresh tubes.
4. Add 5 µL of 2 mg/mL NeutrAvidin to one of the two tubes for each sample tested.
5. Mix and incubate at room temperature for 5 minutes.
6. Add loading dye to all samples to a final concentration of 1X loading dye.
7. Prepare 10 bp and 100 bp DNA ladders (1 µL ladder +7 µL water+2 µL loading dye for each lane).
8. Carefully load samples and two ladders on gel. Each well can hold a maximum of 20 µL.
9. Run the gel at 150 volts until the front dye (red) almost reaches the bottom. The electrophoresis takes approximately 1 hour.
10. While the gel is running, prepare at least 100 mL of a 1X solution of SYBR Gold for staining.

✔ Note

SYBR Gold is light sensitive. Therefore, use caution and shield the staining solution from light. Prepare a new batch of stain at least once a week.

11. After the gel is complete, break open cartridge and stain the gel in 1X SYBR Gold for 10 minutes.
12. Place the gel on the UV light box and produce an image following standard procedure. Be sure to use the appropriate filter for SYBR Gold.



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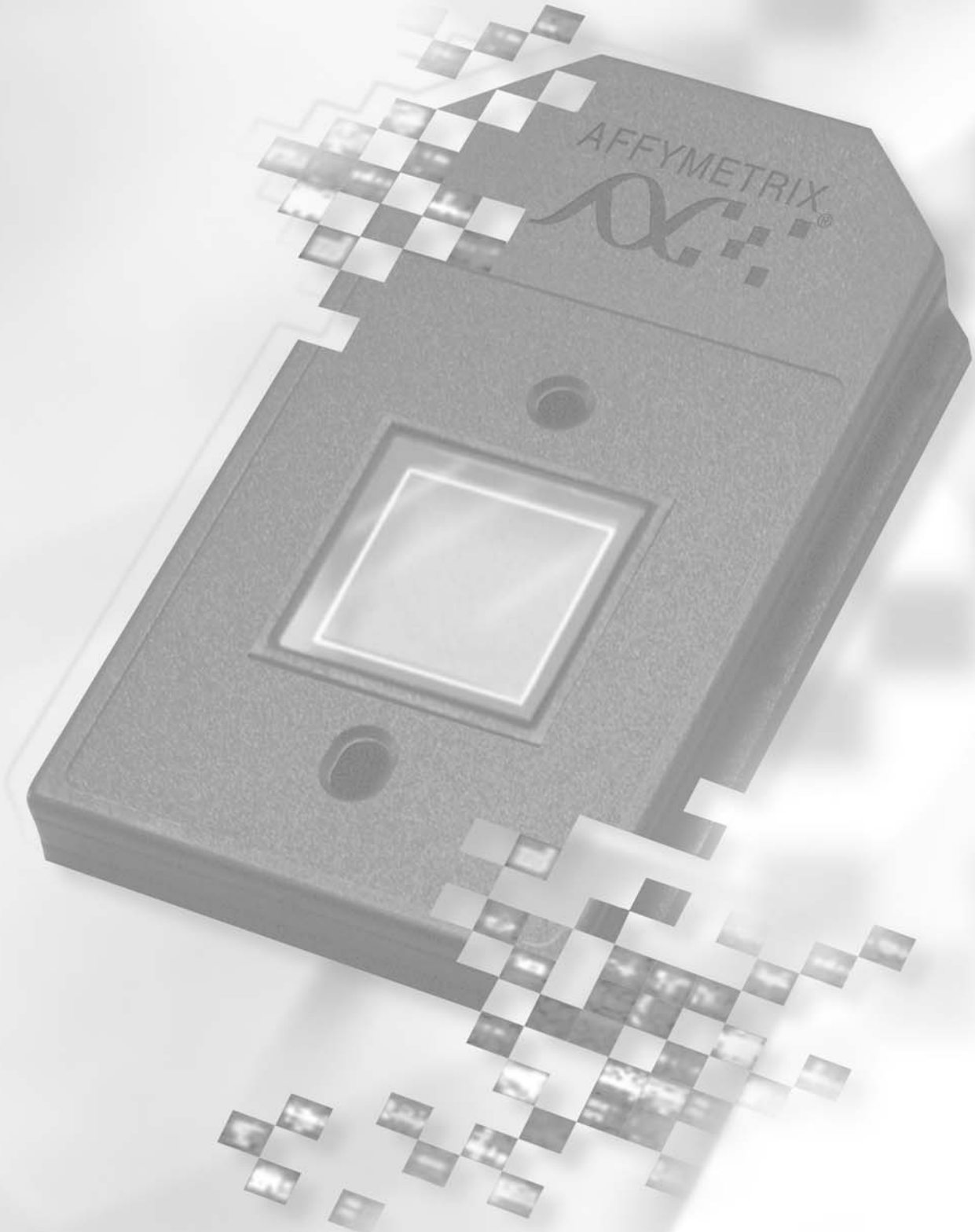


Prokaryotic Target Hybridization

Reagents and Materials Required 3.2.5
Reagent Preparation 3.2.6
Prokaryotic Target Hybridization 3.2.7

This Chapter Contains:

This chapter contains detailed steps for preparing the hybridization mix, and instructions for hybridizing the target mix to the GeneChip® *P. aeruginosa* Genome Array and GeneChip® *E. coli* Antisense Genome Array. The hybridized probe array is then ready for washing, staining, and scanning as detailed in Section 3, 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
- Control Oligo B2, 3 nM, Affymetrix, P/N 900301 (can be ordered separately)
- NaCl, 5M, 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

Miscellaneous Reagents

- Tough-Spots, Label Dots, USA Scientific, P/N 9185 (optional)
- 100% DMSO, Sigma-Aldrich, P/N D2650
- 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

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 (50 mL)

(Final 1X concentration is 100mM 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.

Prokaryotic Target Hybridization

After determining that the fragmented cDNA is labeled with biotin, prepare the hybridization solution mix. The minimum amount of cDNA product required for target hybridization is 1 µg. The solution is stable for approximately 6 to 8 hours at 4°C. The following protocol can be used for freshly prepared or frozen hybridization cocktail. Re-use of prokaryotic sample has not been thoroughly tested and, therefore, is not recommended.

1. Prepare the following hybridization solution mix.

Table 3.2.1
Hybridization Cocktail for Single Probe Array*

Components	169 Format (Mini)	100 Format (Midi)	49 Format (Standard)	Final Concentration
2X Hybridization Buffer	40 µL	65 µL	100 µL	1X
3 nM B2 Control Oligo	1.3 µL	2.2 µL	3.3 µL	50 pM
10 mg/mL Herring Sperm DNA	0.8 µL	1.3 µL	2.0 µL	0.1 mg/mL
50 mg/mL BSA	0.8 µL	1.3 µL	2.0 µL	0.5 mg/mL
100% DMSO	6.2 µL	10.2 µL	-	7.8% (or 0%)
Fragmented and Labeled cDNA	25 µL	Up to 50 µL	Up to 50 µL	0.5 – 7.0 µg
Molecular Biology Grade Water	5.9 µL	-	42.7 µL	
Total Volume	**80 µL**	**130 µL**	**200 µL**	

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

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

✔ Note

It is important to allow the arrays to normalize to room temperature completely. Specifically, if the rubber septa are not equilibrated to room temperature, they may be prone to cracking, which leads to leaks.

3. Add the indicated amount of hybridization solution mix to the probe array. Refer to specific probe array package insert for information on array format.

✔ 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.

4. Place probe array in the hybridization oven set at the temperatures indicated below.

- *P. aeruginosa* 50°C
- *E. coli Antisense* 45°C

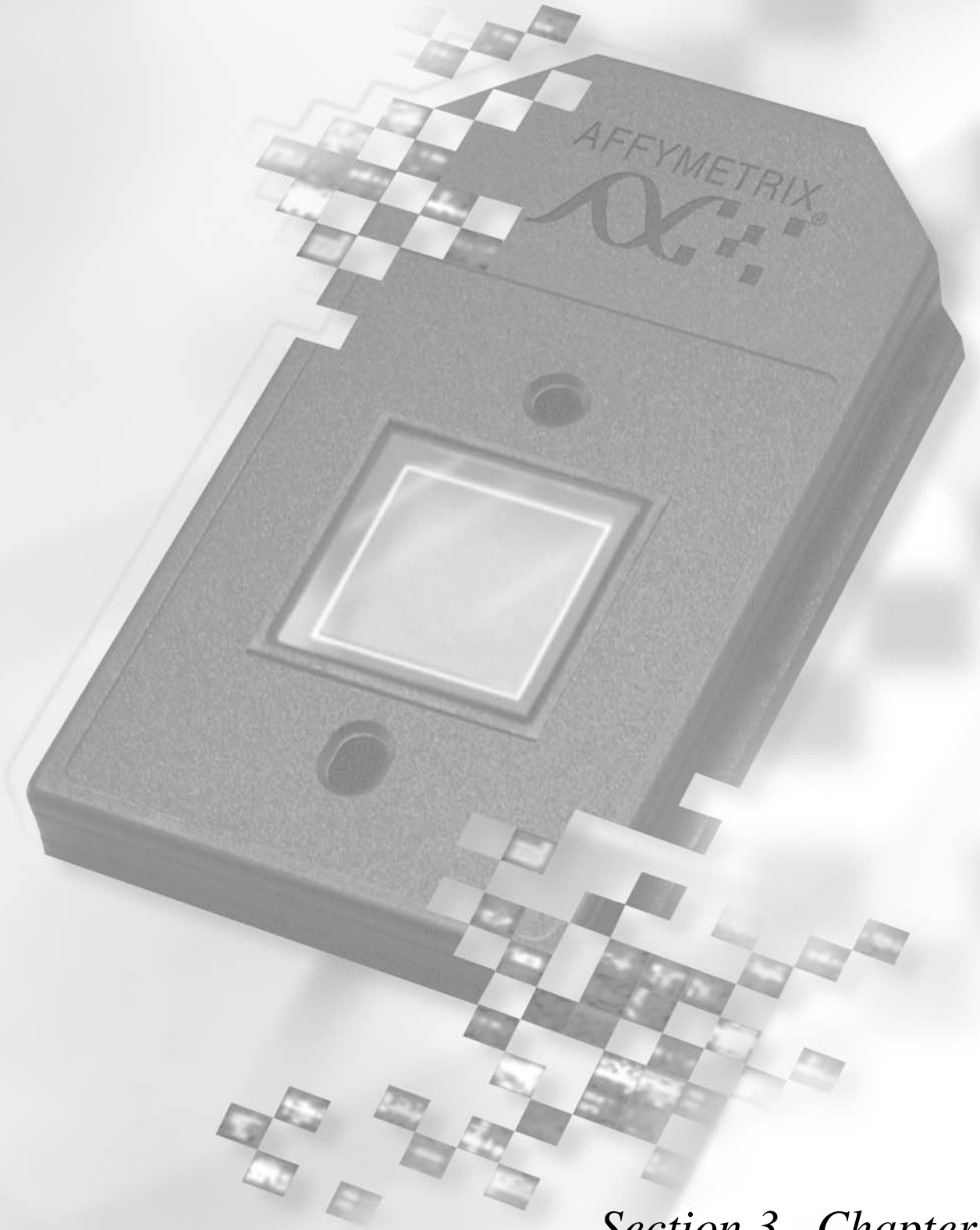
✔ Note

The hybridization temperature of 50°C is higher than that used for other expression assays. The increased hybridization temperature is required due to the high GC content of P. aeruginosa.

5. Avoid stress to the motor; load probe arrays in a balanced configuration around axis. Rotate at 60 rpm.

6. Hybridize for 16 hours.

During the latter part of the 16-hour hybridization, proceed to Section 3, Chapter 3, *Prokaryotic Arrays: Washing, Staining, and Scanning* to prepare reagents required immediately after completion of hybridization.



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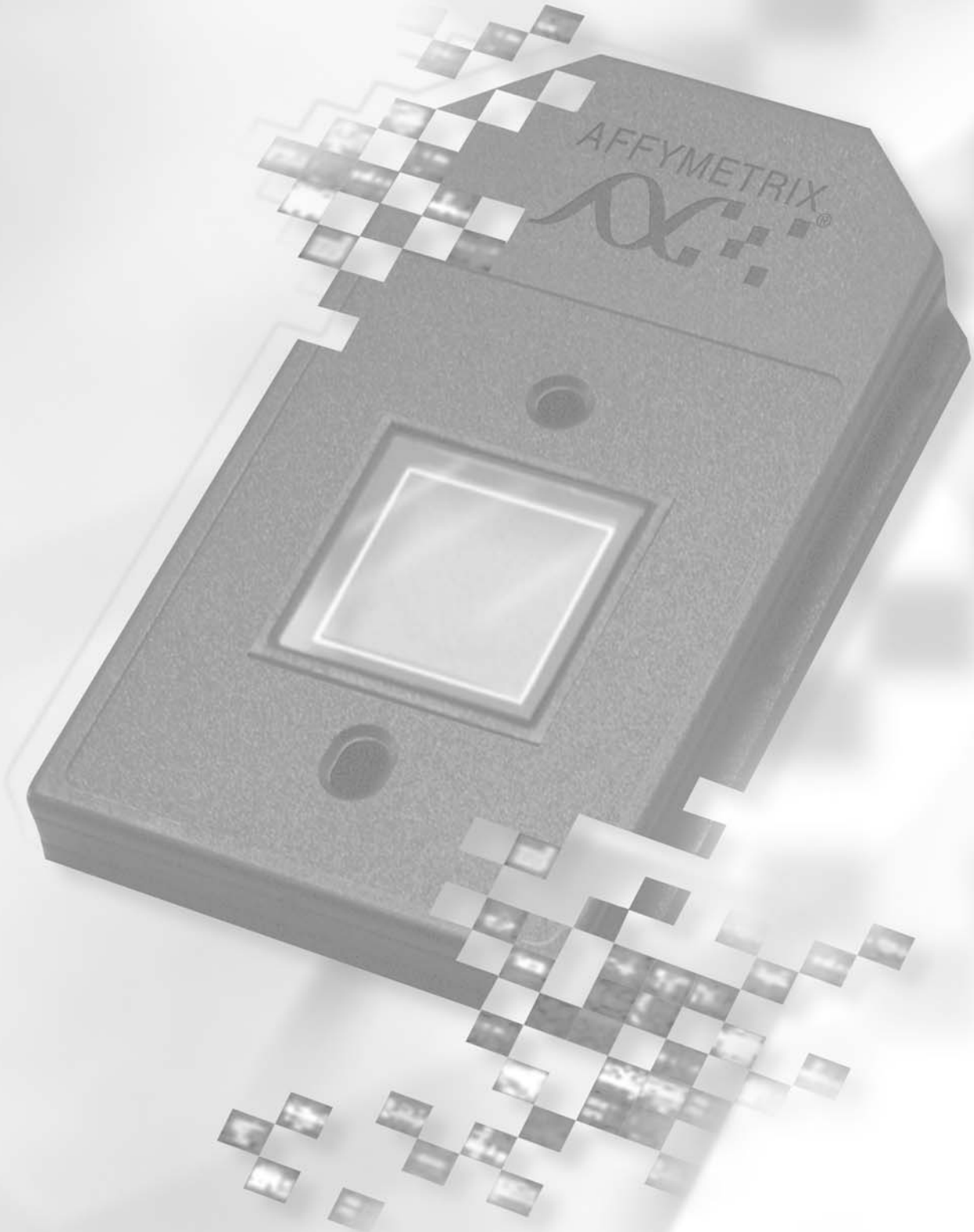


Prokaryotic Arrays: Washing, Staining, and Scanning

Reagents and Materials Required 3.3.5
Reagent Preparation 3.3.6
Experiment and Fluidics Station Setup 3.3.8
Step 1: Defining File Locations 3.3.8
Step 2: Entering Experiment Information. 3.3.8
Step 3: Preparing the Fluidics Station. 3.3.9
Probe Array Wash and Stain 3.3.10
Probe Array Scan 3.3.17
Handling the GeneChip® Probe Array 3.3.17
Scanning the Probe Array 3.3.18
Shutting Down the Fluidics Station 3.3.19
Customizing the Protocol 3.3.20

Prokaryotic

This Chapter Contains:

- Instructions for using the Fluidics Station 400 or 450/250 to automate the washing and staining of GeneChip *P. aeruginosa* and GeneChip *E. coli* Antisense Genome 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
- Nuclease-free Water, Ambion, P/N 9930
- Bovine Serum Albumin (BSA) solution, 50 mg/mL, Invitrogen Life Technologies, P/N 15561-020
- R-Phycoerythrin Streptavidin, Molecular Probes, P/N S-866
- NaCl, 5M, RNase-free, DNase-free, Ambion, P/N 9760G
- PBS, pH 7.2, Invitrogen Life Technologies, P/N 20012-027
- 20X SSPE (3 M 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
- Bleach (5.25% Sodium Hypochlorite), VWR Scientific, P/N 37001-060 (or equivalent)
- ImmunoPure Streptavidin, Pierce Chemical, P/N 21125

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.

