GENE EXPRESSION MONITORING

GeneChip® Expression Analysis

Technical Manual

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701021 Rev. 4

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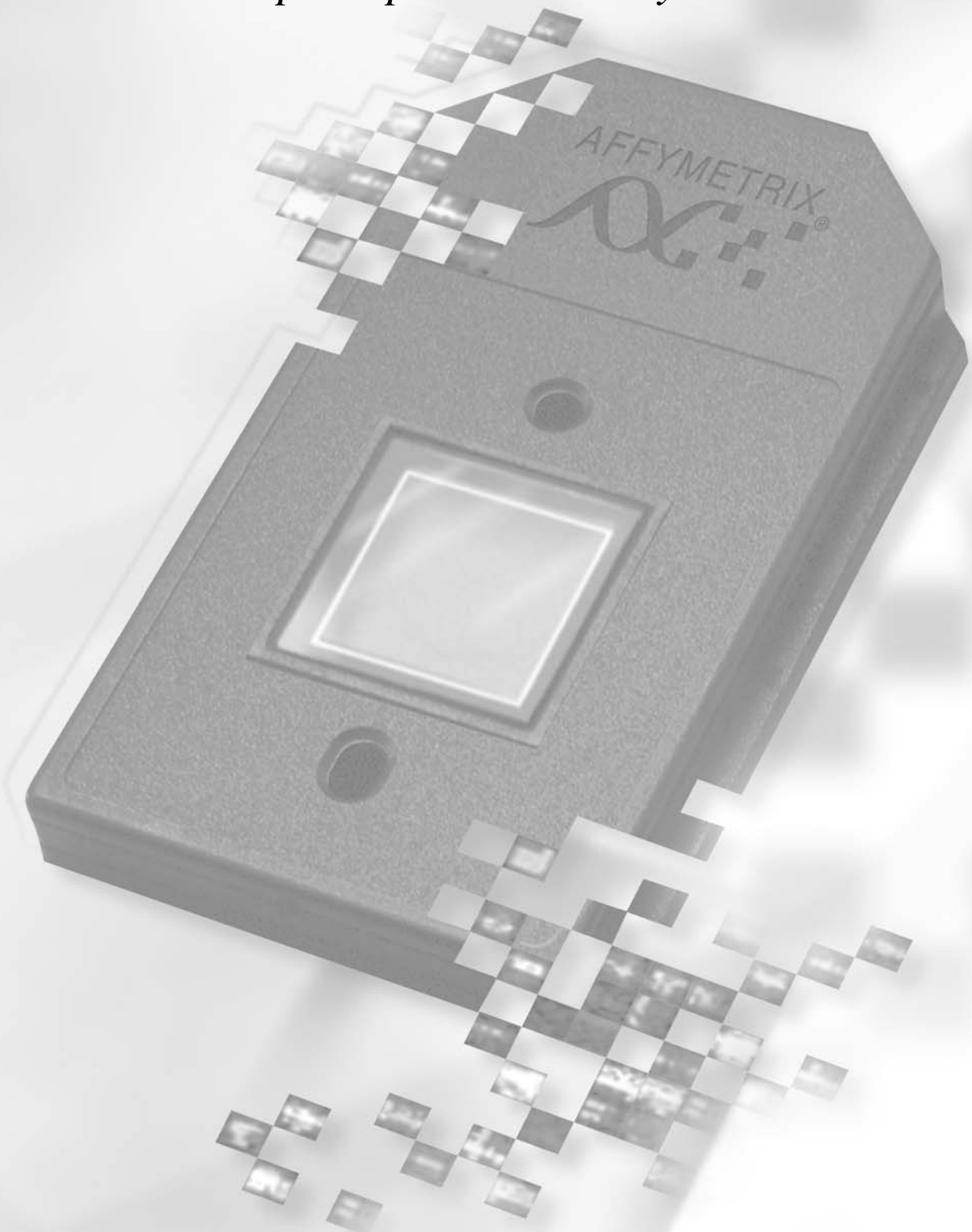

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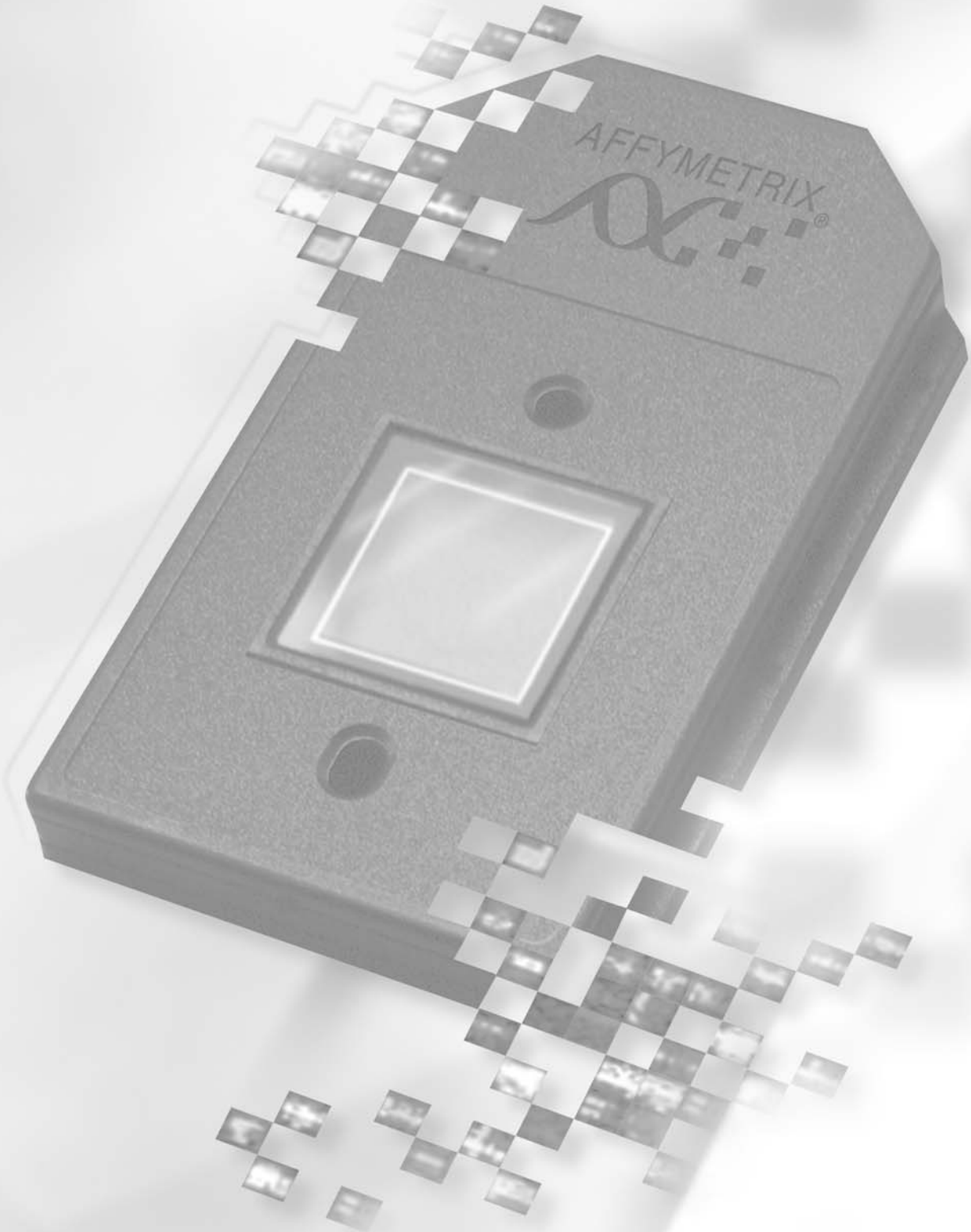