Store at room temperature.

Wash Buffer B: Stringent Wash Buffer

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

For 1,000 mL:

83.3 mL of 12 X MES Stock Buffer (see Section 3, 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 3, Chapter 2)

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 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.

1 mg/mL Streptavidin Stock

Resuspend 5 mg in 5 mL of PBS.

Store at 4°C.

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.

   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)

2. 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:

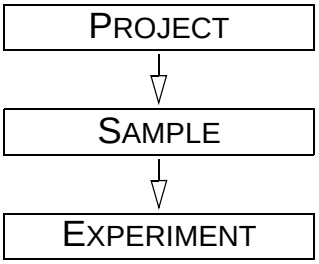
- Experiment Name
- Probe Array Type

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 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 list is accessed for choosing the Protocol for each of the four fluidics station modules.

✔ Note

Refer to the appropriate GeneChip® 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. 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

Following hybridization, the wash and stain procedures are carried out by the Fluidics Station. A modified FlexMidi_euk2v3 fluidics script (FlexMidi_euk2v3_450, if you are using the FS-450) is used for the GeneChip *P. aeruginosa* Genome Array, and the ProkGE-WS2 fluidics script (ProkGE-WS2_450, if you are using the FS-450) is used for the GeneChip *E. coli* Antisense Genome Array. The procedures take approximately 75 and 90 minutes, respectively, to complete. The use of streptavidin in the first part of the stain procedure enhances the overall signal.

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 3.3.1.

✔ 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.

Table 3.3.1
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

Preparing the Staining Reagents

1. Prepare the following stain and wash solutions the day of the procedure. The solutions are stable for approximately 6 to 8 hours at 4°C. Volumes given are sufficient for one probe array.

   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 3.3.2
Streptavidin Solution Mix - Vial 1

Components	Volume	Final Concentration
2X Stain Buffer	300.0 µL	1X
50 mg/mL BSA	24.0 µL	2 mg/mL
1 mg/mL Streptavidin	6.0 µL	10 µg/mL
Nuclease-free H_20	270.0 µL	—
Total Volume	**600 µL**	

Table 3.3.3
Antibody Solution Mix - Vial 2

Components	Volume	Final Concentration
2X MES Stain Buffer	300.0 µL	1X
50 mg/mL BSA	24.0 µL	2 mg/mL
10 mg/mL Normal Goat IgG	6.0 µL	0.1 mg/mL
0.5 mg/mL Anti-streptavidin Antibody, biotinylated	6.0 µL	5 µg/mL
Nuclease-free H_20	264.0 µL	—
Total Volume	**600 µL**	

Table 3.3.4
SAPE Solution Mix - Vial 3

Components	Volume	Final Concentration
2X MES Stain Buffer	300.0 µL	1X
50 mg/mL BSA	24.0 µL	2 mg/mL
1 mg/mL Streptavidin Phycoerythrin	6.0 µL	10 µg/mL
Nuclease-free H_20	270.0 µL	—
Total Volume	**600 µL**	

2. In the Fluidics Station dialog box on the workstation, select the correct experiment name from the drop-down **Experiment** list. The probe array type will appear automatically.

Array	Fluidics Protocol
■ GeneChip *E. coli* Genome 2.0 Array	Mini_prok2v1 (if using FS-450, Mini_prok2v1_450)
■ GeneChip *E. coli* Antisense Genome Array	ProkGE-WS2 (if using FS-450, ProkGE-WS2_450)
■ GeneChip *P. aeruginosa* Genome Array	Modified FlexMidi_euk2v3* (*See Table 3.3.5. If using FS-450, FlexMidi_euk2v3_450)

IMPORTANT *Fluidics protocols are specific to array format and content. Follow procedures below for specific arrays*

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 when using the Fluidics Station 400.

If you are unfamiliar with inserting and removing probe arrays from the fluidics station modules, please refer to the appropriate *User's Guide* for your GeneChip® Fluidics Station 400, or 450/250.

Table 3.3.5
Modification of FlexMidi_euk2v3 for GeneChip® *P. aeruginosa* Array

GeneChip *P. aeurginosa* Genome Array requires a modification to the **FlexMidi_euk2v3** (or the **FlexMidi_euk2v3_450**) protocol. See below for details.

The **FlexMidi_euk2v3** (or the **FlexMidi_euk2v3_450**) fluidics protocol must be modified. Please follow the instructions carefully to make the modifications. Additionally, it is highly recommended that you save your new *P. aeruginosa* fluidics protocol under a different name to avoid confusion.

1. Modify and save the fluidics protocol for the assay:
   a. Modify the fluidics protocol by using **Tools → Edit Protocol** drop-down list and selecting **FlexMidi_euk2v3** (or the **FlexMidi_euk2v3_450**) within the Protocol Name window.
   b. Change the following parameters: (Enter the new parameters by highlighting the default values and typing in the new values.)

      i) **Wash A1 Temperature** from 30°C to 25°C;

      ii) **Number of Wash B Cycles** from 6 to 4;

      iii) **Stain Temperature (C)** from 35°C to 25°C;

      iv) **First Stain Time (seconds)** from 300 to 600 seconds;

      v) **Second Stain Time (seconds)** from 300 to 600 seconds;

      vi) **Third Stain Time (seconds)** from 300 to 600 seconds; and

      vii) **Wash A3 Temperature** from 35°C to 30°C.
   c. Save the modified fluidics protocol by highlighting **FlexMidi_euk2v3** (or the **FlexMidi_euk2v3_450**) within the Protocol Name window and typing over with an assigned protocol name (e.g., Pae_cDNA). Click **Save**.
      The new fluidics protocol should be present in the Protocol drop-down list and is used in the subsequent steps.
2. Select the name of the newly modified protocol (e.g., Pae_cDNA) from the **Protocol** drop-down list in the Fluidics Station dialog box. Select **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 *User's Guide* for your GeneChip® Fluidics Station 400, or 450/250.

Table 3.3.6
Fluidics Scripts Prokaryotic Arrays

Format	Fluidics Scripts
169	Mini_prok2v1
100	modified FlexMidi_Euk2v3
49	ProkGE_WS2

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

Table 3.3.7
Fluidics Protocols

	Mini_prok2v1[a]	FlexMidi_euk2v3[b]	Modified FlexMidi_euk2v3 for *P. aeruginosa* Array	ProkGE_WS2[c]
Post Hyb Wash #1	10 cycles of 2 mixes/cycle with Wash Buffer A at 30°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**	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	**4** cycles of 15 mixes/cycle with Wash Buffer B at 50°C	4 cycles of 15 mixes/cycle with Wash Buffer B at 45°C
1st Stain	Stain the probe array for 300 seconds in Streptavidin Solution Mix at 35°C	Stain the probe array for **300 seconds** in SAPE Solution Mix at **35°C**	Stain the probe array for **600 seconds** in Streptavidin Solution Mix at **25°C**	Stain the probe array for 600 seconds in Streptavidin Solution Mix at 25°C.
Post Stain Wash	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	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 300 seconds in Antibody Solution Mix at 35°C	Stain the probe array for **300 seconds** in Antibody Solution Mix at **35°C**	Stain the probe array for **600 seconds** in Antibody Solution Mix at **25°C**	Stain the probe array for 600 seconds in Antibody Solution Mix at 25°C.
3rd Stain	Stain the probe array for 300 seconds in SAPE Solution Mix at 35°C	Stain the probe array for **300 seconds** in SAPE Solution Mix at **35°C**	Stain the probe array for **600 seconds** in SAPE Solution Mix at **25°C**	Stain the probe array for 600 seconds in SAPE Solution Mix at 25°C.
Final Wash	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.	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 30°C. The holding temperature is 25°C.

a. Mini_prok2v1_450 fot the FS-450/250
b. FlexMidi_Euk2v3_450 for the FS-450/250
c. ProkGE_WS2_450 for the FS-450/250

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 3.3.6.

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 down, or in the eject position. When finished, verify that the cartridge lever is returned to the up, or engaged, position.

5. Remove any microcentrifuge vial(s) 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.

   a. Place one vial containing streptavidin solution in sample holder 1.

   b. Place one vial containing the anti-streptavidin biotinylated antibody solution in sample holder 2.

   c. Place one vial containing the streptavidin phycoerythrin (SAPE) solution in sample holder 3.

   d. 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 the protocol progresses.

7. When the protocol is complete, the LCD window displays the message **EJECT CARTRIDGE**.

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

9. Lift up on the needle lever to disengage the needles from the microcentrifuge vials. Remove the three microcentrifuge vials from the needle holders.

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

    - If bubbles are present, refer to Table 3.3.8.
    - 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 close the washblock and proceed to *Probe Array Scan* on page 3.3.17.

11. If there are no more samples to hybridize, shut down the fluidics station following the procedure in *Shutting Down the Fluidics Station* on page 3.3.19.
12. Keep the probe arrays at 4°C and in the dark until ready for scanning.
13. Lift up on the cartridge lever to close the washblock.

✔ Note

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

Table 3.3.8
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 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 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 3.3.17. 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 3.3.6.
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.

   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).
4. 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.
5. Remove any microcentrifuge tube remaining in the sample holder of the fluidics station module(s) being used.
6. Place a microcentrifuge tube containing the streptavidin solution into the sample holder, making sure that the metal sampling needle is in the tube 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.
7. When the LCD window indicates, replace the microcentrifuge vial containing the streptavidin stain with a microcentrifuge vial containing antibody stain solution into the

Prokaryotic

sample holder, making sure 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 antibody solution with the microcentrifuge vial containing the streptavidin phycoerythrin (SAPE) solution.
9. When the protocol is complete, the LCD window displays the message **EJECT CARTRIDGE**.
10. Remove microcentrifuge vial containing stain and replace with an empty microcentrifuge tube.
11. Remove the probe arrays from the fluidics station modules by first moving the cartridge lever to the **EJECT** position.
12. Check the probe array window for large bubbles or air pockets.
    - If bubbles are present, refer to Table 3.3.9.
    - If the probe array has no large bubbles, it is ready to scan on the GeneChip Scanner 3000 or the GeneArray® Scanner. ENGAGE washblock and proceed to *Probe Array Scan* on page 3.3.17.

    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 3.3.19.

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

Table 3.3.9
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 washblock 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 3.3.17. 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 GeneChip® Scanner 3000. If the 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.

▲ WARNING *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.*

✔ Note *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, follow this procedure to apply Tough-Spots™ to 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-Spot 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 3.3.1.

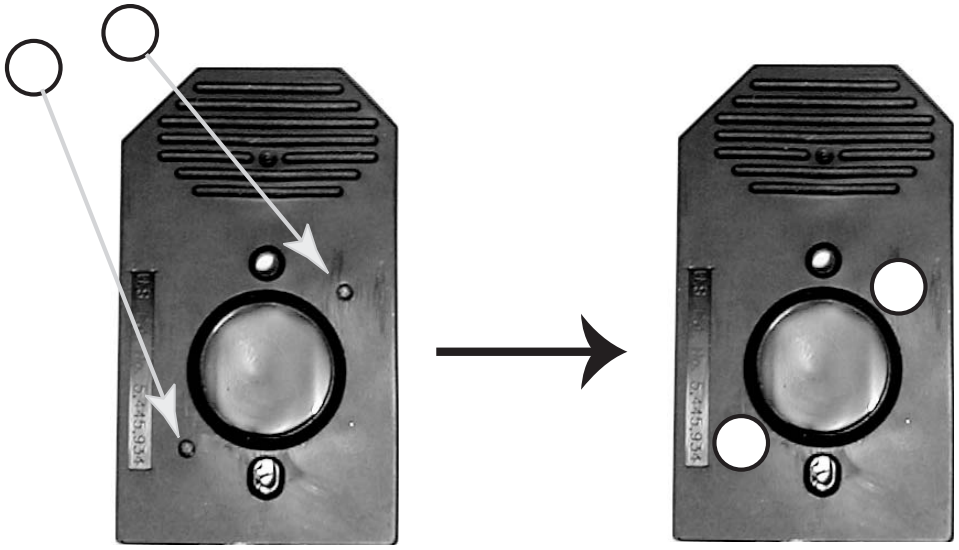


Figure 3.3.1
Applying Tough-Spots™ to the probe array cartridge

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 Agilent® 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 a sample 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. If you are using the GeneChip Scanner 3000, do not attempt to close the door by hand. The door closes automatically through the User Interface when start scan is selected or the scanner goes into stand-by mode.
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. Select **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.
2. In the Edit Protocol dialog box under Protocol Name, click the arrow to open a list of protocols. Click the protocol to be changed.
   ⇒ The name of the protocol is displayed in the **Protocol Name** text 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 parameters within the ranges shown below in Table 3.3.10.

Table 3.3.10
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 86,399
Stain Temperature (°C)	15 to 50
Holding Temperature (°C)	15 to 50

- Wash A1 corresponds to Post Hyb Wash #1 in Table 3.3.7.
- Wash B corresponds to Post Hyb Wash #2 in Table 3.3.7.
- Wash A2 corresponds to Post Stain Wash in Table 3.3.7.
- Wash A3 corresponds to Final Wash in Table 3.3.7.

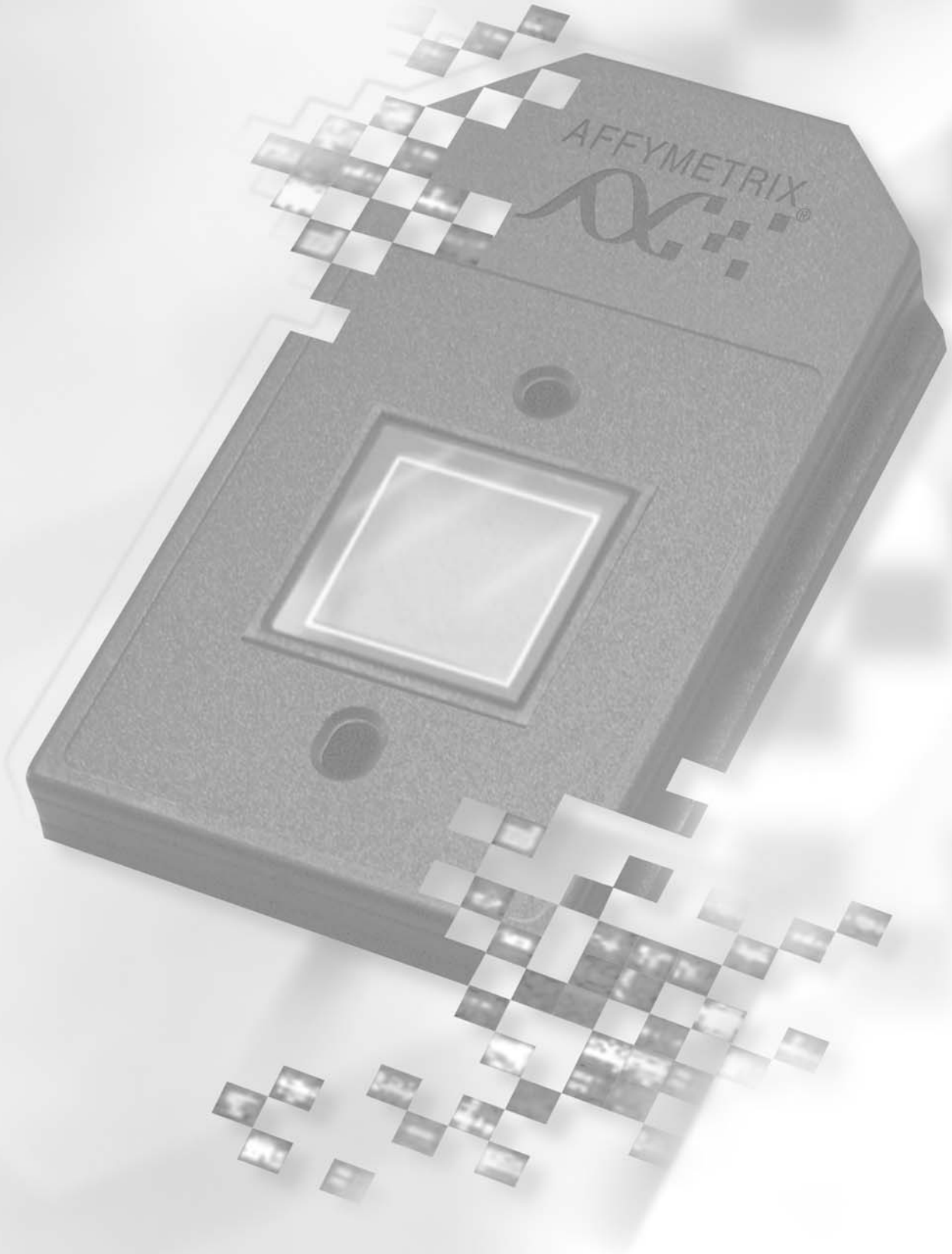
4. To return to the default values for the protocol selected, click the **Defaults** button.
5. Once all the protocol conditions are modified as desired, change the name of the edited protocol in the **Protocol Name** box.

! 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 step is not required.

Section 4:

Fluidics Station Maintenance Procedures



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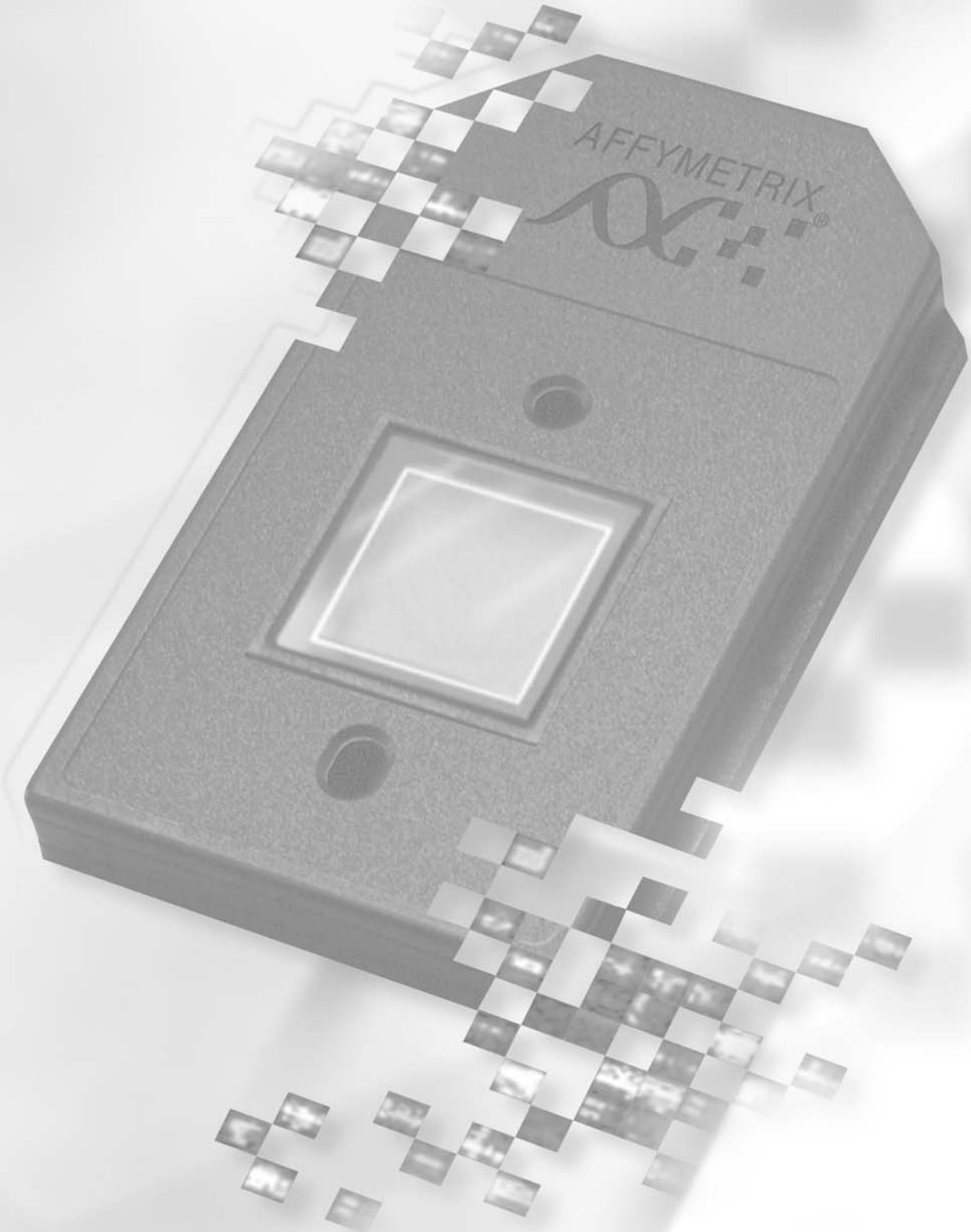

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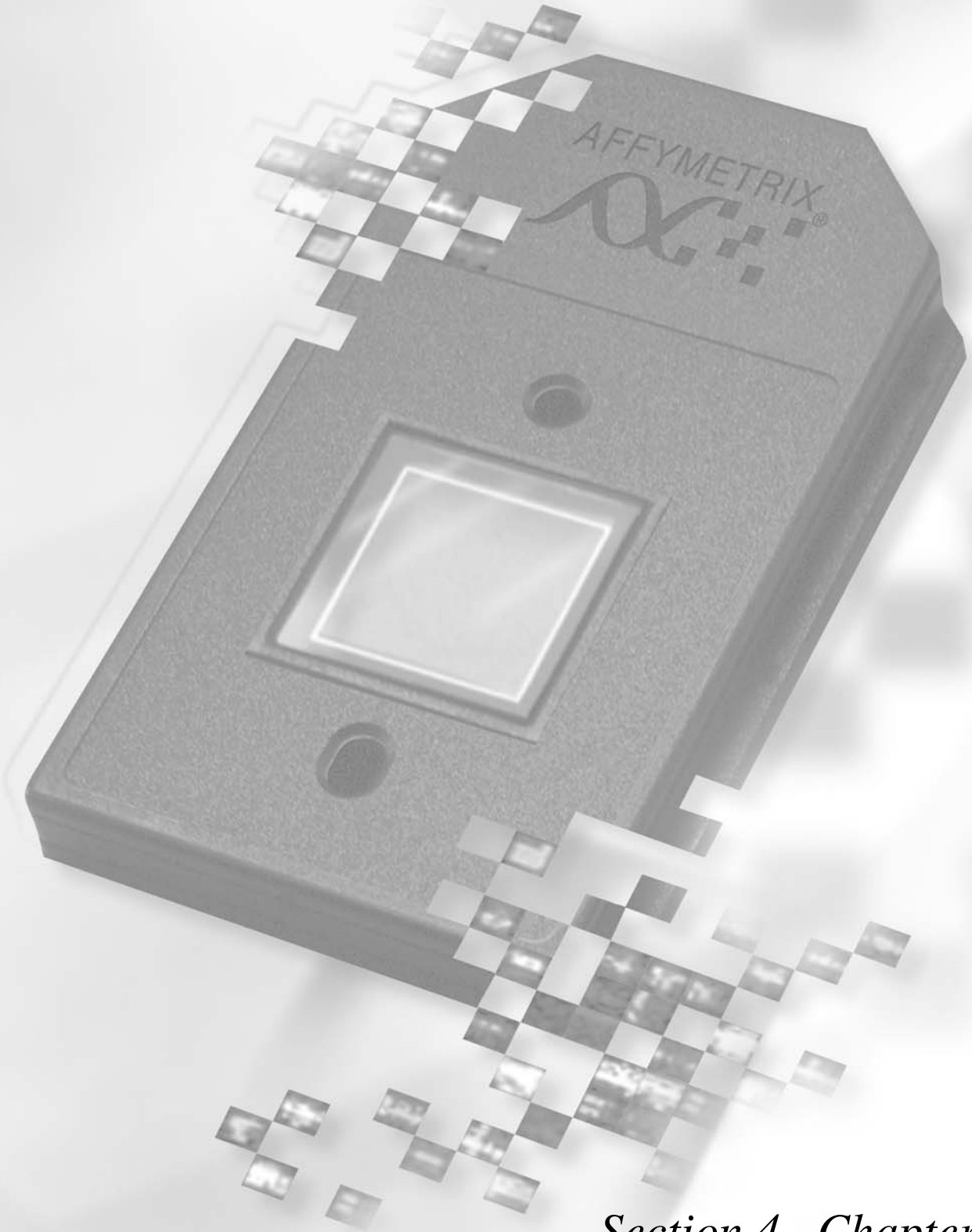

F.S. Maintenance

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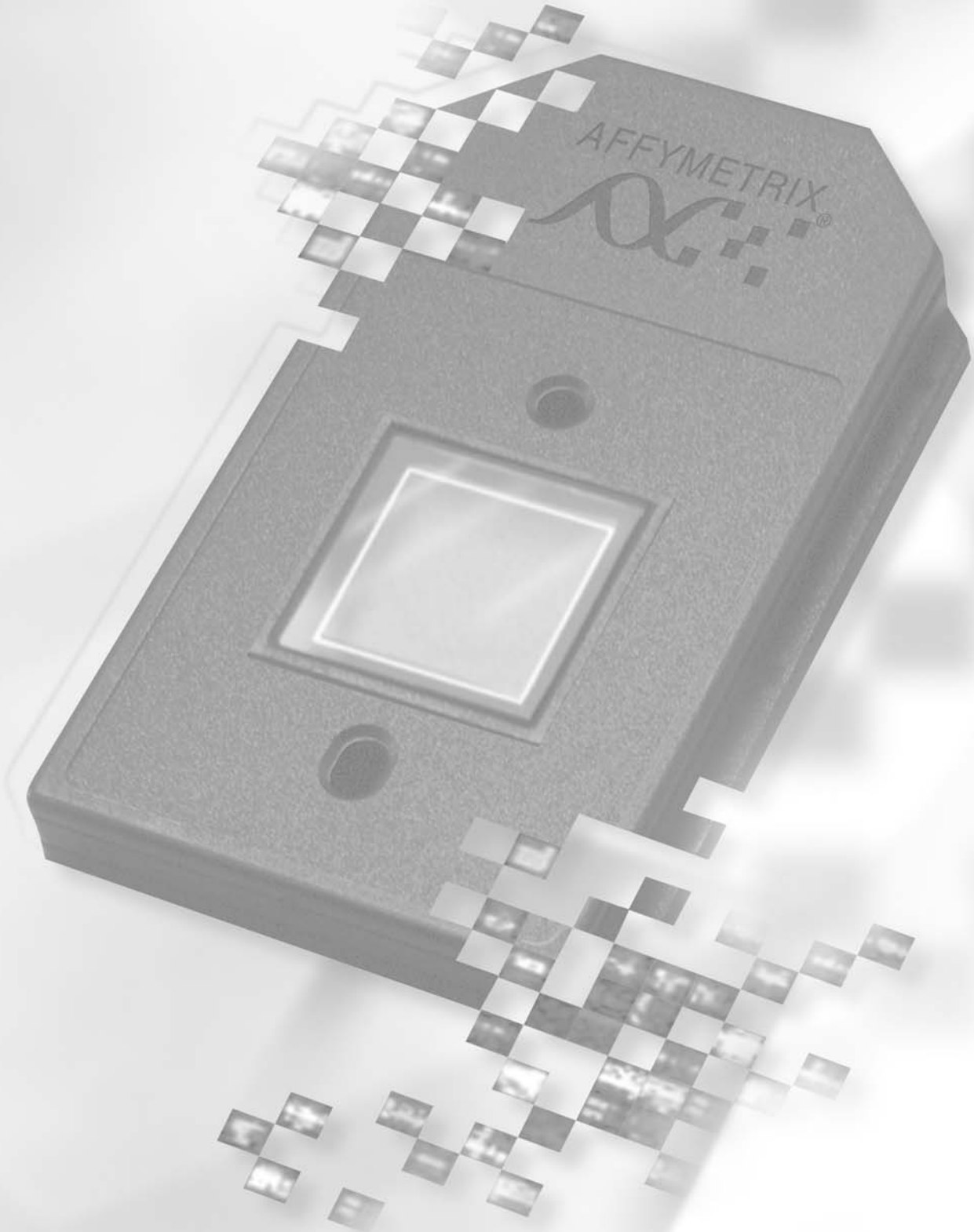


Fluidics Station Maintenance Procedures

Reagents and Materials Required 4.1.5
Weekly Fluidics Station Cleanout. 4.1.7
Bleach Protocol 4.1.7
Monthly Fluidics Station Decontamination Protocol 4.1.13

This Section Contains:

- A weekly fluidics station bleach protocol.
- A monthly fluidics station decontamination protocol.

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.

- Bleach (5.25% Sodium Hypochlorite), VWR Scientific, P/N 37001-060 (or equivalent)
- Tygon Tubing, 0.04″ inner diameter, Cole-Parmer, P/N H-06418-04

Weekly Fluidics Station Cleanout

A cleaning protocol is recommended for fluidics station maintenance if the antibody staining procedure is used. Choose **Bleach** or **Bleach_450** for all modules from the drop-down list in the Fluidics Station dialog box. Click the **Run** button for all modules and follow LCD instructions.

Bleach Protocol

This protocol is designed to eliminate any residual SAPE-antibody complex that may be present in the fluidics station tubing and needles. We recommend running this protocol at least once a week.

1. Prepare 1 liter of 0.525% sodium hypochlorite solution using distilled water. Shake well.

✔ Note

Each fluidics station with four modules requires at least 500 mL of the 0.525% sodium hypochlorite solution.

2. Cut tubing.

FS-450

If you are using the Fluidics Station 450/250:

If you are using the FS-450 or FS-250, cut three pieces of tubing with each piece at least 2.5-3 feet in length (Tygon tubing, 0.04”) for each module of each fluidics station, for a total of 6 tubes (for the FS-250) or 12 tubes (for the FS-450). These can be reused for subsequent BLEACH runs.

FS-400

If you are using the Fluidics Station 400:

If you are using the FS-400, cut four pieces of tubing at least 2.5-3 feet in length.

3. Place all three wash lines (these are not the tubing on the needles but the supply lines from the reagent bottles on the side of the station) of each fluidics station in 1 liter of distilled water.

✔ Note *The BLEACH protocol requires at least 550 mL of distilled water.*

4. Choose **Fluidics** from the **Run** menu. Alternatively, click the down arrow Protocol list on the toolbar.
5. Choose **Bleach** or **Bleach_450** for the respective modules in the **Protocol** drop-down list.
6. Disengage the washblock for each module by pressing down on the cartridge lever (Figure 4.1.1).

Washblocks disengaged with cartridge levers down

Figure 4.1.1
Disengaged washblocks showing cartridge levers in the down position.

✔ Note *Temperature will ramp up to 50°C.*

7. Connect tubing to needles.

FS-450

If you are using the Fluidics Station 450/250:

If you are using the FS-450/250, connect one end of the plastic tubing to each of the three needles. The proper technique is to press down on the cartridge lever until the needles extend a convenient distance from the module, then slip the tube on as you hold the cartridge lever down with the free hand as shown in Figure 4.1.2. Take care not to bend or break the needles.

FS-400

If you are using the Fluidics Station 400:

If you are using the FS-400, connect one end of the plastic tubing to each needle at the bottom of each module.

Figure 4.1.2
Inserting tubes on the needles. Take care not to break or bend the needles.

8. Insert the other ends into 0.525% sodium hypochlorite solution (at least 500 mL for all four modules) as shown in Figure 4.1.3.

✔ Note *Remove cartridges before you start the bleach protocol.*

Figure 4.1.3
The tubes extending from the modules to the bleach bottle. Note that the probe array cartridges must be removed before the protocol can begin.

9. Ensure that all the tube ends remain immersed in the bleach solution by tamping down on the tubes using a dowel or similar object as shown in Figure 4.1.4.

Figure 4.1.4
Tamp down on the tubes to ensure that the ends remain immersed in the solution.

✔ Note *For ease of handling, band the tubes together using a rubber band.*

10. Remove any probe array cartridges and engage the washblock as shown in Figure 4.1.5. The fluidics station will begin the protocol, begin to empty the lines, and perform three cleaning cycles of 10 rinses each using bleach solution.

Figure 4.1.5
Remove probe array cartridges and pull up on the cartridge lever to engage the washblock and begin the protocol.

11. When the fluidics station LCD window displays **Remove Tube from Needles**, carefully remove tubing from each module needle by pushing the tubing down with your fingers while holding the needle with the other.

IMPORTANT *Do not pull the tube outwards, as this may bend and possibly damage the needle in the process.*

12. Load empty microcentrifuge vials onto each module.

The fluidics station will empty the lines and run three cycles with three rinses each. In addition, the fluidics station will rinse the needle 20 times, twice using distilled water, then bring the temperature back to 25°C and drain the lines with air.

13. The LCD display will read **CLEANING DONE**.

Monthly Fluidics Station Decontamination Protocol

To maintain your Fluidics Station in the best possible working condition, we recommend that the following decontamination protocol be performed on your fluidics station **at least once a month**, in addition to the weekly cleaning described above. The protocol requires approximately 2 hours to run.

This protocol ensures that all of the tubing associated with the station is kept thoroughly clean. Keeping this tubing as clean as possible ensures that array images will be optimized and high-quality results will be obtained.