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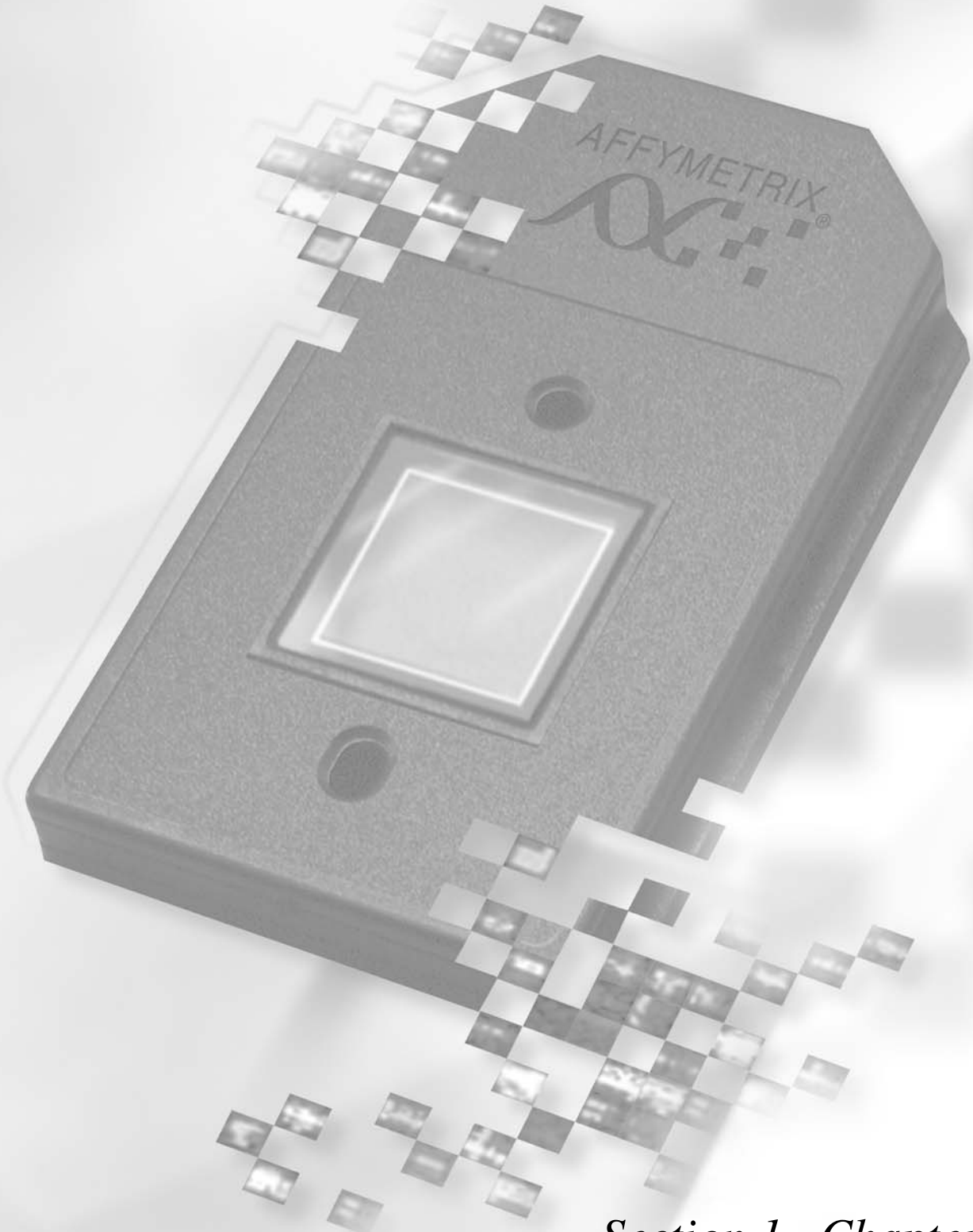

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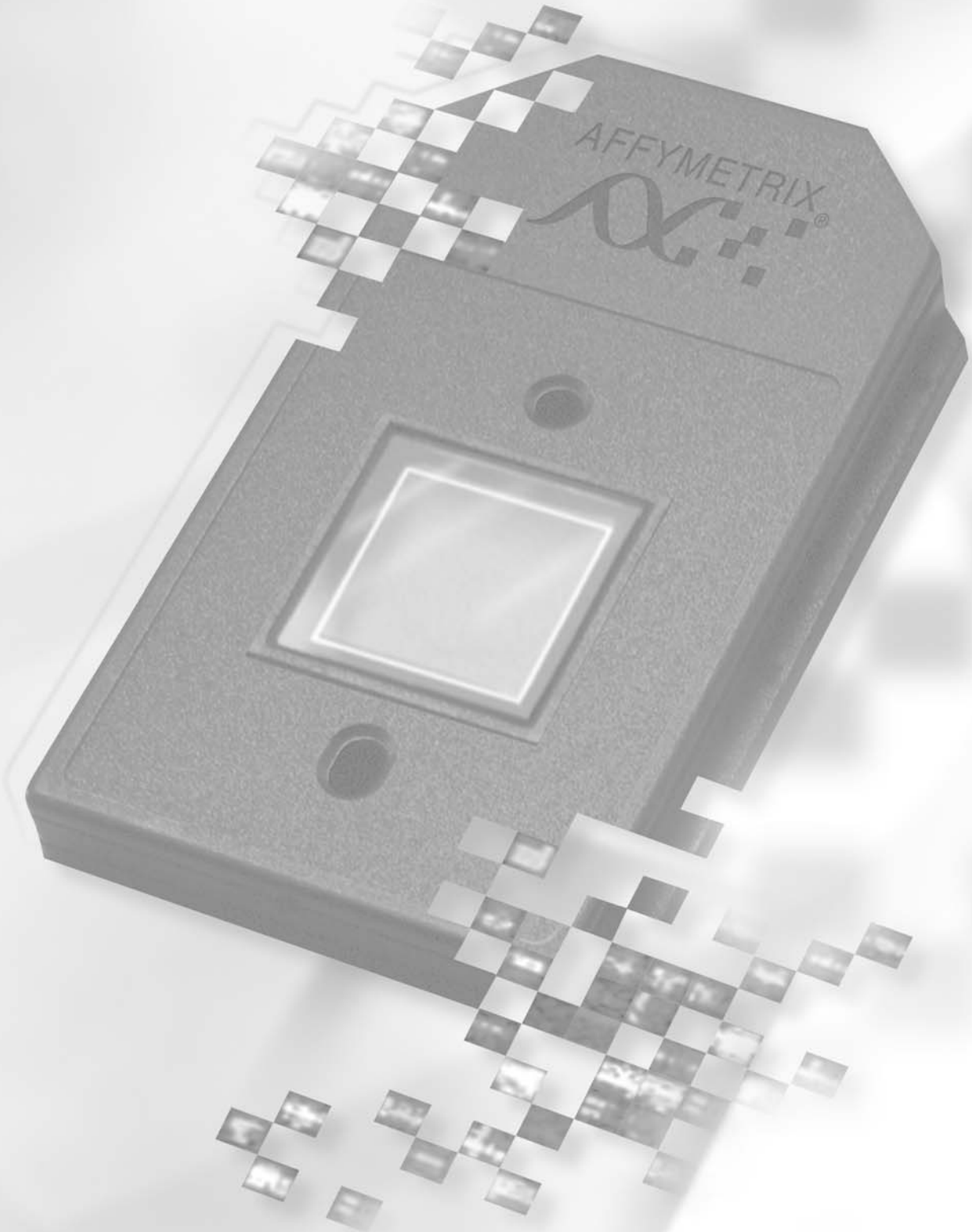


GeneChip® Expression Analysis Overview

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] Tens to hundreds of thousands of 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 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. Experiment and 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, or +44 (0)1628 552550 in Europe.

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: Experiment and Fluidics Station Setup

Specific experimental information is defined using Affymetrix® Microarray Suite on a PC-compatible workstation with a Windows 2000 operating system. The probe array type, sample description, and comments are entered in Microarray Suite 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 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

Data is analyzed using the Microarray Suite Expression Analysis window. 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.