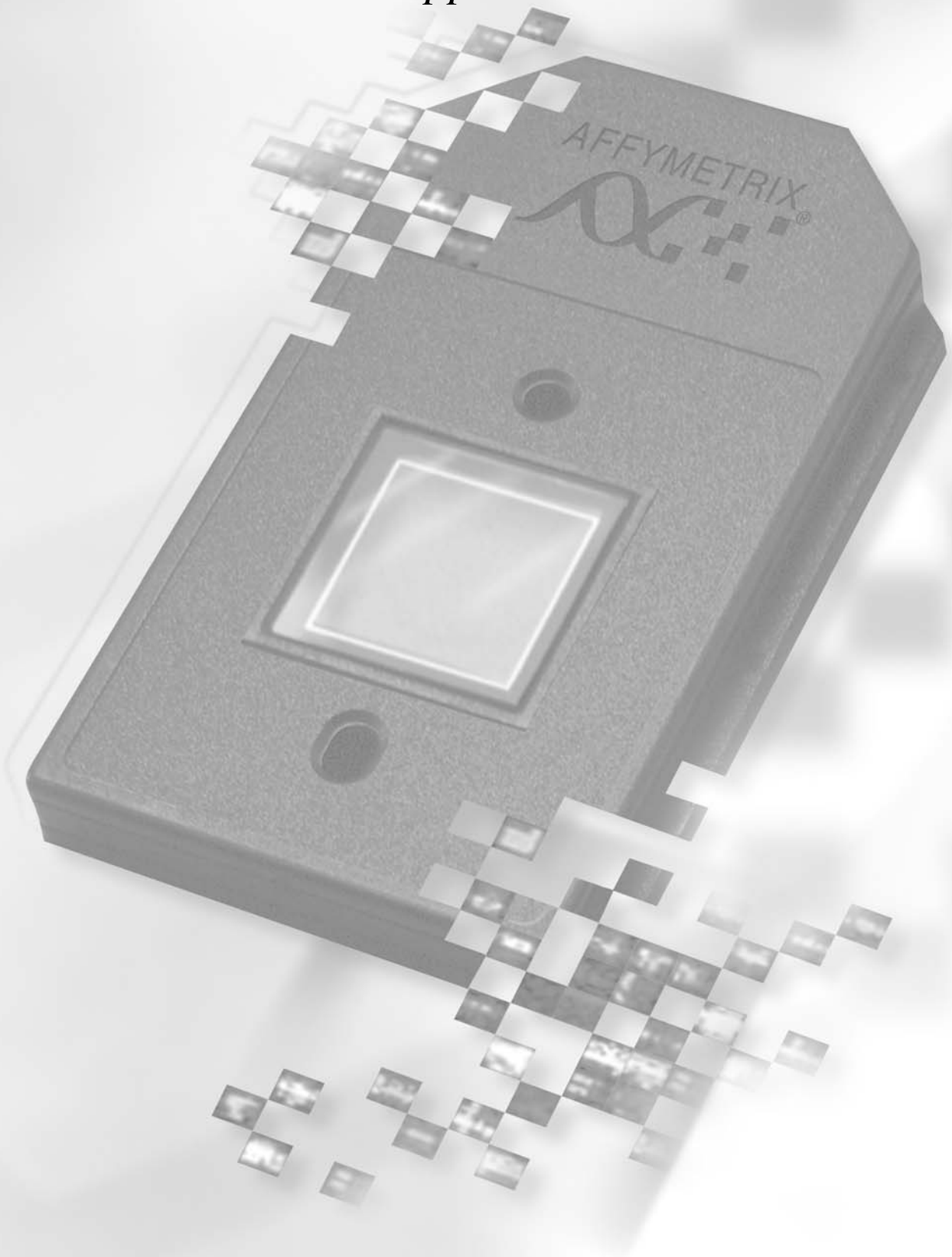
1. Prepare 2 liters of 0.525% sodium hypochlorite solution using distilled water. Mix well.
2. Place all three wash lines of the fluidics station in 1 liter of 0.525% sodium hypochlorite solution.
3. Run the **Prime** protocol (Fluidics Station 400) or the **Prime_450** protocol (Fluidics Station 450/250) on all four modules with wash lines in 0.525% sodium hypochlorite solution (instead of wash buffers A and B).
4. Run the **Shutdown** protocol (Fluidics Station 400) or the **Shutdown_450** (Fluidics Station 450/250) on all four modules with wash lines in 0.525% sodium hypochlorite solution (instead of distilled water).
5. Follow Bleach Protocol (as described on page 4.1.7) with the following change in Step 3: place the three wash lines of the fluidics station in 1 liter of 0.525% sodium hypochlorite solution instead of distilled water.
6. Change intake tubing and peristaltic tubing, if required (as described in the *Fluidics Station 400 User's Guide* or the *Fluidics Station 450/250 User's Guide*).
7. Run the **Bleach** protocol (Fluidics Station 400) or the **Bleach_450** protocol (Fluidics Station 450/250) with three wash lines of the fluidics station in distilled water.
8. Run the **Prime** protocol (Fluidics Station 400) or the **Prime_450** protocol (Fluidics Station 450/250) with wash lines in distilled water (instead of wash buffers A and B).
9. Run the **Shutdown** protocol (Fluidics Station 400) or the **Shutdown_450** protocol (Fluidics Station 450/250) with wash lines in distilled water.
10. Run the **Prime** protocol (Fluidics Station 400) or the **Prime_450** protocol (Fluidics Station 450/250) with wash lines in distilled water (instead of wash buffers A and B).
11. Run the **Shutdown** protocol (Fluidics Station 400) or the **Shutdown_450** protocol (Fluidics Station 450/250) with distilled water.

✔ Note

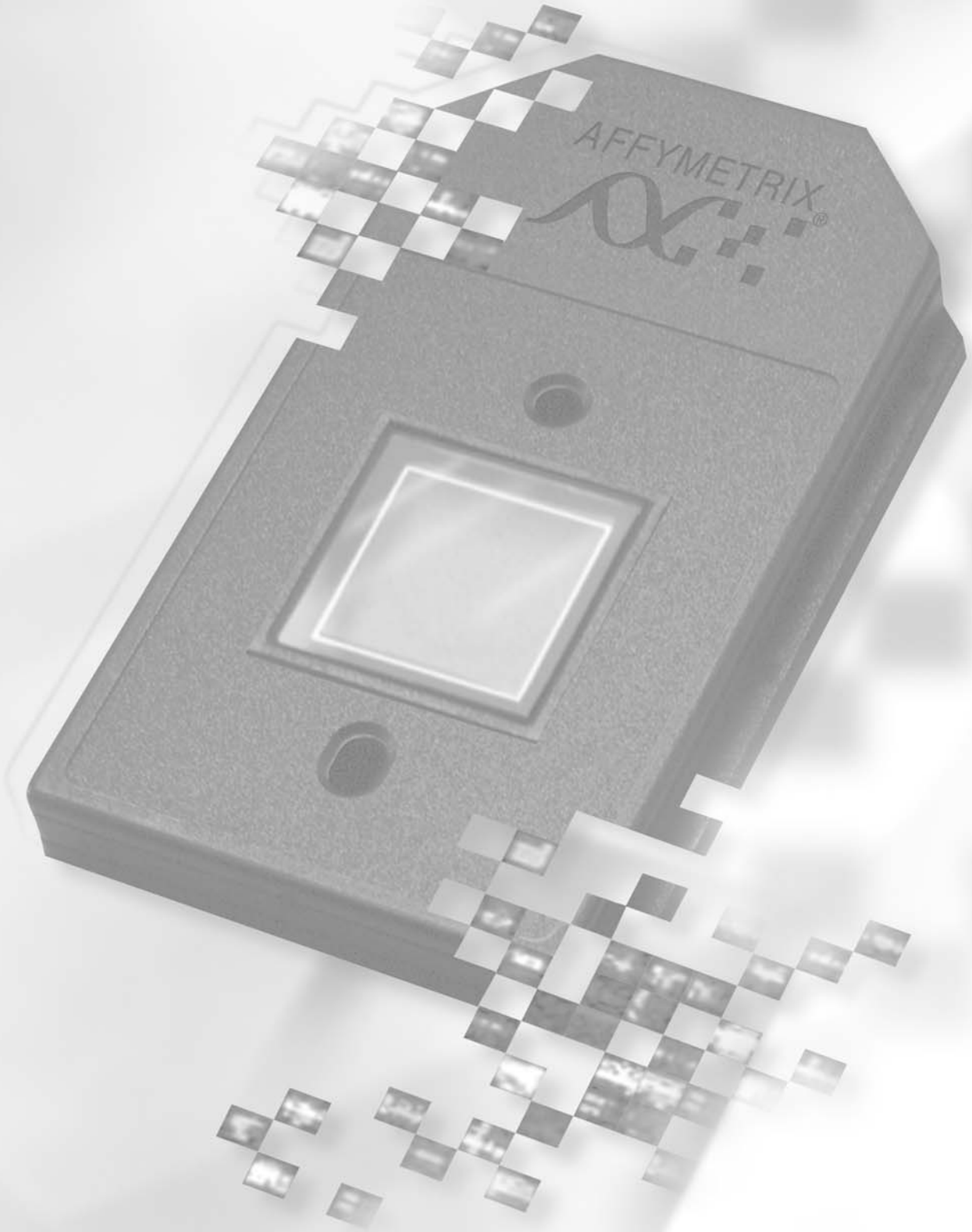
At the end of each step, the fluidics station will indicate a 'ready' status. The fluidics station should not be used until this entire procedure (steps 1-11) is complete.

Section 5:

Appendices



Section 5



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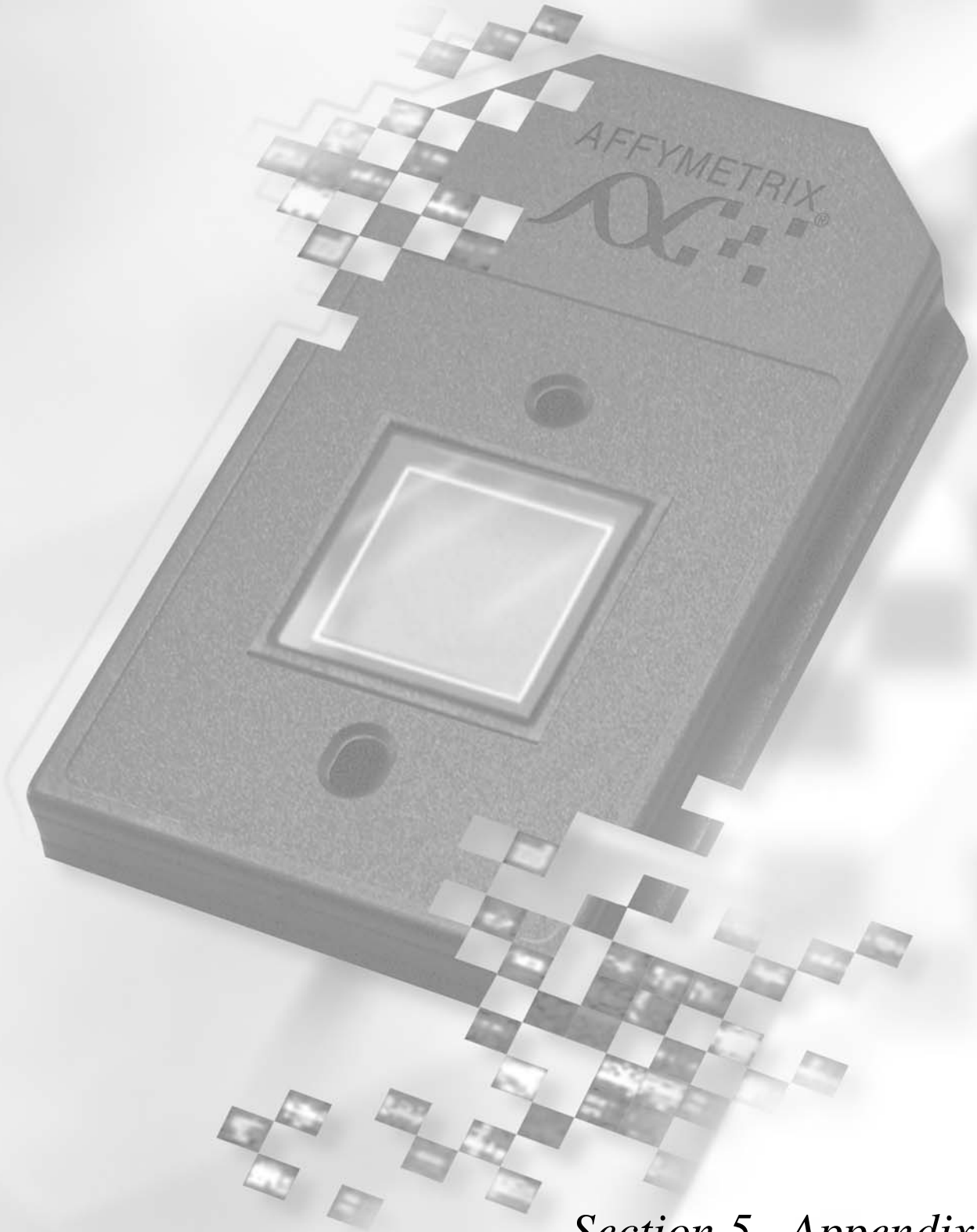
Appendix B *FAQs & Troubleshooting* **5.B.3**

Appendix C *List of Controls on GeneChip Probe Arrays* **5.C.3**



Section 5, Appendix A

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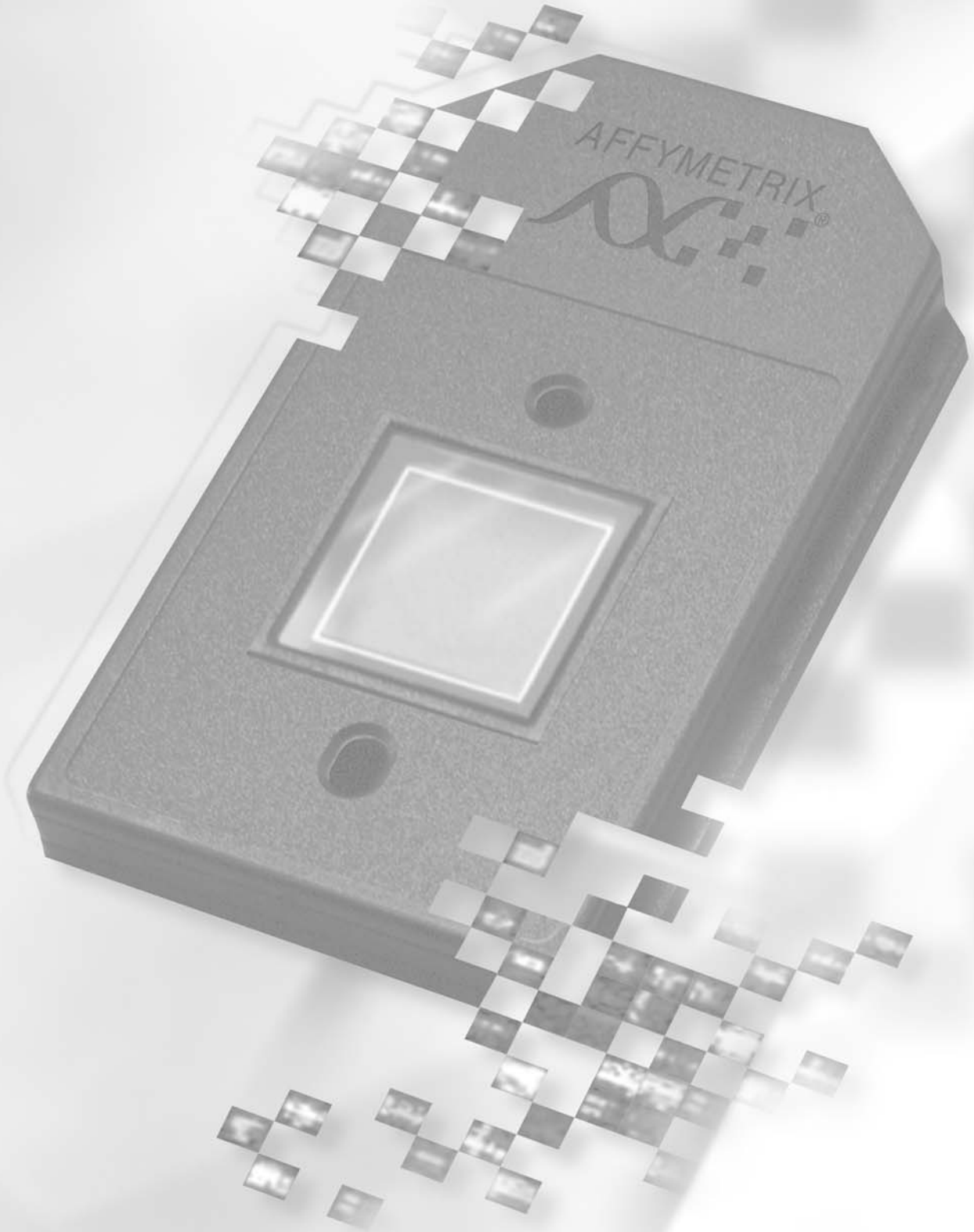