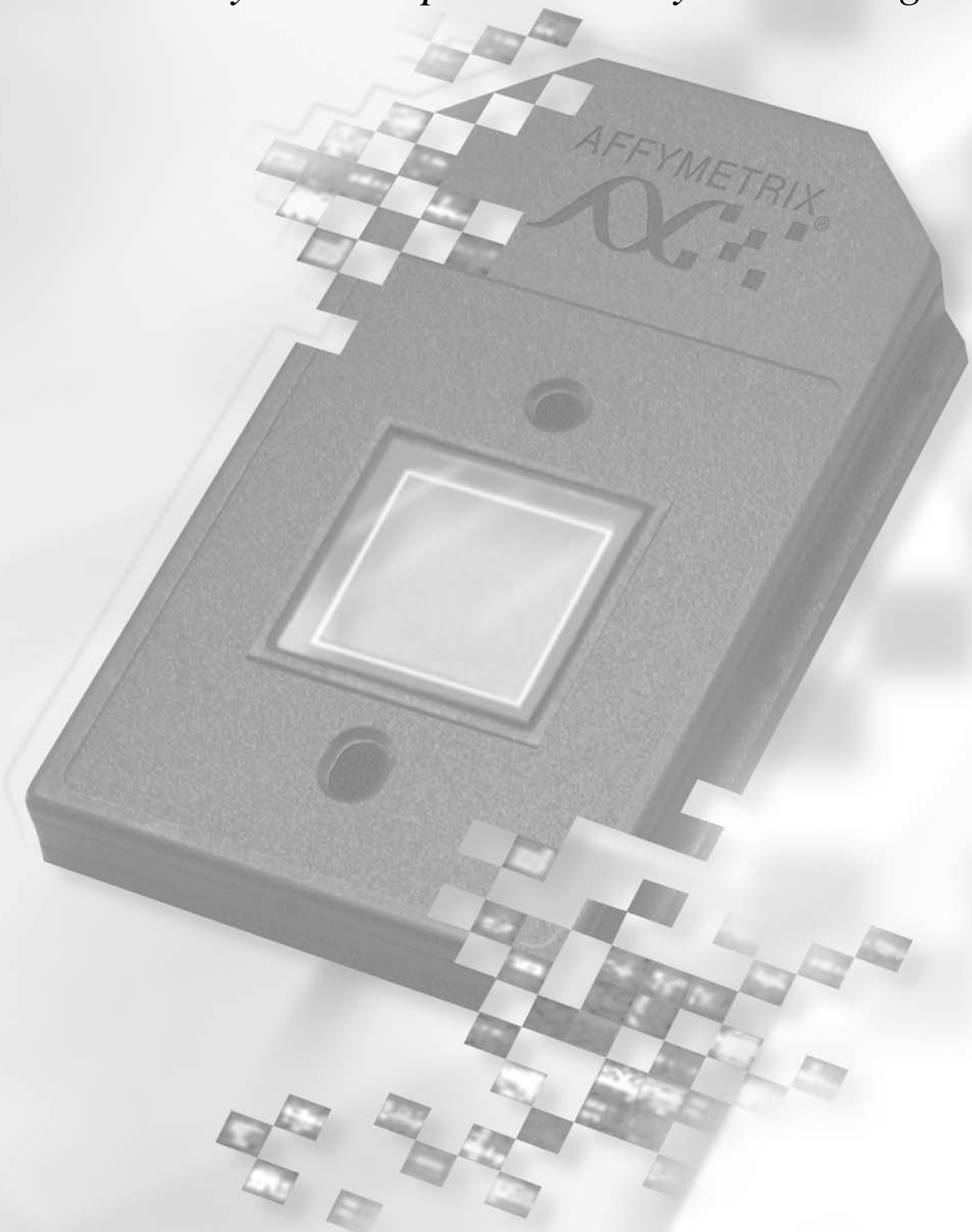
Limitations

- The results of the assay kit 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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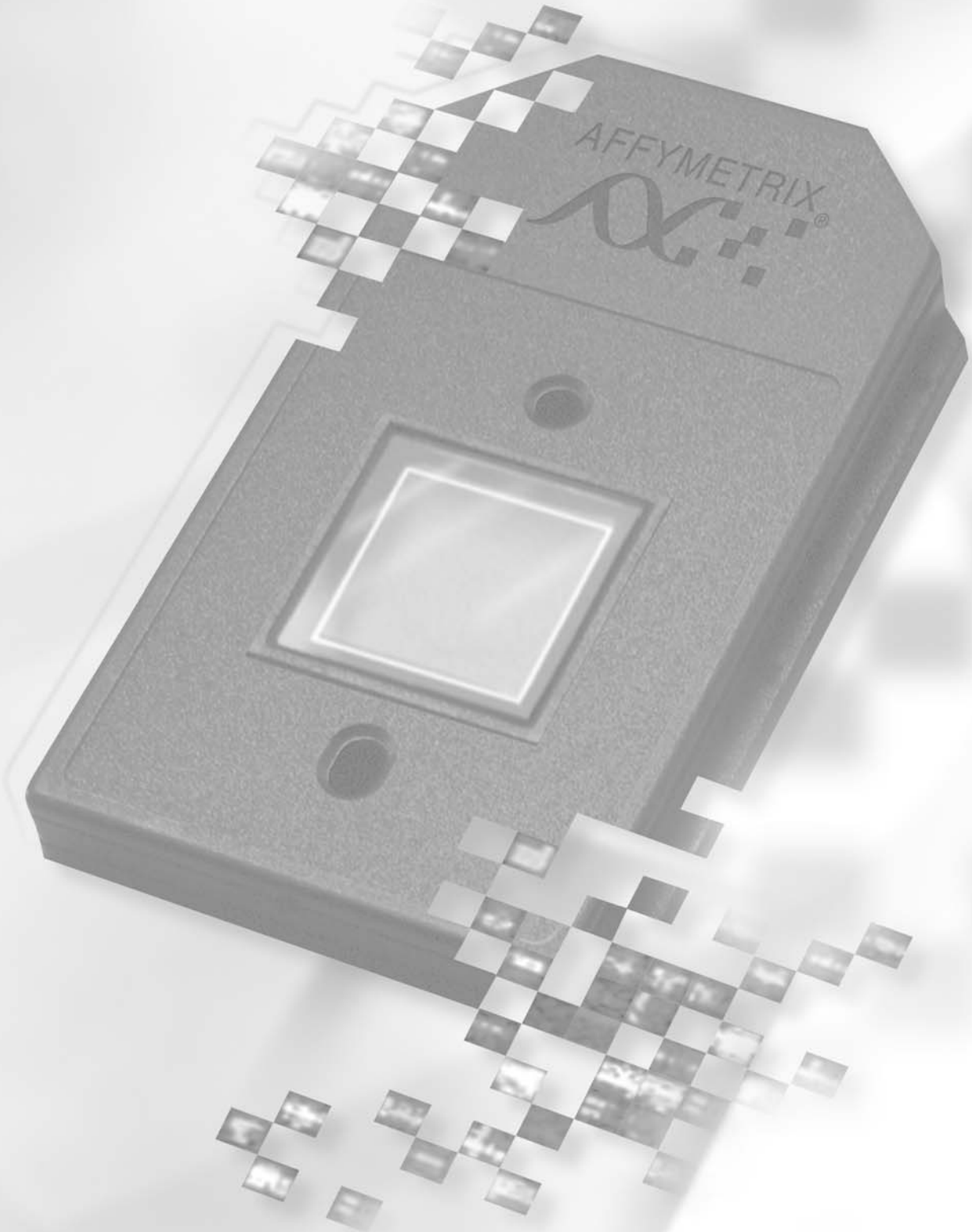

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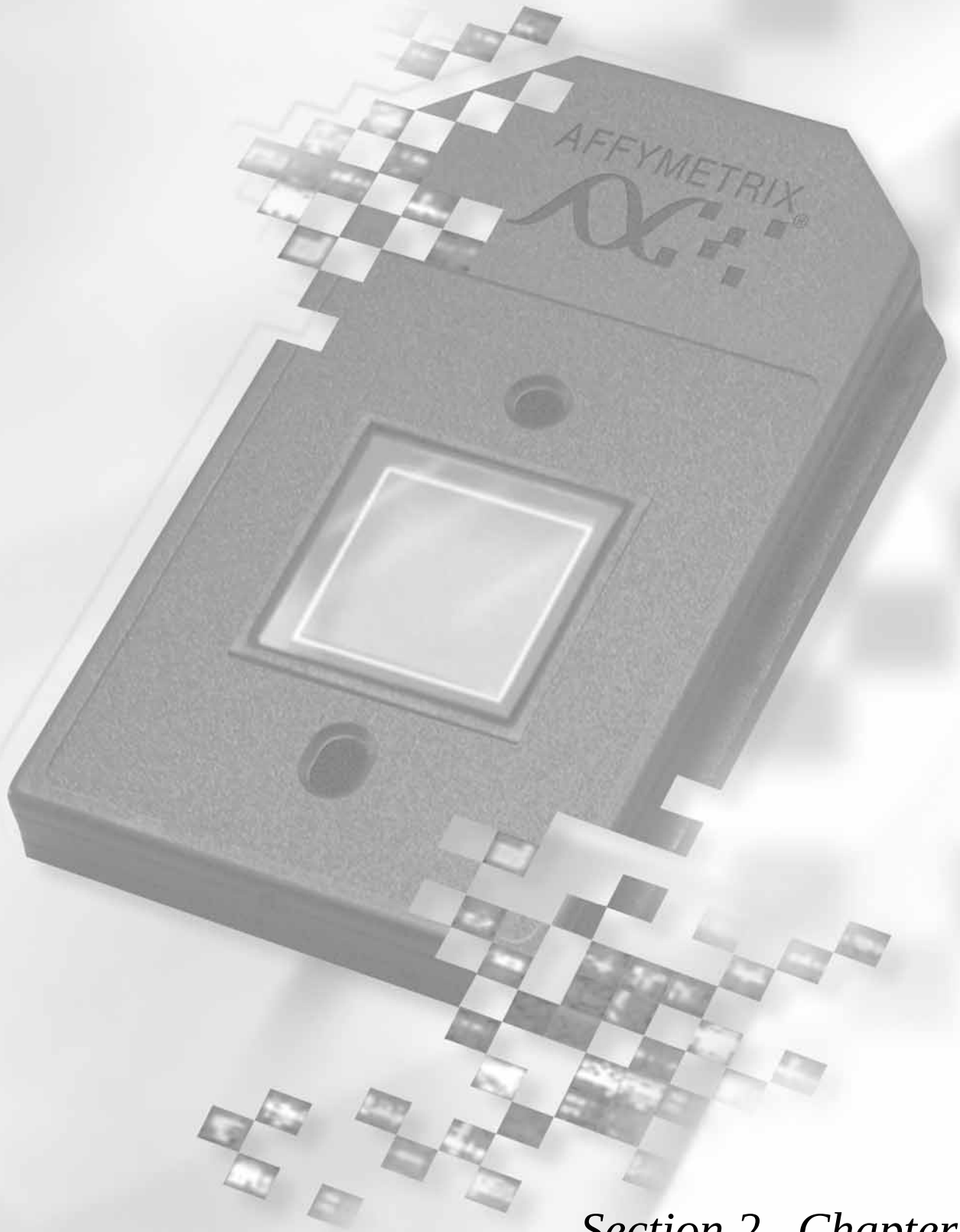