Supplier and Reagent Reference List

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Affymetrix Technical Support

Affymetrix provides technical support via phone or e-mail. To contact Affymetrix Technical Support:

Affymetrix Inc.
3380 Central Expressway
Santa Clara, CA 95051
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Tel: 1-888-362-2447 (1-888-DNA-CHIP)
Fax: 1-408-731-5441
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Fax: +81-(0)3-5730-8201
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Supplier Contact Information

Supplier	Web Site
Ambion	www.ambion.com
Amersham Biosciences	www.amershambiosciences.com
Amresco	www.amresco-inc.com
BD Biosciences	www.bdbiosciences.com/
BioWhittaker Molecular Applications / Cambrex	www.cambrex.com
Brinkmann Instruments	www.brinkmann.com
Cole-Parmer	www.coleparmer.com
Corning Life Sciences	www.corning.com/lifesciences/
CLONTECH	www.bdbiosciences.com/clontech/
Epicentre Technologies	www.epicentre.com
Eppendorf	www.eppendorf.com
Invitrogen Life Technologies	www.invitrogen.com
Millipore Corp	www.millipore.com
Molecular Probes	www.probes.com
New England Biolabs	www.neb.com
Novagen	www.emdbiosciences.com
Pierce Chemical	www.piercenet.com
Promega Corporation	www.promega.com
Proligo	www.gensetoligos.com
QIAGEN	www.qiagen.com
Rainin	www.rainin.com
Roche Diagnostics	www.roche-applied-science.com
Sigma-Aldrich	www.sigma-aldrich.com
USA Scientific	www.usascientific.com
Vector Laboratories	www.vectorlabs.com
VWR Scientific Products	www.vwrsp.com

Reagents and Materials Quick List for Hybridization and Staining

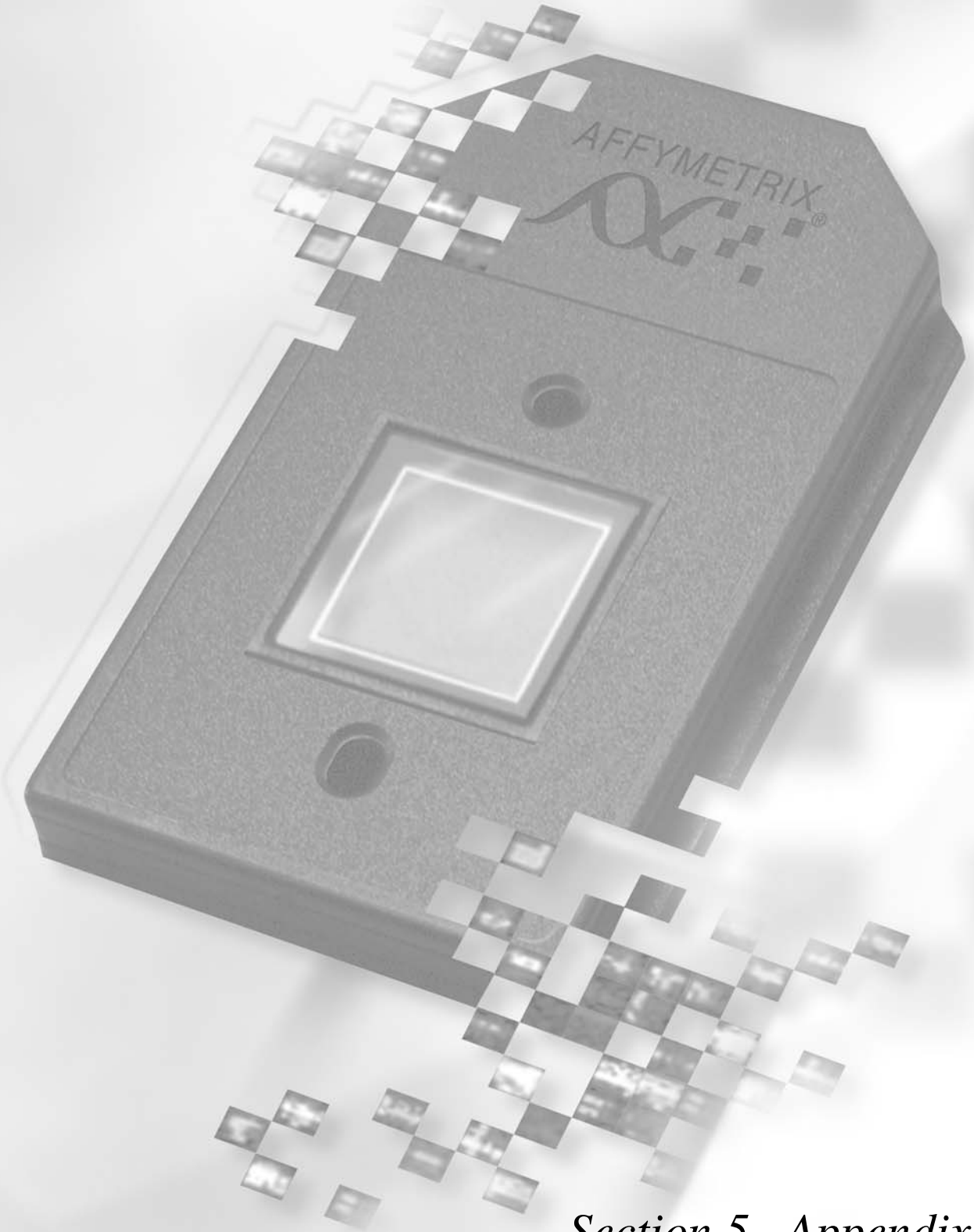
	Reagent	Vendor	P/N	Volume per Rxn	Volume in Kit	Rxns per Kit
Hybridization Cocktail (Following quantities are for 49 Format (Standard) Arrays, see **Table 2.2.1** for details on other array formats.)						
	GeneChip® Eukaryotic Hybridization Control Kit[1]	Affymetrix	900454 or 900457	---	---	30 150
	Herring Sperm DNA	Promega	D1811	3 µL	1 mL	333
	BSA[2]	Invitrogen	15561-020	3 µL	3 mL (3 X 1)	1,000
	2x Hybridization Buffer[3]	---	---	150 µL	50 mL	333
	Mol. Bio. or DEPC-Water	---	---	---	---	---
	DMSO 100%	Sigma-Aldrich	D5879			
Stain Reagents (Following quantities are for the Antibody Amplification Staining Protocol)						
	2x Stain Buffer[4]	---	---	900 µL	250 mL	277
	DI water	---	---	806.4 µL	---	---
	BSA[2]	Invitrogen	15561-020	72 µL	3 mL (3 X 1)	41
	Streptavidin Phycoerythrin (SAPE)	Molecular Probes	S-866	12 µL	1 mL	83
	Goat IgG	Sigma-Aldrich	I5256	6 µL	1 mL	166
	Biotinylated Antibody	Vector Labs	BA-0500	3.6 µL	1 mL	277

[1] The GeneChip Eukaryotic Hybridization Control Kit contains 20x Eukaryotic Hybridization Controls and Oligo B2.

[2] Total Bovine Serum Albumin (BSA) used for hybridization and stain reagents is 75 µL. Each order of BSA contains 3 mL, so each order is sufficient for 40 samples.

[3] See page 2.2.6, for 50 mL preparation.

[4] See page 2.3.6, for 250 mL preparation.



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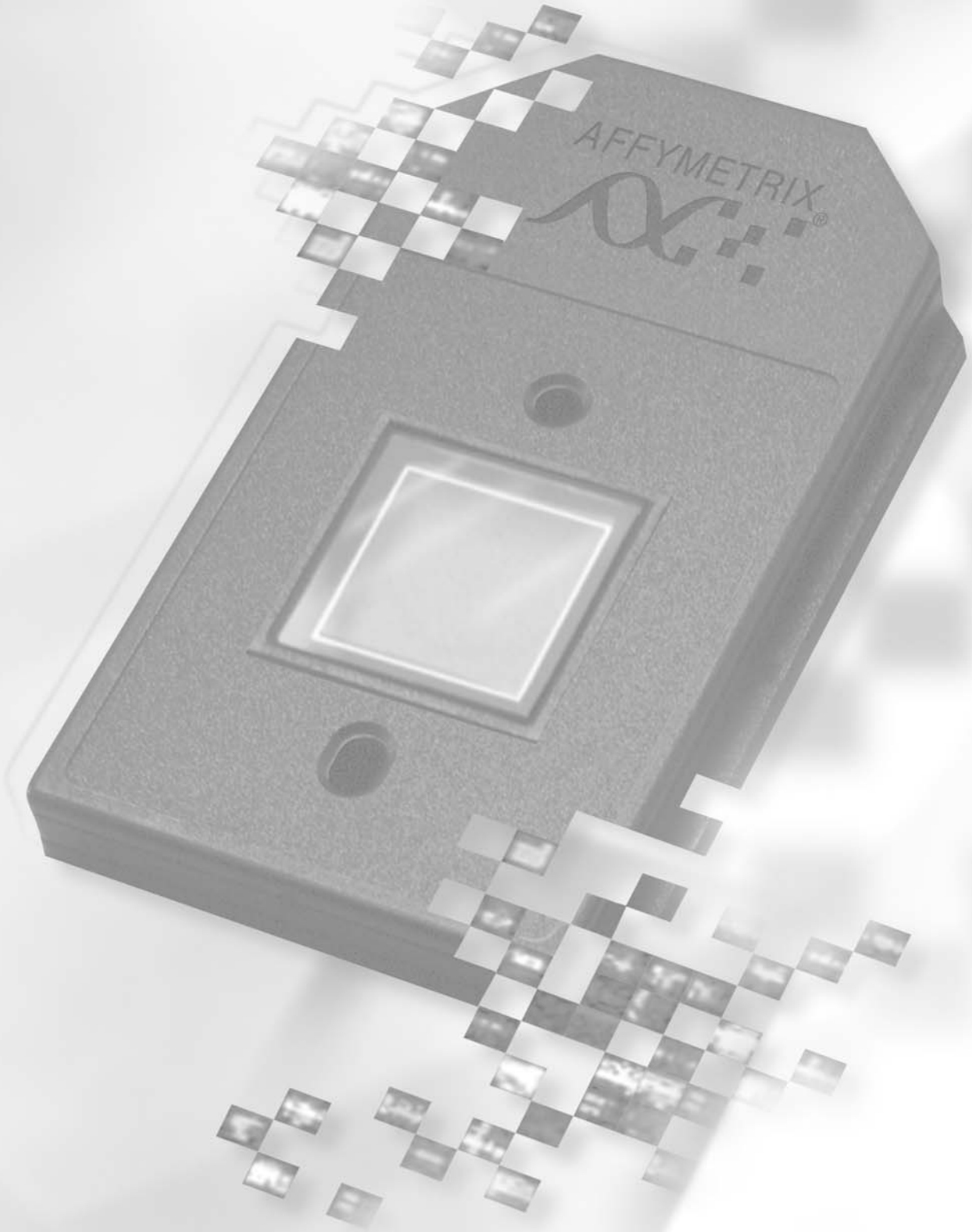


FAQs & Troubleshooting

FAQs

This section contains frequently asked questions related to GeneChip® expression analysis.

Sample Preparation

What is the minimum amount of total RNA I can use for each microarray experiment?

Two protocols have been developed that are optimized for different amounts of starting materials. For 1 to 15 µg of total RNA, or 0.2 to 2 µg of poly-A RNA, the One-Cycle Target Labeling Assay is recommended. For smaller amounts of total RNA, from 10 ng to 100 ng, follow the Two-Cycle Target Labeling Assay. Detailed procedures are described in the Expression Analysis Technical Manual. Follow the instructions carefully for optimal results.

What is the least amount of labeled eukaryotic cRNA target I can put on an array?

You should always use the recommended quantity of cRNA described in this manual. Please refer to Table 2.2.1 for detailed instructions on the amount of cRNA needed for different array formats. Although there is a tolerance for some variation in quantity, we have found that hybridization of significantly less cRNA results in reduced sensitivity, particularly for low-copy transcripts.

How long can I store my eukaryotic cRNA target in the hybridization cocktail after its first hybridization?

Assuming no RNase contamination, the fragmented cRNA targets in the hybridization cocktail can be stored for at least five months at -80°C without significant loss of signal intensity. The fact that the cRNA is fragmented prior to hybridization reduces the effects of any subsequent degradation.

What parameters should I use to QC my GeneChip® probe array data?

Quality assessments are critical in obtaining highly reproducible GeneChip probe array results. QC procedures should be performed at various key checkpoints:

1. RNA sample quality: As described in the Expression Analysis Technical Manual, the quality of starting RNA is very important. Ratio of 260/280 absorbance values, as well as appearance of samples by gel electrophoresis, or Bioanalyzer Total RNA assay are suggested methods to detect any degradation of your RNA samples.
2. Target labeling: Various QC protocols described in the Expression Analysis Technical Manual can be employed at different stopping points of the assay. For example, gel electrophoresis after cDNA synthesis (if using poly-A mRNA as starting material), after cRNA synthesis, and after fragmentation is helpful in estimating quantity and size

distribution. Spectrophotometric measurements are also important after cRNA synthesis. Low cRNA yield can be a sensitive indicator of problematic labeling procedures and/or starting material. You may also want to experiment with using real-time PCR analysis on housekeeping genes after each of these reactions to monitor the efficiency of each step.

3. GeneChip array image and basic data analysis. Routine QC parameters to monitor include visual array inspection, B2 signal, intensity of poly-A RNA and Hybridization controls, background, scaling factor, noise, 3'/5' GAPDH and β-Actin ratios, and % Present calls. Reference the GeneChip® Expression Analysis Data Analysis Fundamentals (P/N 701190) for more detailed information.

Can I hybridize samples to an array from a species other than the organism for which the array was designed?

Affymetrix has not validated the use of GeneChip expression arrays with alternate species. Although there may be high homology between different species, the sequence differences may be sufficient to interfere with hybridization, and more importantly, data interpretation. However, some customers have explored this approach. The following publication is an example of this type of study. Please note that this reference is listed for the convenience of our customers and is not endorsed or supported by Affymetrix.

Kayo, T., Allison, D.B., Weindruch, R., Prolla, T.A. Influences of aging and caloric restriction on the transcriptional profile of skeletal muscles from rhesus monkeys. *Proceedings of the National Academy of Sciences of the USA* **98**:5093-5098 (2001).

When I follow your recommended protocol of isolating total RNA from mammalian tissues, first using TRIzol reagents, then with RNeasy columns, I sometimes see a reduced recovery off the RNeasy columns.

TRIzol reagents and RNeasy columns are based on very different principles for nucleic acids purification. RNeasy columns exclude certain contaminants that may give rise to a falsely higher spectrophotometric reading, including carried-over phenol and transcripts shorter than 200 nucleotides in length. These shorter transcripts include the 5S rRNA and tRNA molecules that may account for 10% or more of the total RNA isolated.

To verify that the RNA of interest has been cleaned up efficiently during column purification, it may be helpful to run aliquots of your samples on a gel or perform some gene-specific real-time PCR quantitation. In addition, you can estimate how much total RNA you anticipate to recover since the yield is highly dependent on tissue type. These reference numbers can be obtained through your own experience or can be found in published literature, for example, the RNeasy Mini HandBook.

If you continue to observe significant loss of material on RNeasy columns, please contact QIAGEN Technical Support directly.

Sample Cleanup Module

Does the GeneChip® Sample Cleanup Module generate comparable results relative to the previously recommended phenol/chloroform extraction for cDNA purification?

Highly concordant results have been obtained during our product development process by comparing global array hybridization results obtained from samples cleaned up with both protocols. The concordance was determined based on the overall signal intensity, as well as the qualitative calls. However, due to the different mechanisms associated with each cleanup procedure, there will be minor differences in the data obtained. For example, cDNA cleanup column reduces the recovery of fragments of 100 nucleotides or less, whereas these fragments are retained in the phenol/chloroform method. However, we do believe these differences are minor in magnitude. Customers are encouraged to perform their own comparisons and analysis to determine when to adopt the Sample Cleanup Module into their laboratories.

The storage condition of this kit has recently been changed. What is the change?

The two columns included in this kit are now recommended to be stored at 2 - 8°C, compared with previously recommended storage at room temperature.

Should I store the rest of the kit components (i.e., the buffers) at the lower temperature as well?

The buffers can be stored at either room temperature or 2 - 8°C. However, if the entire kit is kept at refrigerated temperatures, the buffers should be warmed up to room temperature before use, and all buffers should be checked to make sure that any possible precipitation in the buffer is completely dissolved.

What happens if the recommended storage condition is not followed?

The cDNA Cleanup Columns may age faster than the indicated expiration date. The expired columns may lead to reduced yield and recovery of cDNA at the cleanup step.

Poly-A RNA Control Kit

What is this kit used for?

This kit contains exogenous spike-in poly-A transcripts designed specifically for GeneChip® expression profiling as:

- internal positive controls for both the One- and Two-Cycle Target Labeling Assays.
- a convenient way to monitor the success of the entire labeling process, independent from the starting material quality.
- an evaluation of the assay sensitivity, dynamic range of amplification, as well as data consistency.

What components are in each kit?

Four independent poly-A RNA controls are provided conveniently in a pre-mixed stock solution at staggered concentrations. After spiking directly into eukaryotic RNA samples, labeled cRNA targets are prepared and hybridized onto GeneChip expression arrays. The resultant Signal intensities for the poly-A RNA controls serve as sensitive indicators of the efficiency of the labeling reaction.

Poly-A RNA Controls	Final Concentration* in Samples
lys	1:100,000
phe	1:50,000
thr	1:25,000
dap	1:6,667

*Concentrations are defined as the number of copies of a particular poly-A RNA control to the total number of poly-A RNA molecules in the complex sample.

Volume	Components
16 µL	Poly-A Control Stock
3.8 mL	Poly-A Control Dil Buffer

What are the assumptions you have used in estimating the concentrations in terms of complexity ratio of these control transcripts in a sample?

The concentrations in terms of complexity ratios are calculated based on the following assumptions:

- Average transcript length = 2,000 bases
- Average MW of a single base = 330 g/mole
- mRNA constitutes approximately 2% of the total RNA sample

Based on these assumptions, how did you calculate the concentrations in terms of complexity ratios from the starting concentrations of the poly-A RNA controls in the Poly-A Control Stock?

The concentrations of the poly-A RNA controls in the stock solution are as follows:

lys	7.6 nM
phe	15.2 nM
thr	30.4 nM
dap	114.0 nM

A complexity ratio is the ratio of the number of copies of poly-A control transcript to the total number of mRNA transcripts in the sample. Complexity can also be expressed as a ratio of molar amounts (since 1 mole contains 6.02×10^{23} molecules).

Here are the calculations for the 1:100,000 concentration in terms of complexity ratio for *lys* when spiked into 5 µg of starting total RNA:

Calculations for lys:

Stock concentration of *lys*: 7.6 nM (7.6 nmoles/L)

Total dilution in final input (from Product Insert): 1:10,000 (or (1:20) * (1:50) * (1:10))

Amount of diluted poly-A spike-in volume (from Product Insert): 2 µL

(7.6 nmoles/L)*(1/10,000)*(1 L/1x10^6 µL)*(2 µL) = 1.52 x 10^{-9} nmoles

Calculations for starting RNA:

Starting total RNA: 5 µg

% of mRNA in total RNA: 2%

Starting mRNA: (5 µg)*(0.02) = 0.1 µg

Average transcript length: 2,000 bases

Average MW of single base: 330 g/mole

Average MW of transcript: (2,000 bases)*((330 g/mole)/base) = 660,000 g/mole

(660,000 g/mole)*(1 x 10^6 µg/g)*(1 mole/1 x 10^9 nmoles) = 660 µg/nmole

(0.1 µg)*(1 nmole/660 µg) = 0.000152 nmole = 1.52 x 10^{-4} nmoles

Concentration in Terms of Complexity Ratio Calculation:

Complexity ratio, *lys* = (1.52 x 10^{-9} nmoles)/(1.52 x 10^{-4} nmoles) = 1 x 10^{-5}

1 x 10^{-5} = 1/100,000

How many reactions does each kit contain?

Depending on the quantity of the starting material and if the poly-A controls are used in the One- or Two-Cycle Target Labeling Assays, the amount of poly-A controls used in each sample will vary, causing the number of reactions contained in the kit to vary as well. In addition, it is related to the number of samples being processed at the same time since the Poly-A Control Stock needs to be diluted serially before adding to the sample. The intermediate dilutions should be prepared fresh each time and, therefore, depending on the number of samples to be labeled at the same time, the amount of Poly-A Control Stock used may vary.

Our rough estimation is that each kit is sufficient for approximately 100 reactions.

Where can I find the protocol for diluting the Poly-A Control Stock before adding to the samples?

Detailed protocols for performing the serial dilutions of the Poly-A Control Stock for the One- and Two-Cycle Target Labeling Assays, as well as example data, can be found in Section 2, Chapter 1 of the *GeneChip Expression Analysis Technical Manual*.

At what temperature should the kit be stored?

The recommended storage temperature of the kit is -20°C. If possible, storing the poly-A controls at -80°C will help ensure the kit's long-term stability.

What is the shelf life of the kit?

The kit's shelf life is 15 months from the date of manufacturing. We will continue to perform real-time stability studies and anticipate extending the shelf life as we accumulate more real-time data.

How do I evaluate the sensitivity and efficiency of the labeling reaction based on the Poly-A Controls?

First, examine the Present call rate of the 3' probe set of the poly-A RNA control transcripts. Following the recommended dilutions, all four poly-A RNA controls should be routinely called as Present in a One-Cycle Target Labeling Assay. The poly-A RNA control present at the lowest concentration, *lys*, is expected to be called as Present > 70-percent of the time for the Two-Cycle Target Labeling Assay, and the other three controls should be routinely called as Present.

Next, the relative Signal intensities of the four transcripts should also be checked such that they follow the same order of increasing poly-A RNA control concentration as expected.

Should I anticipate the same results using the poly-A controls with all eukaryotic GeneChip® expression arrays?

The Signal intensity representative data (globally scaled) shown in the Expression Analysis Technical Manual are typical results for the most current probe design from Affymetrix (3' AFFX-r2-Bs). These probe sets are available on catalog arrays including the GeneChip® Human Genome U133, Mouse 430, and Rat 230 arrays.

However, with previous generations of probe sets, the Signal values observed may vary from the current ones. Nevertheless, utilization of these controls still serves as a good indicator of the experimental process quality, low-end sensitivity, and assay dynamic range. Some of the arrays that have the older probe sets include the Drosophila Genome Array and Yeast Genome S98 Array.

How does this kit perform with labeling kits other than those recommended in the Affymetrix protocols?

We have not tested the performance of this kit extensively with any other kits/protocols other than the One- and Two-Cycle Target Labeling Assays currently recommended by Affymetrix. Therefore, we are not able to provide any data or support if this kit is used with any other protocols.

Can this Kit be used with prokaryotic samples and arrays?

This product was designed and tested for eukaryotic arrays. The presence of poly-A tails on the transcripts is not relevant to prokaryotic samples. Since the eukaryotic and prokaryotic assays are fundamentally different, this kit may not provide similar array results with prokaryotic assays.

Probe sets for all four controls are available on prokaryotic arrays including the *E. coli* (antisense) and *P. aeruginosa* arrays. Adding the Poly-A RNA Controls to these samples will also serve as a valuable positive control. Follow the instructions in Section 3, Chapter 1 of the Expression Analysis Technical Manual carefully for preparing dilutions for the Prokaryotic Assay. Read the entire protocol before starting since the dilutions of the Poly-A RNA Control stock may vary depending on the type of prokaryotic arrays used.

Can I use the Signal intensities of the poly-A controls as standard curve for transcript abundance quantitation?

These controls are only designed to provide a qualitative assessment on efficiency of the labeling assays, and NOT for the purpose of serving as a generic quantitative standard curve.

The Poly-A Control Dil Buffer comes in one big bottle of 3.8 mL and it is inconvenient to freeze/thaw each time since it freezes when stored at -20°C. What do you recommend and can you change the packaging so the buffer comes in smaller aliquots?

Since the number of samples processed each time may vary drastically at different customer sites, we have decided to provide the Dil Buffer in one bottle. Upon receipt, users are encouraged to thaw the buffer, and then aliquot it in more convenient volumes for long-term storage based on their own usage rate.

What is in the Poly-A Control Dil Buffer?

The Dil Buffer has been formulated specifically for diluting the poly-A controls to ensure stability and consistent performance. Do not substitute the Dil Buffer with any other buffer when carrying out the dilutions.

Do you have any specific recommendation on the type of Non-Stick Tubes to use for diluting the Poly-A RNA Control Stock?

The following tubes were used at Affymetrix during product development of this Poly-A RNA Control Kit, and we have not tested any other tubes at this time:

- Non-stick RNase-free 0.5 mL microfuge tubes, Ambion, cat #12350
- Non-stick RNase-free 1.5 mL microfuge tubes, Ambion, cat #12450

Can I purchase the Poly-A Control Stock and the Poly-A Control Dil Buffer separately?

The Control Stock and the Dilution Buffer are packaged in the same kit to provide convenience to customers. Currently they cannot be purchased as separate products.

Can I purchase one, or all, of the four poly-A controls separately?

These four controls are only available as a pre-mixed cocktail with the defined concentrations and relative ratios. This combination of concentrations has been tested to provide qualitative information on both sensitivity and dynamic range of the labeling assay. Providing these controls in a pre-mixed format increases ease of use and reduces the number of manual dilution steps.

One-Cycle cDNA Synthesis Kit

What components does the GeneChip One-Cycle cDNA Synthesis Kit contain?

The One-Cycle cDNA Synthesis Kit contains all necessary reagents (see the following table) to carry out the cDNA synthesis reaction for thirty samples in a "standard" assay for GeneChip arrays. From total RNA starting materials (1 µg to 15 µg), using this kit, sufficient double-stranded cDNA can be synthesized, purified on the cDNA Cleanup Column, and used as template in the subsequent *in vitro* transcription labeling reaction.

Component Name	Concentration	Volume	Quantity
T7-Oligo(dT) Primer, 50 µM	50 µM	120 µL	1
5X 1st Strand Reaction Mix	5X	120 µL	1
DTT, 0.1M	0.1M	60 µL	1
dNTP, 10 mM	10 mM	120 µL	1
SuperScript™ II	200 U/µL	60 µL	1
5X 2nd Strand Reaction Mix	5X	900 µL	1
E. coli DNA Ligase	10 U/µL	30 µL	1
E. coli DNA Polymerase I	10 U/µL	120 µL	1
RNase H	2 U/µL	30 µL	1
T4 DNA Polymerase	5 U/µL	60 µL	1
EDTA, 0.5M	0.5M	300 µL	1
RNase-free Water		3.1 mL	1

What is the shelf life of the kit?

The One-Cycle cDNA Synthesis Kit has about 18 months of dating from the date of manufacturing.

How are the kits shipped and what are the storage conditions?

The kits are shipped on dry ice, and should be stored at -20°C.

Are the data obtained from the One-Cycle cDNA Synthesis Kit concordant with the previous Standard GeneChip Target Labeling Assay?

The One-Cycle cDNA Synthesis Kit is formulated following precisely the standard GeneChip Target Labeling Assay and there is no change to the major reagents used in the protocol. Therefore, the performance of the assay and data obtained should be highly concordant.

Do you have any data showing that high-quality results can be obtained using 1 µg of total RNA as starting material following the One-Cycle Target Labeling Assay?

Extensive analysis was carried out at Affymetrix to examine the sensitivity and reproducibility of the protocol for 1 µg of starting material. The results demonstrate comparable cRNA length (by Bioanalyzer electrophoresis), sensitivity (percent Present call and spike-ins), and reproducibility (by Signal correlation, call concordance, false change) compared with using 5 µg of total RNA as starting material. In addition, the Change calls analyzing two different samples were also highly consistent within the assay.

For large-scale studies involving many different samples, it is still advisable to normalize the starting materials, and always use the same starting quantity to obtain the most direct and reliable comparison analysis results.

Two-Cycle cDNA Synthesis Kit

What components does the GeneChip® Two-Cycle cDNA Synthesis Kit contain?

The Two-Cycle cDNA Synthesis Kit contains all necessary reagents (see the following table) to carry out the two cycles of cDNA synthesis for thirty samples. Note that the reagents for the intermediary *in vitro* transcription (IVT) amplification using un-labeled ribonucleotides, between the two cDNA synthesis reactions, is not included in the kit. The Ambion MEGAscript® T7 Kit is recommended.

Component Name	Concentration	Volume	Quantity
T7-Oligo(dT) Primer, 50 µM	50 µM	120 µL	1
5X 1st Strand Reaction Mix	5X	180 µL	1
DTT, 0.1M	0.1M	90 µL	1
dNTP, 10 mM	10 mM	200 µL	1
SuperScript™ II	200 U/µL	60 µL	1
RNase Inhibitor	40 U/µL	45 µL	1
5X 2nd Strand Reaction Mix	5X	900 µL	1
Random Primers	3 µg/µL	60 µL	1
$MgCl_2$, 1M	1M	60 µL	1
E. coli DNA Polymerase I	10 U/µL	200 µL	1
RNase H	2 U/µL	55 µL	1
T4 DNA Polymerase	5 U/µL	60 µL	1
RNase-free Water		9.5 mL	1

What is the shelf life of the kit?

The Two-Cycle cDNA Synthesis Kit has about 18 months of dating from the date of manufacturing.

How are the kits shipped and what is the storage condition?

The kits are shipped on dry ice, and should be stored at -20°C.

Are the data obtained following the Small Sample Target Labeling vII (SSTLvII) comparable to those obtained using the Two-Cycle cDNA Synthesis Kit?

The array results are highly comparable with respect to cRNA length and yield, percent Present call rate, Signal intensity value, 3'/5' ratios, false change analysis, and poly-A RNA control spike performance. However, since the protocol has been modified (see detail below), it is recommended NOT to compare data obtained from the two protocols directly.

What are the major differences and improvements comparing the new Two-Cycle cDNA Synthesis Kit with the Affymetrix® Small Sample Target Labeling Version II Protocol?

The Two-Cycle cDNA Synthesis Kit was developed primarily based on SSTLvII, with some additional optimization focusing on a more streamlined procedure. More information on these changes is summarized as follows:

- The new Two-Cycle cDNA Synthesis Kit protocol utilizes larger reaction volume for the 1st-cycle cDNA synthesis step, allowing more flexibility in the starting material concentration and reducing potential pipetting variations.
- The new Two-Cycle cDNA Synthesis Kit protocol eliminates the cDNA cleanup step in the 1st cycle, increasing ease of use.
- The GeneChip® Sample Cleanup Module is used for all cDNA and cRNA cleanup steps, replacing all previously recommended precipitation or speed vacuum protocols.
- Overnight incubations are recommended for both IVT steps following the 1st- and 2nd-cycle cDNA synthesis, reducing the total target labeling time to 2½ days.

Can you compare the data from the Two-Cycle Target Labeling Assay directly with the One-Cycle Target Labeling Assay?

Although there is good agreement between the two protocols with respect to Present/Absent calls, the absolute Signal values of individual probe sets may still differ. It is not recommended to perform direct comparisons of Signal data obtained using different protocols.

I have never performed any small sample target labeling before. Do you have any suggestions for incorporating the Two-Cycle cDNA Synthesis Kit into our routine GeneChip microarray analysis?

The basic principles of the Two-Cycle Target Labeling Assay are very similar to the One-Cycle Standard Protocol, and many of the steps involved may look familiar. However, since it does require manipulation of very small amounts of materials in the 1st cycle, even the most experienced GeneChip microarray users may not obtain ideal data when using the Two-Cycle Target Labeling Assay the first time.

Therefore, it is suggested that users first read the entire protocol very carefully, including all notes and tips, and then practice initially with diluted samples that you have previously characterized using the One-Cycle Standard Protocol. This way, you will be starting with materials of good quality and you already know what results to anticipate. Follow common laboratory practices that minimize RNase contamination and use caution at each step to prevent sample loss.

Does this Two-Cycle cDNA Synthesis Kit/protocol work well on partially degraded RNA samples?

No. Similar to previous recommendations for the standard protocol and SSTLvII, the Two-Cycle Target Labeling Assay also requires high-quality total RNA as starting material. Most commonly, customers assess $OD_{260/280}$ ratios together with electrophoretic analysis results when sufficient material is available. When sample quantity is limiting, the combination of RiboGreen for quantitation and Bioanalyzer for quality assessment has worked quite well.

To better distinguish whether the starting material quality has affected the target labeling process, it is recommended to use the GeneChip® Eukaryotic Poly-A RNA Control Kit and spike the positive controls into the starting materials for efficient troubleshooting.

How does your Two-Cycle cDNA Synthesis Kit protocol perform compared with other commercially available alternative target labeling kits?

GeneChip assays are an integrated part of the complete system solution and are the **only** reagent kits developed and fully validated on GeneChip brand arrays. With a thorough understanding of the performance characteristics of the GeneChip reagent kits on GeneChip arrays, users should have more confidence in obtaining meaningful results.