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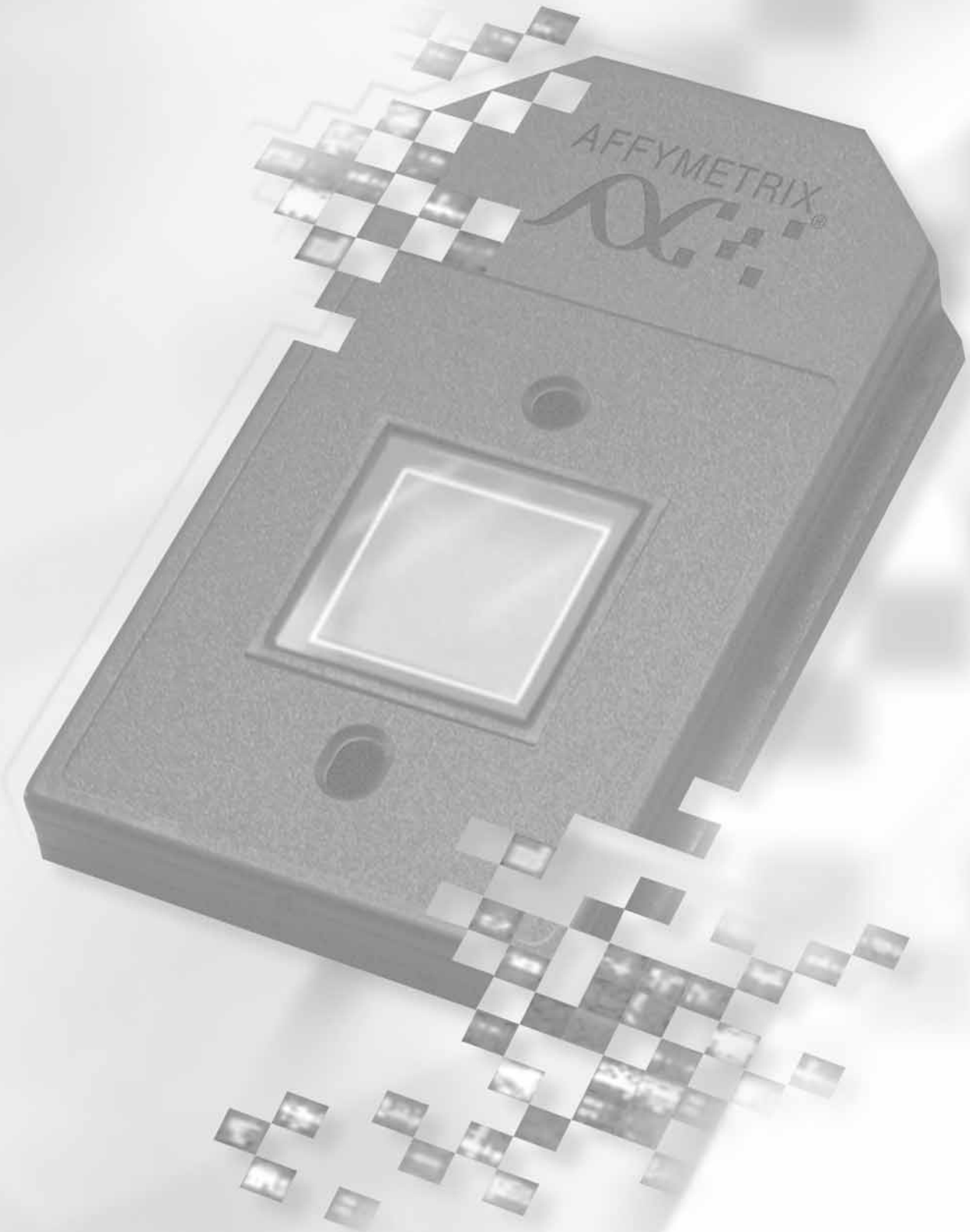


Eukaryotic Target Preparation

This Chapter Contains:

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

Introduction

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

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

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

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

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

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

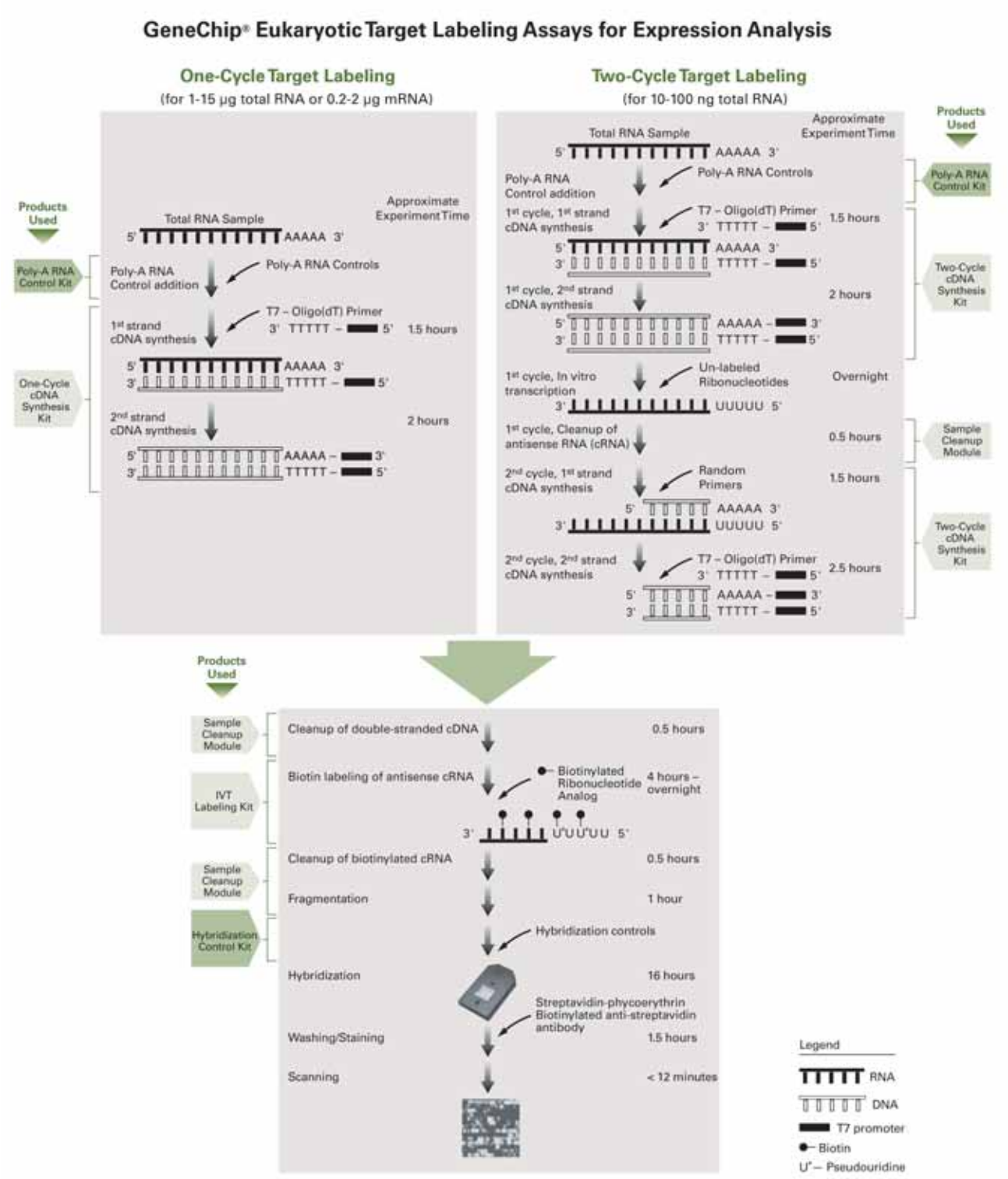


Figure 2.1.1
GeneChip Eukaryotic Labeling Assays for Expression Analysis

Reagents and Materials Required

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

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

Total RNA Isolation

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

Poly-A mRNA Isolation

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

One-Cycle Target Labeling and Control Reagents

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

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

Two-Cycle Target Labeling and Control Reagents

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

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

One-Cycle cDNA Synthesis

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

Two-Cycle cDNA Synthesis

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

Cleanup of Double-Stranded cDNA

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

Synthesis of Biotin-Labeled cRNA

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

IVT cRNA Cleanup and Quantification

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

cRNA Fragmentation

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

Miscellaneous Reagents

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

Miscellaneous Supplies

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

Alternative Protocol for One-Cycle cDNA Synthesis

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

✔ Note

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

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

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

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

Please review precautions and interfering conditions in Section 1.

IMPORTANT

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

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

Isolation of RNA from Yeast

Total RNA

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

Poly-A mRNA

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

Isolation of RNA from Arabidopsis

Total RNA

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

Poly-A mRNA

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

Isolation of RNA from Mammalian Cells or Tissues

Total RNA

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

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

IMPORTANT

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

Poly-A mRNA

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

Precipitation of RNA

Total RNA

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

Poly-A mRNA

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

Precipitation Procedure

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

***Addition of Carrier to Ethanol Precipitations**

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

- *Pellet Paint*

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

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

Quantification of RNA

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

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

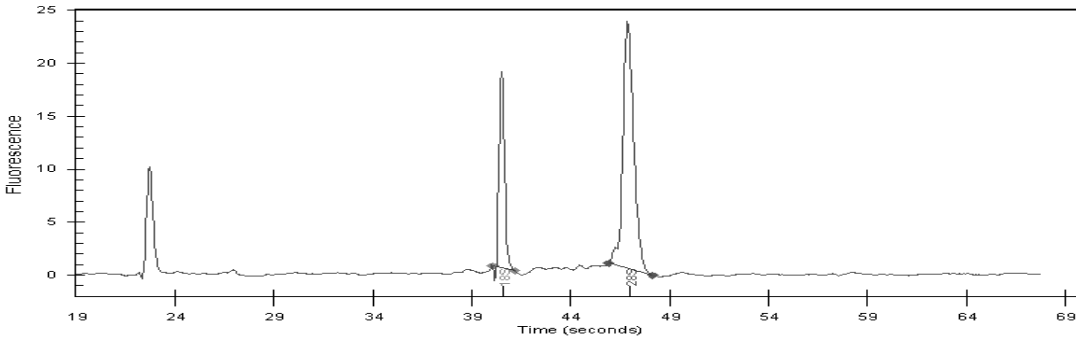


Figure 2.1.2
Electropherogram (from the Agilent 2100 Bioanalyzer) for HeLa Total RNA. For a high-quality total RNA sample, two well-defined peaks corresponding to the 18S and 28S ribosomal RNAs should be observed, similar to a denaturing agarose gel, with ratios approaching 2:1 for the 28S to 18S bands.

Total RNA Isolation for Two-Cycle Target Labeling Assay

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

One-Cycle cDNA Synthesis[1]

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

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

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

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

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

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

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

✔ Note

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

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

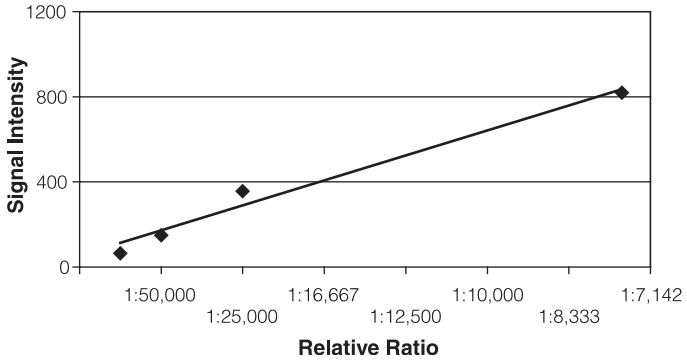


Figure 2.1.3
Poly-A RNA spikes amplified using a complex human Jurkat total RNA sample. Evaluation was performed using Affymetrix® Microarray Suite (MAS) 5.0 software.

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

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

Table 2.1.2
Serial Dilutions of Poly-A RNA Control Stock

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

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

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

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

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

✔ Note

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

Step 2: First-Strand cDNA Synthesis

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

✔ Note

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Table 2.1.5
Preparation of First-Strand Master Mix

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

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

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

4. Incubate for 2 minutes at 42°C.

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

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

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

IMPORTANT

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

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

Step 3: Second-Strand cDNA Synthesis

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

✔ Note

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

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

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

✔ Note

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

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

Table 2.1.6
Preparation of Second-Strand Master Mix

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

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

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

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

3. Incubate for 2 hours at 16°C.

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

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

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

Two-Cycle cDNA Synthesis[2]

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

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

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

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

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

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

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

Note

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

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

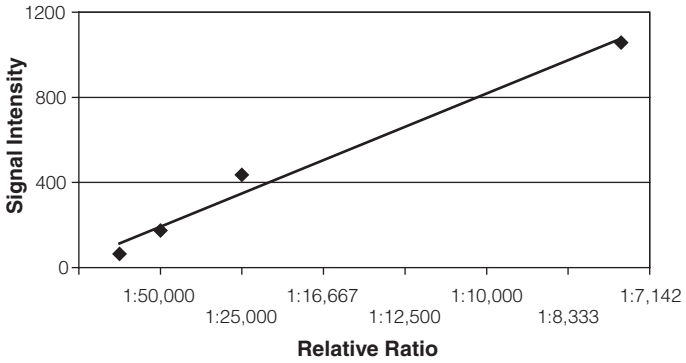


Figure 2.1.4
Poly-A RNA spikes amplified using a complex human Jurkat total RNA sample. Evaluation was performed using MAS 5.0 software.

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

IMPORTANT

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

Table 2.1.8
Serial Dilutions of Poly-A RNA Control Stock

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

Recommendation

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

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

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

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

✔ Note

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

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

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

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

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

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

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

✔ Note

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

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

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

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

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

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

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

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

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

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

   e. Incubate for 6 minutes at 70°C.

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

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

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

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

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

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

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

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

4. Incubate for 1 hour at 42°C.

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

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

IMPORTANT

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

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

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

✔ Note

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

16°C 2 hours
75°C 10 minutes
4°C hold

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

Recommendation

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

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

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

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

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

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

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

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

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

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

✔ Note

No cDNA cleanup is required at this step.

Step 4: First-Cycle, IVT Amplification of cRNA

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

✔ Note

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

37°C 16 hours
4°C hold

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

Table 2.1.13
Preparation of First-Cycle, IVT Master Mix

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

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

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

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

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

Step 5: First-Cycle, Cleanup of cRNA

Sample Cleanup Module is used for this step.

Reagents to be Supplied by User

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

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

IMPORTANT

***BEFORE STARTING** please note:*

- *IVT cRNA Wash Buffer is supplied as a concentrate. Before using for the first time, add 20 mL of ethanol (96-100%), as indicated on the bottle, to obtain a working solution, and checkmark the box on the left-hand side of the bottle label to avoid confusion.*
- *IVT cRNA Binding Buffer may form a precipitate upon storage. If necessary, redissolve by warming in a water bath at 30°C, and then place the buffer at room temperature.*
- *All steps of the protocol should be performed at room temperature. During the procedure, work without interruption.*

1. Add 50 µL of **RNase-free Water** to the IVT reaction and mix by vortexing for 3 seconds.
2. Add 350 µL **IVT cRNA Binding Buffer** to the sample and mix by vortexing for 3 seconds.
3. Add 250 µL ethanol (96-100%) to the lysate, and mix well by pipetting. Do not centrifuge.
4. Apply sample (700 µL) to the **IVT cRNA Cleanup Spin Column** sitting in a **2 mL Collection Tube**. Centrifuge for 15 seconds at ≥ 8,000 x g (≥ 10,000 rpm). Discard flow-through and Collection Tube.
5. Transfer the spin column into a new **2 mL Collection Tube** (supplied). Pipet 500 µL **IVT cRNA Wash Buffer** onto the spin column. Centrifuge for 15 seconds at ≥ 8,000 x g (≥ 10,000 rpm) to wash. Discard flow-through.

✔ Note

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

6. Pipet 500 µL 80% (v/v) ethanol onto the spin column and centrifuge for 15 seconds at ≥ 8,000 x g (≥ 10,000 rpm). Discard flow-through.
7. Open the cap of the spin column and centrifuge for 5 minutes at maximum speed (≤ 25,000 x g). Discard flow-through and Collection Tube.