Affymetrix has been very diligent in investigating alternative target labeling protocols to ensure that researchers have access to the most robust and standardized assays when running GeneChip arrays. Affymetrix believes that the current set of GeneChip reagents are the most optimized solutions and can help customers extract the most reproducible and reliable data on GeneChip arrays.

Affymetrix will continue to research alternative procedures to evolve the assay for improved ease of use and more streamlined assays.

What is the detection sensitivity of the Two-Cycle Target Labeling Assay?

One way to assess the assay sensitivity is to monitor the global array metric of percent Present calls. From the same high-quality starting material, the Two-Cycle Target Labeling Assay demonstrates highly comparable percent Present calls as the results generated from the One-Cycle Target Labeling Assay.

Another way to estimate the detection sensitivity is to use poly-A RNA spike-in controls. Following the recommended protocol included in the GeneChip® Eukaryotic Poly-A RNA Control Kit, the Two-Cycle cDNA Synthesis Kit generally amplifies the samples sufficiently to detect the 3'-Probe Set of *lys* (the transcript with the lowest concentration, at 1:100,000 complexity ratio) as Present over 70% of the time.

Which RNA isolation method do you recommend for the Two-Cycle Target Labeling Assay?

Different tissue types may have different requirements for RNA extraction. 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 most suitable for the processing of your samples and follow the vendor-recommended procedures closely. Assess the quality and integrity of obtained total RNA samples carefully by gel electrophoresis or Bioanalyzer before starting the GeneChip target labeling protocol.

If I have only between 100 ng and 1 µg of total RNA, which protocol would you recommend, the One-Cycle or the Two-Cycle Target Labeling Assay?

Since it is recommended to choose one protocol consistently for all samples to be compared within the same study, the procedure you choose may depend on the amount of RNA obtained from the majority of the samples you plan on including in the entire study. If most of the samples fall into the 100 ng to 1 µg range, it is reasonable to use the Two-Cycle Target Labeling Assay for the whole set of experiments.

How do I know if my 1st-cycle cDNA synthesis reaction has worked well?

It is challenging to assess the success of the cDNA synthesis reaction and this applies to both the One-Cycle and Two-Cycle protocols. This is because the amount of double-stranded cDNA obtained is quite small, and it is mixed in with the starting total RNA materials. The best way to QC this step is to carry out the subsequent IVT reaction and quantitate the cRNA yield.

How do I know if my 1st-cycle IVT reactions have worked well?

After the first cycle of amplification, it is often possible to quantitate the cRNA yield at this stage. Routinely, more than 600 ng of cRNA can be obtained from 50 to 100 ng of starting material. However, it is difficult to use the cRNA yield as the only metric to determine the success of the assay. Therefore, it may be most informative to combine the results from the 1st-cycle IVT yield with the 2nd-cycle IVT yield, as well as array-quality metrics and data on various positive controls, to obtain a comprehensive view of how the assay has proceeded.

In the Two-Cycle Target Labeling Assay, the 1st-cycle cDNA cleanup step is omitted. Is there any impact on the data by removing this step?

In this more streamlined version of the Two-Cycle protocol, the 1st-cycle cDNA cleanup step is removed since the amount of material is very limiting and it has been shown that omitting this step does not affect the final array results.

In the Two-Cycle Target Labeling Assay, after the first reaction, you are not recommending using the ADJUSTED cRNA yield to estimate the amount of cRNA to be used in the 2nd-cycle cDNA synthesis reaction. Why is this?

This is because the amount of starting material is rather small in the Two-Cycle Target Labeling Assay and the amount of cRNA to be used in the 2nd-cycle does not have to be precise to make it successful. For simplification purposes, we are recommending using the actual cRNA as an estimation at this step.

IVT Labeling Kit

What does the GeneChip® IVT Labeling Kit contain?

The IVT Labeling Kit contains all necessary reagents (see the following table) to perform thirty *in vitro* transcription (IVT) reactions from double-stranded cDNA templates. The template cDNA containing the T7 promoter sequence can be obtained following either the One- or Two-Cycle cDNA Synthesis protocols.

Component Name	Quantity	Volume
10X IVT Labeling Buffer	1	120 µL
IVT Labeling Enzyme Mix	1	120 µL
IVT Labeling NTP Mix	1	360 µL
3′-Labeling Control (0.5 µg/µL)	1	10 µL
RNase-free Water	1	910 µL

Why are you recommending the overnight incubation for the IVT reaction?

There are two major benefits for this change in protocol. One benefit is that it provides the option for starting with as little as 1 µg of total RNA using just the standard One-Cycle Labeling protocol. The other benefit is that it incorporates a more convenient and efficient experimental workflow.

Previously, IVT was carried out with 4-5 hours of incubation on the second day of a standard One-Cycle Target Labeling protocol. This made the procedure on the second day rather hectic, since users typically performed the cRNA cleanup (~30 minutes) and fragmentation (~1 hour) after the IVT in order to hybridize their samples on the arrays overnight.

In order to make the workflow more flexible, the new GeneChip IVT Labeling Kit provides a general guideline of using an overnight incubation. However, for customers who prefer the previous protocol, they have the option to spike in additional T7 RNA Polymerase (Ambion, P/N 2085, as described in the product insert) to reduce the IVT reaction time to 4-5 hours.

Should I be concerned about getting shorter cRNA targets because of the overnight IVT incubation?

It has been shown using gel electrophoresis that the length of the cRNA products change only slightly with increasing IVT reaction time. In addition, array results generated after increasing the length of IVT incubation are highly concordant with shorter incubation times and show little or no detectable difference.

Does this IVT Labeling Kit work for both the One- and Two-Cycle Target Labeling protocols?

This kit has been tested to be compatible with both the One- and Two-Cycle Target Labeling protocols to be used in the last step of amplification and biotin-labeling.

What is the typical cRNA yield of the IVT Labeling Kit?

Using this IVT Labeling Kit, over 30 µg of biotinylated cRNA is usually generated from high-quality materials following the recommended One- or Two-Cycle Target Labeling protocols.

How do I know if the kit is working well without hybridizing my targets to GeneChip arrays?

A linearized plasmid DNA is included in this kit as a positive control. Using this as template, users can test the transcriptional activity of the kit components. Following the protocol outlined in the Expression Analysis Technical Manual, the control can be used to track yield and consistency of product size generated by the kit (a distinct band of approximately 2 kb).

How do the labels in the GeneChip IVT Labeling Kit work?

The IVT Labeling Kit contains a proprietary biotinylated nucleotide analog (patent pending). This analog is incorporated in the T7 RNA Polymerase-mediated IVT amplification and labeling reaction as a pseudouridine reagent to generate cRNA targets.

The recommended starting amount of total RNA for the One-Cycle protocol has been reduced from 5 µg to 1 µg. Do you have any data to support that the procedure works well with the reduced amount of starting material?

Studies were conducted at Affymetrix to compare the data obtained from 1 µg vs. 5 µg of starting total RNA, and the array results were highly concordant with respect to detection sensitivity (percent Present call) and reproducibility (Signal correlation, Absolute call concordance). However, subtle differences were observed. Therefore, for best comparability of results, it is generally recommended to use the same amount of starting RNA in studies where the data are intended to be directly compared.

Can I compare GeneChip array data from samples labeled with Enzo to those labeled with the new GeneChip IVT Labeling Kit?

It is not recommended to change labeling protocols in the middle of a study. Comparisons between the two kits have shown comparable array data with respect to intra-assay reproducibility and sensitivity. However, differences between the methods do exist, and depending on a user's individual applications and data analysis criteria, customers are encouraged to perform their own evaluation to make the final determination about whether or not they can directly compare the data.

Why have you modified the hybridization cocktail composition by including 10% DMSO and changed the Fluidics Scripts?

Since the labeling molecules are different between the two protocols, optimized hybridization cocktail and Fluidics Scripts have been developed to be compatible with the new IVT Labeling Kit. These instructions should be followed closely in order to obtain the best results on GeneChip arrays.

How can I get the modified fluidics scripts that are compatible with the new kit?

The new fluidics script, EukGE-WS2v5, uses higher wash stringency for the higher density (11 µm feature size) 49- and 64-format arrays. It can be downloaded from the Affymetrix web site at the following link: www.affymetrix.com/support/technical/fluidics_scripts.affx.

The 49- and 64-format, 18-µm feature size arrays, will continue to use the existing fluidics script, EukGE-WS2v4.

The current fluidics script for the 100 format arrays, Midi_euk2, already uses the same degree of stringency, and therefore remains unchanged for use with the IVT Labeling Kit.

What is the storage condition of the IVT Labeling Kit?

The kit should be stored at -20°C.

What end user license will be provided with the new Labeling Reagent?

Affymetrix will provide an end user license with the kit allowing customers freedom of operation for all research use applications.

Hybridization, Washing, and Staining

What happens if the hybridization time is extended beyond 16 hours?

The standard gene expression hybridization time is 16 hours at 45°C. At high temperatures or with longer incubation times, the sample will evaporate. Loss of sample is undesirable for several reasons:

- Low volume of hybridization solution in the probe array can lead to dry spots that will show up as uneven hybridization and, thus, compromise data.
- Sample loss compromises the possibility of repeating the experiment with the identical sample.
- Sample evaporation can lead to changes in the salt concentration of the solution, which can affect the stringency conditions for hybridization.

How many times can I scan an array before the data is affected?

It is always best to capture the data on the initial scan. Scanning bleaches the fluorophore and will result in reduction in signal intensity of 10-20% with each scan of the GeneArray® Scanner and 3-5% with each scan of the GeneChip® Scanner 3000. Therefore, subsequent scans will not give signals equivalent to the initial scan.

How often do I need to do maintenance on the fluidics station?

With normal use (e.g., 20 arrays/module/week), we recommend the following schedule: every week, the needle bleaching protocol (i.e., “Bleach” fluidics protocol) should be performed; on a monthly basis, the full-fluidics bleaching protocol (i.e., “Monthly Decontamination” protocol) should be performed and the peristaltic-pump tubing replaced. Please refer to Section 4, *Fluidics Station Maintenance Procedures*, for more detail.

What fluidics script should I use?

The appropriate fluidics script is specific to the array format and the organism (eukaryotic or prokaryotic), the type of labeling reagents used to prepare the cRNA target, and the model of Fluidics Station being used. Scripts for FS-450 and FS-250 are identified by a '_450' suffix. Information on the array format and appropriate script is contained in the package insert that comes with each array package. Please refer to the reference table available from the Affymetrix web site for the appropriate fluidics script to be used for your experiment: www.affymetrix.com/support/technical/fluidics_scripts.affx

Is there a possibility of contaminating the fluidics station with RNase when gene expression and genotyping applications are being performed on a shared station?

It is extremely important to change the vials each time a sample is removed or loaded onto a probe array. This prevents cross-contamination, as well as sample loss. RNase contamination is not an issue with gene expression applications due to the fact that the cRNA sample is fragmented prior to hybridization and is removed prior to array processing on the fluidics station.

I have a bubble in the array. How do I get rid of it?

After the final wash on the fluidics station, if the door is still open, place the array in the probe array holder and close the door. The fluidics module will automatically run a drain and fill protocol with buffer A. If one cycle does not remove the bubble, repeat the process and try again. If this doesn't work or the door has already been closed, manually drain the array and refill with buffer A.

What are the safe stopping points in the assay?

It is safe to stop work after each of the major steps in the sample preparation process: second-strand cDNA synthesis, IVT, fragmentation, or after preparing the hybridization cocktail. If possible, work with extracted RNA samples immediately rather than freezing them. Although it is common practice to use stored, frozen RNA samples in the process, eliminating freeze-thaws will most likely yield higher quality cRNA.

Data Analysis

I have observed on occasion that multiple _at probe sets are mapped to the same gene but give different expression results. How do I reconcile the difference?

There are various reasons why this happens. With increasing knowledge of the genome, the unique probe sets (_at probe sets) that were initially designed may turn out to represent subclusters that have collapsed into a single cluster in a later design. Therefore, it may seem that multiple "unique" _at probe sets now correspond to a single gene.

Different results from the probe sets could be observed due to the following reasons:

1. They represent splice variants or may cross-hybridize to different members that belong to a highly similar gene family or transcripts with different poly-A sites
2. One probe set is more 5' than the other
3. One probe set is better designed than the other

In these cases, it is important to use the resources available on the NetAffx™ Analysis Center (www.affymetrix.com) to understand if any of the above scenarios apply. Other expression analysis techniques may also be used to confirm which probe set reflects the transcript level more accurately.

What 3'/5' ratio for control genes, for example GAPDH and β-Actin, should I anticipate to obtain on GeneChip probe arrays?

In addition to the conventional probe sets designed to be within the most 3' 600 bases of a transcript, additional probe sets in the 5' region and middle portion (M) of the transcript have also been selected for certain housekeeping genes, including GAPDH and β-Actin. Signal intensity ratio of the 3' probe set over the 5' probe set is often referred to as the 3'/5' ratio. This ratio gives an indication of the integrity of your starting RNA, efficiency of first-strand cDNA synthesis, and/or *in vitro* transcription of cRNA. The signal of each probe set reflects the sequence of the probes and their hybridization properties. A 1:1 molar ratio of the 3' to 5' transcript regions will not necessarily give a signal ratio of 1.

There is no single threshold cutoff to assess sample quality for all of the diverse organisms and tissues. This is due to the presence of different isoforms of these housekeeping genes and their different expression patterns in various tissues and organisms. Although we routinely refer to a threshold ratio of less than 3 for the most common tissues, such as mammalian liver and brain, this may not be applicable to all situations. It may be more appropriate to document the 3'/5' ratios within a particular study and flag the results that deviate, therefore representing an unusual sample that deserves further investigation.

Can results from different laboratories and different times be compared with each other directly, and how do you control the variables in this type of experiment?

Array results can potentially be compared directly. However, it is important to check the following important elements before doing so:

- Experimental design strategy should be the same at various sites.
- Identical target labeling protocols should be followed, and yields from cDNA and IVT reactions should be within the same range as specified for that study.
- Same algorithm parameters are used.
- Similar results from 3'/5' ratios, background, noise, and scaling factors. Check arrays for scratches and even hybridization/staining.
- Comparability of results obtained from different operators should be evaluated before including their results in the same study.

Affymetrix® Microarray Suite (MAS) is on the C: drive, which is low on space. How can I create more room on the hard drive?

The library and data files can be moved to another drive, then deleted from the C: drive. After moving the files, remember to change your library file default settings in MAS to the appropriate directory by clicking on the **Tools** tab, and then select **Defaults** in the drop-down menu, then **File locations** tab in the **Defaults** window.

✔ NOTE

These instructions do not apply for GeneChip Operating Software (GCOS). To free up space on workstations running GCOS, it is important to routinely create backup (.CAB) files of the data, using GCOS Administrator, and move these backup files to alternative storage media. The data files can then be archived or deleted using GCOS Manager. Please review the appropriate sections of the GCOS User's Guide for details.

What is the difference between scaling and normalization when I scale or normalize my data to all genes on the array?

With scaling, you select an arbitrary target intensity and scale the average intensity of all genes (minus the highest 2% and lowest 2% Signal values) on each array within a data set to that number. This enables you to compare multiple arrays within a data set. The scaling factor remains the same for a particular array as long as you use the same arbitrary target intensity for scaling. Scaling can be performed independent of the comparison analysis.

On the other hand, normalization can only be done when performing a comparison analysis. It compares an experimental array with a baseline array and normalizes the average intensity of all genes (minus the highest 2% and lowest 2% Signal values) of the experimental array to the corresponding average intensity of the baseline array when running a comparison analysis in MAS. The normalization factor for a particular array changes when you change the comparison baseline array.

How important is it to evaluate the value of the Scaling Factor between different arrays?

Scaling Factor is the multiplication factor applied to each Signal value on an array. A Scaling Factor of 1.0 indicates that the average array intensity is equal to the Target Intensity. Scaling Factors will vary across different samples and there are no set guidelines for any particular sample type. However, if they differ by too much within a set of experiments, approximately 3-fold or more, this indicates wide variation in the .dat files. Therefore, the analyzed data (in the .chp file) should be treated with caution.

Should I always anticipate the hybridization controls, *bioB, bioC, bioD,* and *cre*, to be called as Present?

The four transcripts are added to the hybridization cocktail at staggered concentrations. At 1.5 pM, *bioB* is at the detection limit for most expression arrays and is anticipated to be called Present at least 70% of the time. In contrast, the other controls should be called Present all of the time, with increasing Signal values (*bioC*, *bioD*, and *cre*, respectively). Absent calls, or relatively low Signal values, indicate a potential problem with the hybridization reaction or subsequent washing and staining steps. Check to see if the hybridization cocktail was prepared correctly, if the recommended hybridization temperature and Fluidics Protocol were used, and make sure the SAPE staining solution did not deteriorate.

Other than qualitative calls and Signal values, the 3'/5' ratio data for these controls are not as informative since they do not relate to the quality of the samples and data.