   Place columns into the centrifuge using every second bucket. Position caps over the adjoining bucket so that they are oriented in the opposite direction to the rotation (i.e., if the microcentrifuge rotates in a clockwise direction, orient the caps in a counterclockwise direction). This avoids damage of the caps.

Recommendation

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

Centrifugation with open caps allows complete drying of the membrane.

8. Transfer spin column into a new **1.5 mL Collection Tube** (supplied), and pipet 13 µL of **RNase-free Water** directly onto the spin column membrane. Ensure that the water is dispensed directly onto the membrane. Centrifuge 1 minute at maximum speed (≤ 25,000 x g) to elute. The average volume of eluate is 11 µL from 13 µL RNase-free Water.

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

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

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

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

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

✔ Note

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

✔ Note

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

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

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

IMPORTANT

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

Recommendation

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

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

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

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

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

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

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

4. Incubate for 2 hours at 16°C.

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

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

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

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

Sample Cleanup Module is used for cleaning up the double-stranded cDNA.

Reagents to be Supplied by User

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

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

IMPORTANT

BEFORE STARTING, please note:

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

1. Add 600 µL of **cDNA Binding Buffer** to the double-stranded cDNA synthesis preparation. Mix by vortexing for 3 seconds.
2. Check that the color of the mixture is yellow (similar to cDNA Binding Buffer without the cDNA synthesis reaction).

Note

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

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

Note

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

6. Open the cap of the spin column and centrifuge for 5 minutes at maximum speed (≤ 25,000 x g). Discard flow-through and Collection Tube.

Recommendation

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

Place columns into the centrifuge using every second bucket. Position caps over the adjoining bucket so that they are oriented in the opposite direction to the rotation

(i.e., if the microcentrifuge rotates in a clockwise direction, orient the caps in a counterclockwise direction). This avoids damage of the caps.

Centrifugation with open caps allows complete drying of the membrane.

7. Transfer spin column into a 1.5 mL Collection Tube, and pipet 14 µL of **cDNA Elution Buffer** directly onto the spin column membrane. Incubate for 1 minute at room temperature and centrifuge 1 minute at maximum speed (≤ 25,000 x g) to elute.

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

✔ Note

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

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

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

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

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

GeneChip IVT Labeling Kit is used.

✔ Note

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

IMPORTANT

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

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

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

Table 2.1.16
IVT Reaction Set Up

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

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

Table 2.1.17
IVT Reaction

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

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

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

Note

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

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

Cleanup and Quantification of Biotin-Labeled cRNA

Sample Cleanup Module is used for cleaning up the Biotin Labeled cRNA.

Reagents to be Supplied by User

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

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

Step 1: Cleanup of Biotin-Labeled cRNA

IMPORTANT

***BEFORE STARTING** please note:*

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

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

✔ Note

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

6. Pipet 500 µL 80% (v/v) ethanol onto the spin column and centrifuge for 15 seconds at ≥ 8,000 x g (≥ 10,000 rpm). Discard flow-through.
7. Open the cap of the spin column and centrifuge for 5 minutes at maximum speed (≤ 25,000 x g). Discard flow-through and Collection Tube.

Place columns into the centrifuge using every second bucket. Position caps over the adjoining bucket so that they are oriented in the opposite direction to the rotation (i.e., if the microcentrifuge rotates in a clockwise direction, orient the caps in a counterclockwise direction). This avoids damage of the caps.

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

Centrifugation with open caps allows complete drying of the membrane.

8. Transfer spin column into a new **1.5 mL Collection Tube** (supplied), and pipet 11 µL of **RNase-free Water** directly onto the spin column membrane. Ensure that the water is dispensed directly onto the membrane. Centrifuge 1 minute at maximum speed (≤ 25,000 x g) to elute.
9. Pipet 10 µL of **RNase-free Water** directly onto the spin column membrane. Ensure that the water is dispensed directly onto the membrane. Centrifuge 1 minute at maximum speed (≤ 25,000 x g) to elute.

   For subsequent photometric quantification of the purified cRNA, we recommend dilution of the eluate between 1:100 fold and 1:200 fold.

Step 2: Quantification of the cRNA

Use spectrophotometric analysis to determine the cRNA yield. Apply the convention that 1 absorbance unit at 260 nm equals 40 µg/mL RNA.

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

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

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

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.

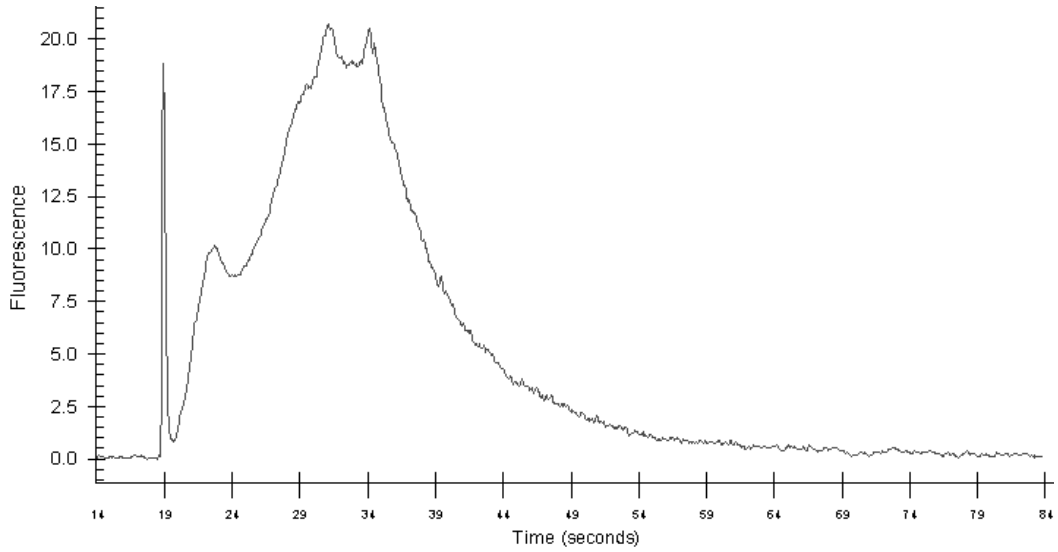


Figure 2.1.5
Biotin-labeled cRNA from One-Cycle cDNA Synthesis Kit. Bioanalyzer electropherogram for labeled cRNA from HeLa total RNA using the One-Cycle Kit. This electropherogram displays the nucleotide size distribution for 400 ng of labeled cRNA resulting from one round of amplification. The average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

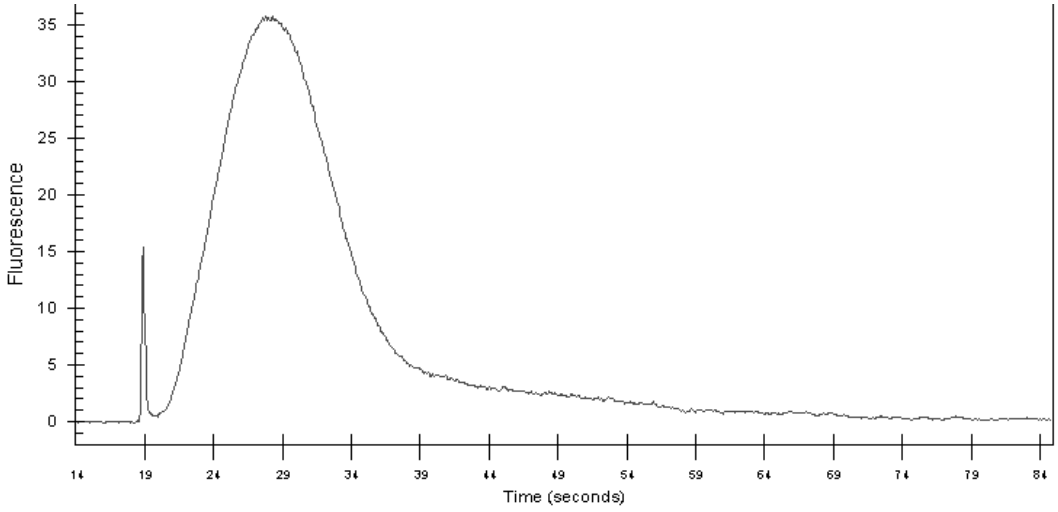


Figure 2.1.6
Biotin-labeled cRNA from Two-Cycle cDNA Synthesis Kit. Bioanalyzer electropherogram for labeled cRNA from HeLa total RNA using the Two-Cycle Kit. This electropherogram displays the nucleotide size distribution for 400 ng of labeled cRNA resulting from two rounds of amplification. The average 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 and gel analysis (refer to the *Eukaryotic Target Hybridization* chapter in Section 2).

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

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

Table 2.1.18
Sample Fragmentation Reaction by Array Format*

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

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

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

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

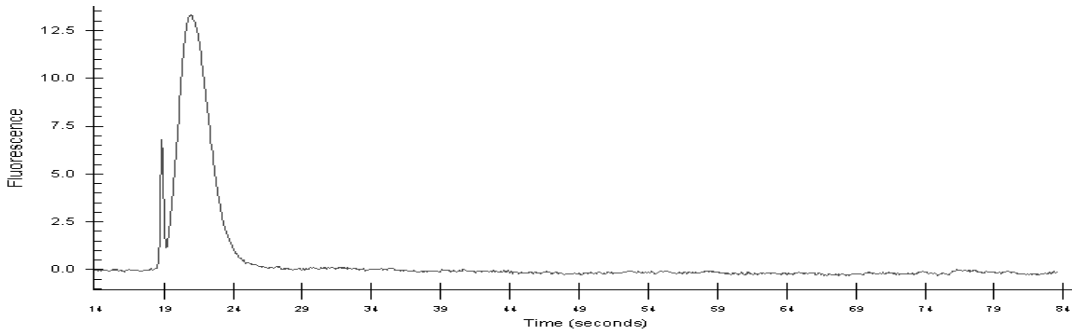


Figure 2.1.7
Fragmented cRNA. Bioanalyzer electropherogram for fragmented labeled cRNA from HeLa total RNA. This electropherogram displays the nucleotide size distribution for 150 ng of fragmented labeled cRNA resulting from one round of amplification. The average size is approximately 100 nt.

Alternative Protocol for One-Cycle cDNA Synthesis from Total RNA

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

- Read all information and instructions that come with reagents and kits.
- Use the GeneChip T7-Oligo(dT) Promoter Primer Kit[3] for priming first-strand cDNA synthesis in place of the oligo(dT) or random primers provided with the SuperScript Choice kit. The GeneChip T7-Oligo(dT) Promoter Primer Kit provides high-quality HPLC-purified T7-Oligo(dT) Primer, which is essential for this reaction.

T7-Oligo(dT) Primer

5´ - GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-$(dT)_{24}$ - 3´

Step 1: First-Strand cDNA Synthesis

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

Note

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

Note

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

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

Before starting cDNA synthesis, the correct volumes of DEPC-treated H_2O and Reverse Transcriptase (RT) must be determined. These volumes will depend on both the concentration and total volume of RNA that is being added to the reaction.

IMPORTANT

Use Table 2.1.19 and Table 2.1.20 for variable component calculations. Determine the volumes of RNA and SuperScript II RT required in Table 2.1.19, then calculate the amount of DEPC-treated H_2O needed in Step 1 Table 2.1.20 to bring the final First-Strand Synthesis volume to 20 µL.

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

Table 2.1.19
Reverse Transcriptase Volumes for First-Strand cDNA Synthesis Reaction

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

✔ Note

The combined volume of RNA, DEPC-treated H_2O and SuperScript II RT should not exceed 11 µL as indicated in Table 2.1.20.

Table 2.1.20
First-Strand cDNA Synthesis Components

	Reagents in reaction	*Volume*	*Final Concentration or Amount in Reaction*
1: Primer Hybridization Incubate at 70°C for 10 minutes Quick spin and put on ice	DEPC-treated H_2O (variable) T7-Oligo(dT) Primer, 50 µM RNA (variable)	for final reaction volume of 20 µL 2 µL 5.0 to 20 µg	 100 pmol 5.0 to 20 µg
2: Temperature Adjustment Add to the above tube and mix well Incubate at *42*°C for 2 minutes	5X First-Strand cDNA buffer 0.1 M DTT 10 mM dNTP mix	4 µL 2 µL 1 µL	1X 10 mM DTT 500 µM each
3: First-Strand Synthesis Add to the above tube and mix well Incubate at *42*°C for 1 hour	SuperScript II RT (variable) (200 U/µL)	See Table 2.1.19	200 U to 1000 U
Total Volume		*20 µL*	

✔ Note

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

Step 2: Second-Strand cDNA Synthesis

1. Place First-Strand reactions on ice. Centrifuge briefly to bring down condensation on sides of tube.
2. Add to the First-Strand synthesis tube the reagents listed in the following Second-Strand Final Reaction Composition Table (Table 2.1.21).

Table 2.1.21
Second-Strand Final Reaction Composition

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

3. Gently tap tube to mix. Then, briefly spin in a microcentrifuge to remove condensation and incubate at 16°C for 2 hours in a cooling waterbath.
4. Add 2 µL [10 U] T4 DNA Polymerase.
5. Return to 16°C for 5 minutes.
6. Add 10 µL 0.5M EDTA.
7. Proceed to cleanup procedure for cDNA, *Cleanup of Double-Stranded cDNA for Both the One-Cycle and Two-Cycle Target Labeling Assays* on page 2.1.32, or store at -20°C for later use.

Alternative Protocol for One-Cycle cDNA Synthesis from Purified Poly-A mRNA

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

- Read all information and instructions that come with reagents and kits.
- Use the GeneChip T7-Oligo(dT) Promoter Primer Kit[4] for priming first-strand cDNA synthesis in place of the oligo(dT) or random primers provided with the SuperScript Choice kit. The GeneChip T7-Oligo(dT) Promoter Primer Kit provides high-quality HPLC-purified T7-Oligo(dT) Primer, which is essential for this reaction.
- It is recommended that each step of this protocol is checked by gel electrophoresis.

T7-Oligo(dT) Primer

5´ - GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-$(dT)_{24}$ - 3´

Step 1: First-Strand cDNA Synthesis

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

Note

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

Before starting cDNA synthesis, the correct volumes of DEPC-treated H_2O and Reverse Transcriptase (RT) must be determined. These volumes will depend on both the concentration and total volume of mRNA that is being added to the reaction. For every µg of mRNA, you will need to add 1 µL of SuperScript II RT (200 U/µL). For mRNA quantity ≤ 1 µg, use 1 µL of SuperScript II RT. Synthesis reactions should be done in a polypropylene tube (RNase-free).

IMPORTANT

Use Table 2.1.22 for variable component calculations. Determine volumes of mRNA and SuperScript II RT required, and then calculate the amount of DEPC-treated H_2O needed in the ***Primer Hybridization Mix*** *step to bring the final First-Strand Synthesis reaction volume to 20 µL.*

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

Table 2.1.22
First-Strand cDNA Synthesis Components

	Reagents in Reaction	*Volume*	*Final Concentration or Amount in Reaction*
1: Primer Hybridization Incubate at 70°C for 10 minutes Quick spin and put on ice	DEPC-treated H_2O (variable) T7-Oligo(dT) Primer, 50 µM mRNA (variable)	for final reaction volume of 20 µL 2 µL 0.2 to 2 µg	 100 pmol 0.2 to 2 µg
2: Temperature Adjustment Add to the above tube and mix well Incubate at 37°C for 2 minutes	5X First-Strand cDNA buffer 0.1 M DTT 10 mM dNTP mix	4 µL 2 µL 1 µL	1X 10 mM 500 µM each
3: First-Strand Synthesis Add to the above tube and mix well Incubate at 37°C for 1 hour	SuperScript II RT (variable) (200 U/µL)	1 µL per µg mRNA	200 U to 1000 U
Total Volume		*20 µL*	