What does high background mean?

A high background implies that impurities, such as cell debris and salts, are binding to the probe array in a nonspecific manner, and that these substances are fluorescing at 570 nm (the detection wavelength). This nonspecific binding causes a low signal-to-noise ratio (SNR), meaning that genes for transcripts present at very low levels in the sample may be incorrectly called as Absent. High background creates an overall loss of sensitivity in the experiment.

What are masks?

Masks are rarely used features in MAS. There are three types of mask files:

Image mask files: You may want to use an image mask if there is a large visible aberration on an image. You define the image mask based on the physical location of the image. Probe pairs included in the mask are excluded from the analysis. Image masks are associated with a given .dat/.cel file and cannot be used on other images.

Probe mask files: Probe masks are defined by the probe set and probe pair number. Probe pairs included in this type of probe mask are excluded from the analysis when the probe mask is used. Probe masks can be applied across a data set.

A second type of probe mask defines a select group of probe sets that can be used in normalization or scaling.

For detailed descriptions of mask files, please refer to *Affymetrix® Microarray Suite User's Guide* (P/N 701099), or the *Affymetrix® GeneChip® Operating Software User's Guide* (P/N 701439).

If I realign the grid, how do I create a new .cel file?

If manual adjustment of the grid is necessary, the corresponding .cel file present at the time of adjustment will no longer be a valid representation of the realigned image data. Microarray Suite/GCOS automatically detects this situation either on initial reopening of the readjusted .dat file or during the analysis process. Once the readjusted .dat file is opened, the .cel file is automatically created. The user does not need to carry out any overt steps to accomplish this.

How do I add additional probe sets in the .rpt file?

Use the **Report Settings** dialog on the shortcut menu in Microarray Suite/GCOS to open the **Expression Report**. You may add any probe sets desired by simply typing in the probe set name(s) you wish to add (this can also be accomplished by cutting and pasting from a text file). Keep in mind that the probe set name must be entered exactly as it appears in the analysis file, including the suffixes, such as "12345_s_at".

Why can I not analyze data files stored on a CD?

Files in CD-ROM format are copied to the hard drive in read-only mode. MAS requires this attribute to be removed. To do this, open Windows Explorer and select the file(s) you copied from the CD. Click the right mouse button and select **Properties**. Clear the **Read-only** check box near the bottom of the **Properties** screen and click **OK**.

In order for data from a CD to be analyzed in GCOS, the data files must first be imported into the GCOS database using either GCOS Manager or GCOS Batch Importer. If the data is backed up as a .CAB file, the data must be restored using GCOS Administrator. Please review the appropriate sections of the *GCOS User's Guide* for details on importing and restoring data.

How can the mismatch probe cell have a higher intensity than its corresponding perfect match probe cell?

There could be a number of causes for this. It is possible that this probe sequence has high homology with another unknown sequence, resulting in a high mismatch-to-perfect match ratio. Another possibility is a mutation or set of mutations in the sequence of the target transcript, which causes specific binding to the Mismatch. Regardless of the cause, the built-in redundancy using multiple probe pairs to represent a single sequence on the probe array mitigates any significant impact on the final interpretation of the data.

There are too many files showing in the file window in Microarray Suite. What can I do?

By placing files for projects in their own directories and changing the default settings for data in Microarray Suite appropriately, you can manage large numbers of files.

In addition, with the Windows 2000 operating system, users can specify their own directory defaults in Microarray Suite while logging on and create their own directories for data. To do so, each user should have a unique logon name and organize files in subdirectories, for example, by project, user, date, or lab. Each user can then set the data default to a subdirectory of choice.

✔ NOTE

*These instructions are not applicable to GCOS. In order to reduce the number of experiments that appear in the GCOS file window, use the Filters function in the **Tools** menu. Please review the appropriate section of the GCOS User's Guide for details on applying filters.*

Experimental Design

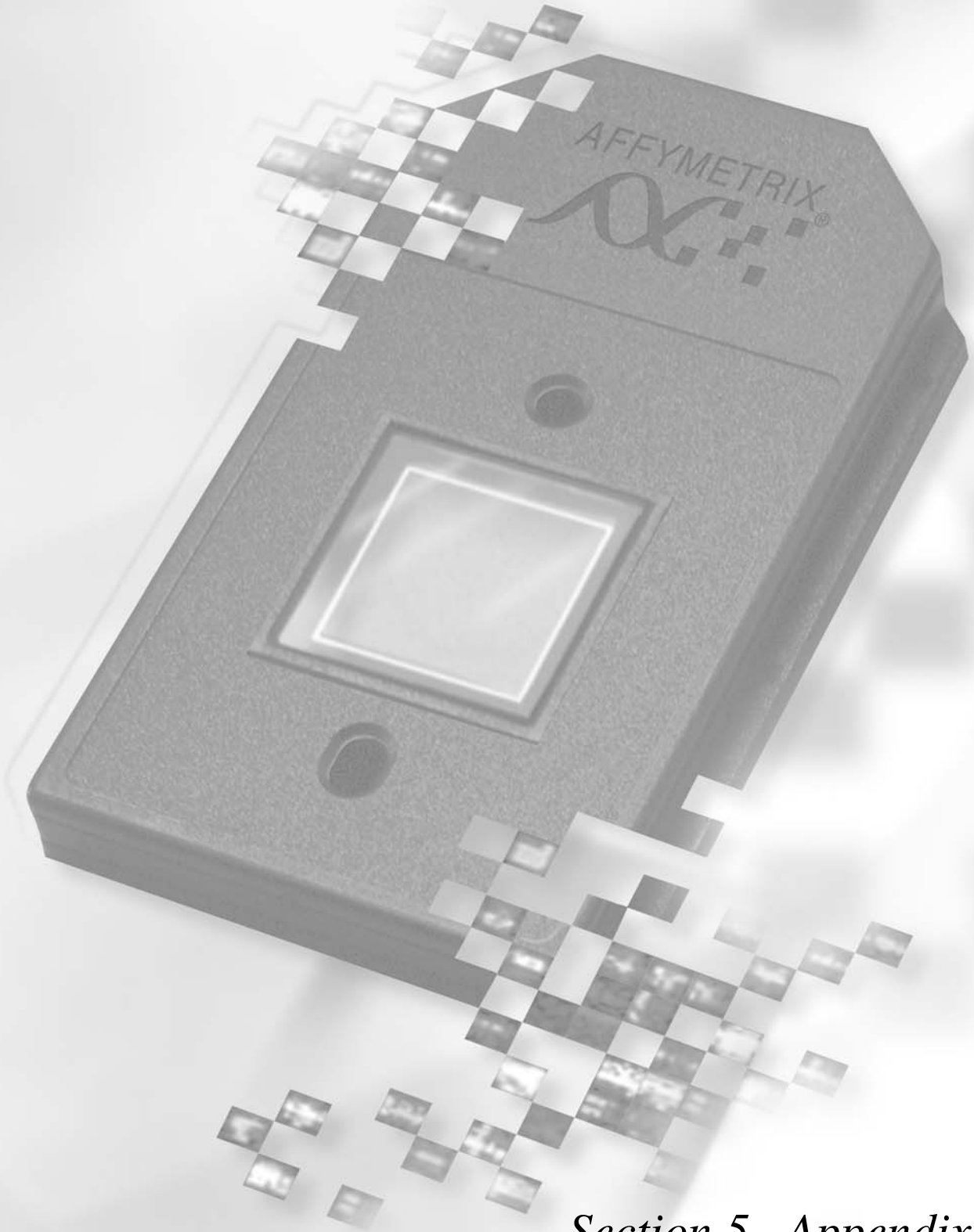
Which is greater, sample or assay variability?

Sample variability, which arises mainly from biological heterogeneity, is certainly higher than assay variability, and has been estimated to be at least 10-fold greater. We recommend that researchers run multiple samples per data point to account for sample-to-sample variability. In addition, carefully design the experiment in order to minimize potential variation associated with the samples.

Troubleshooting

Problem	Likely Cause	Solution
Sample Quality		
High 3′/5′ ratio	Most often caused by degradation of the RNA during the isolation process.	Start with a fresh sample and minimize the possibility of RNase activity. Look for the presence of Ribosomal RNA bands on a non-denaturing agarose gel.
Low cRNA yield	Low RNA quality, which interferes with reverse transcription and subsequent labeling.	It sometimes helps to do a TRIzol-based isolation followed by cleanup with an RNeasy column. For samples with a high lipid content, such as brain, use procedures to reduce the lipid content prior to the reverse transcription reaction.
Target Labeling		
Low yield	Poor quality template.	Check starting material quality.
	Loss in enzyme activity.	Repeat IVT.
	Reaction assembled on ice. Spermidine in the 10X IVT Labeling Buffer can lead to precipitation of the template cDNA.	Repeat IVT.
	Reaction temperature is not set appropriately.	Repeat IVT.
Image / Array Quality		
Low or absent Oligo B2 hybridization	Addition of control Oligo B2 and hybridization, washing or staining.	Make sure that the Control Oligo B2 has been added to the hybridization cocktail at the correct concentration. Also, check the makeup of the hybridization buffer, the stain solution, and hybridization temperature.
Dim Corners	In need of fluidics maintenance.	Bleach the fluidics as recommended and change the peristaltic pump tubing. If the problem persists, call Affymetrix Technical Support.
Dim Arrays	Hybridization problems.	Check the signal from control Oligo B2 to see if the signals are also weak. If it appears to be a hybridization issue, check all hybridization reagents and equipment settings before running another assay. Test arrays can be useful for troubleshooting this issue.
	Sample preparation problems.	Re-check each of the quality control procedures recommended in the manual, such as absorbance measurement and running an aliquot on gel, to ensure that there is no significant loss of sample during target preparation due to manipulation of the sample or RNase contamination. Also see above for "low cRNA yield".

Problem	Likely Cause	Solution
Image / Array Quality (continued)		
Leaking septa	Leaking septa are most often created during the array filling with a pipette.	Be sure to use pipette tips without a beveled end. When filling the arrays, be careful to push the pipette tip straight through the septum and maintain a constant perpendicular angle during filling and draining of the array.
MAS Software Problems		
In Microarray Suite (MAS), I received the error message, "Could not find the .cif file."	The default path for the library files in MAS is incorrect.	Set the correct path for the library files.
	The library files for those specific arrays are not installed on the computer.	Install the library files for that array, making sure to check the box appropriate for that array during the installation process.
The probe array type is missing from the pull-down menu when creating an .exp file.	The default path for the library files in MAS is incorrect.	Set the correct path for the library files.
	The library files for those specific arrays are not installed on the computer.	Install the library files for that array, making sure to check the box appropriate for that array during the installation process.
The fluidics protocols are missing from the pull-down menu in the Fluidics control window.	The default path for the protocol files in MAS is incorrect.	Check that the location of the fluidics files on the hard drive corresponds to the default protocol path in MAS.
	The library files are not installed on the computer.	Install the library files, making sure the protocols are in the same directory as the default path set in MAS.
After putting the computer on the network, the probe array descriptions are not available and a SQL error message appears.	When networking computers, the name of the computer is often changed to correspond to an organization's standard conventions. This results in a breakdown of the connection between MAS and the Microsoft Data Engine (MSDE).	After the computer is renamed, uninstall MAS and MSDE and reinstall MAS.
Microarray Suite is on the C: drive and it's filling up.		The library and protocol files can be moved (or dragged) to another, larger drive. Remember to change the default path for the library and protocol files in MAS, and modify this path for each log in name. In addition, GeneChip data should always be stored locally on the largest available drive on the workstation.
The gene descriptions show up for some users and not for others.	This is a result of different security settings between users and administrators of the workstation.	Call Affymetrix Technical Support for information on how to change the registry to correct this.



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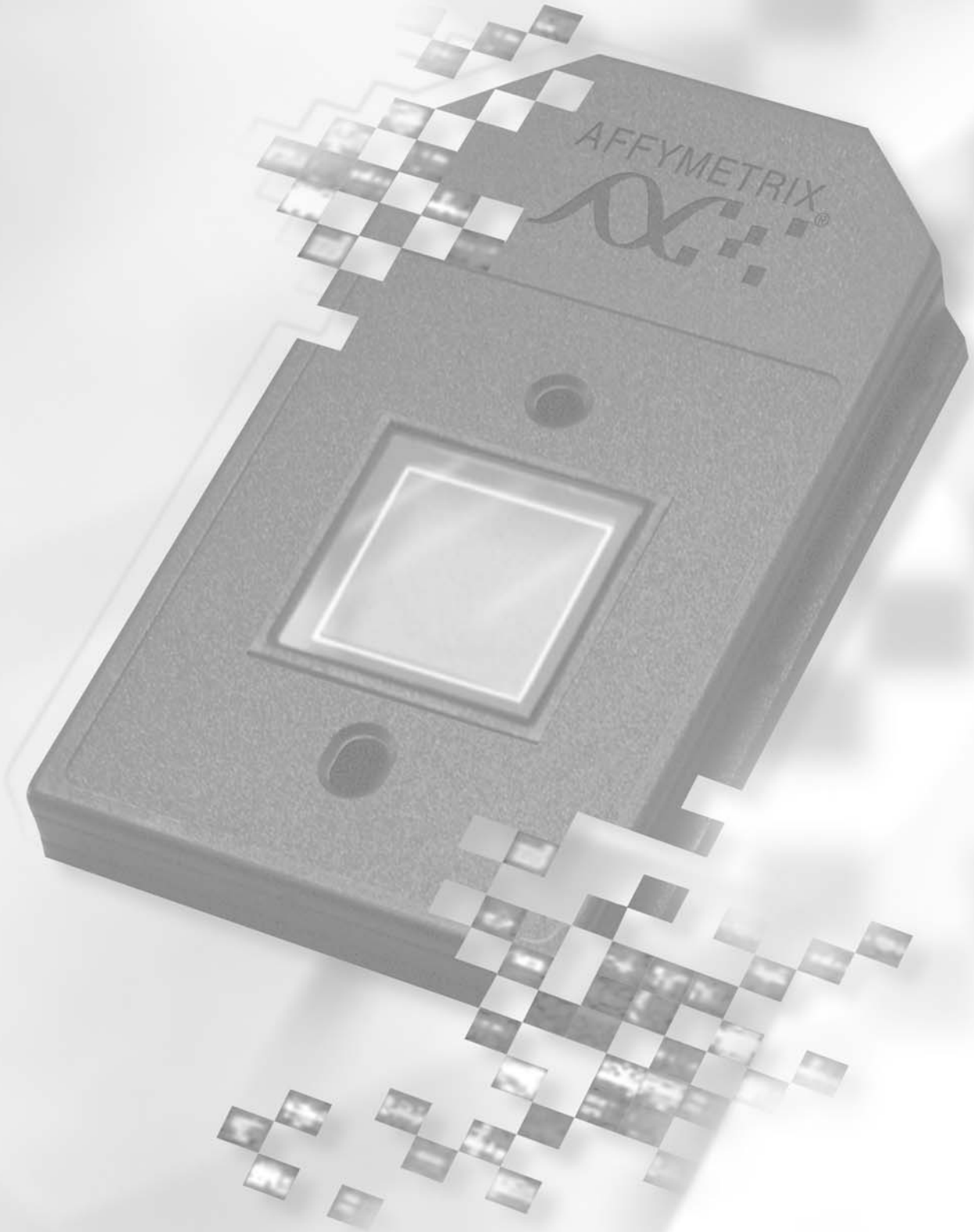

List of Controls on GeneChip Probe Arrays

Table 5.C.1
Control Genes on GeneChip® probe arrays

Array Type	Origin of Organism	Control Gene Name	Utility for GeneChip® Experiments	Associated Affymetrix Products
Eukaryotic Arrays	synthetic	B2 Oligo	Grid alignment.	Control Oligo B2, P/N 900301, also as part of the Hybridization Control Kit P/N 900454 or 900457
	E. coli P1 Bacteriophage	*bioB* *bioC* *bioD* *cre*	Antisense biotinylated cRNA are used as hybridization controls.	GeneChip Eukaryotic Hybridization Control Kit, P/N 900454 or P/N 900457
	B. subtilis	*lys* *phe* *thr* *dap*	Poly-A-tailed sense RNA can be spiked into isolated RNA samples as controls for the labeling and hybridization process. The spikes can also be used to estimate assay sensitivity.	GeneChip Eukaryotic Poly-A RNA Control Kit, P/N 900433
Prokaryotic Arrays	synthetic	B2 Oligo	Grid alignment.	Control Oligo B2, P/N 900301, also as part of the Hybridization Control Kit P/N 900454 or 900457
	B. subtilis	*lys* *phe* *thr* *dap*	Sense RNA can be spiked into purified sample RNA as control for the labeling and hybridization process. The spikes can also be used to estimate assay sensitivity.	GeneChip Eukaryotic Poly-A RNA Control Kit, P/N 900433