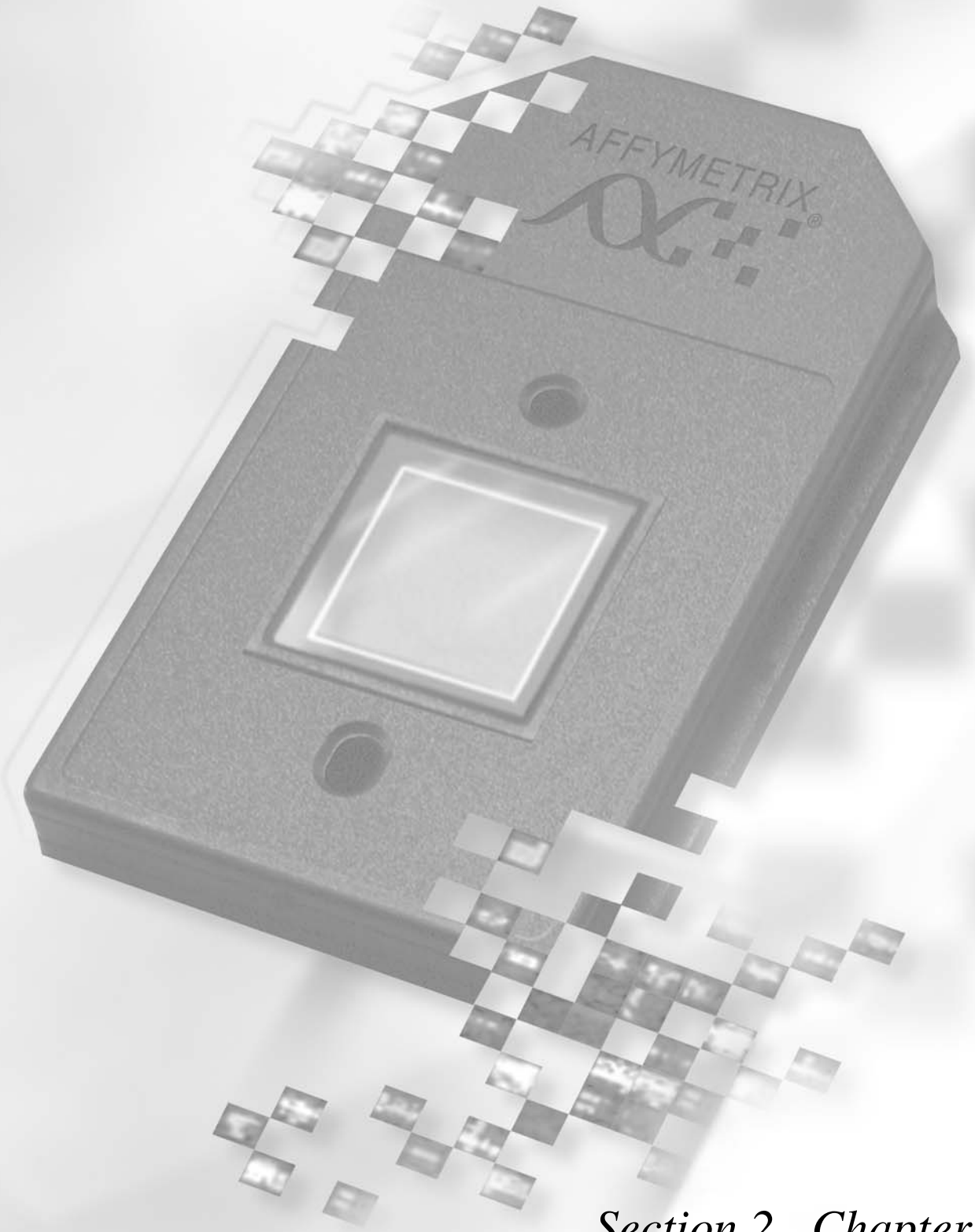
Step 2: Second-Strand cDNA Synthesis

1. Place First-Strand reactions on ice. Centrifuge briefly to bring down condensation on sides of tube.
2. Add to the First-Strand synthesis tube the reagents listed in the following Second-Strand Final Reaction Composition Table (Table 2.1.23).

Table 2.1.23
Second-Strand Final Reaction Composition

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

3. Gently tap tube to mix. Then, briefly spin in a microcentrifuge to remove condensation and incubate at 16°C for 2 hours in a cooling waterbath.
4. Add 2 µL [10 U] T4 DNA Polymerase.
5. Return to 16°C for 5 minutes.
6. Add 10 µL 0.5M EDTA.
7. Proceed to cleanup procedure for cDNA, *Cleanup of Double-Stranded cDNA for Both the One-Cycle and Two-Cycle Target Labeling Assays* on page 2.1.32, or store at -20°C for later use.



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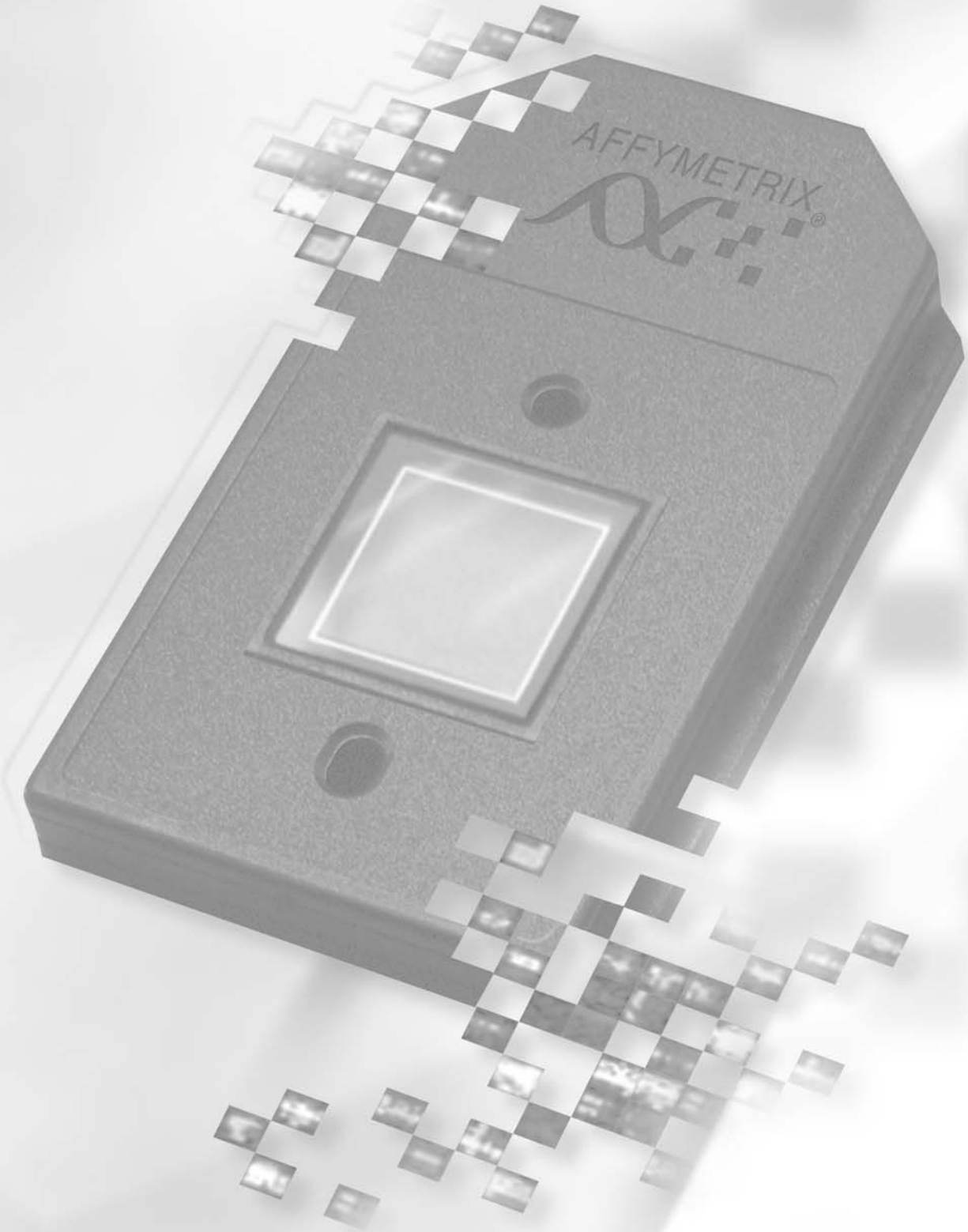


Eukaryotic Target Hybridization

This Chapter Contains:

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

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

Reagents and Materials Required

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

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

Miscellaneous Supplies

- Hybridization Oven 640, Affymetrix, P/N 800138 (110V) or 800139 (220V)
- Sterile, RNase-free, microcentrifuge vials, 1.5 mL, USA Scientific, P/N 1415-2600 (or equivalent)
- Micropipettors, (P-2, P-20, P-200, P-1000), Rainin Pipetman (or equivalent)
- Sterile-barrier pipette tips and non-barrier pipette tips
- Heatblock

Reagent Preparation

12X MES Stock Buffer

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

For 1,000 mL:

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

IMPORTANT

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

2X Hybridization Buffer

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

For 50 mL:

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

Eukaryotic Target Hybridization

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

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

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

Table 2.2.1
Hybridization Cocktail for Single Probe Array*

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

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

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

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

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

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

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

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

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

✔ Note

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

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

Table 2.2.2
Probe Array Cartridge Volumes

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

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

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

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

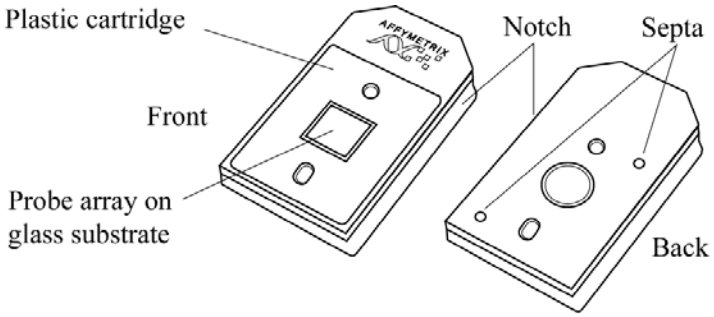
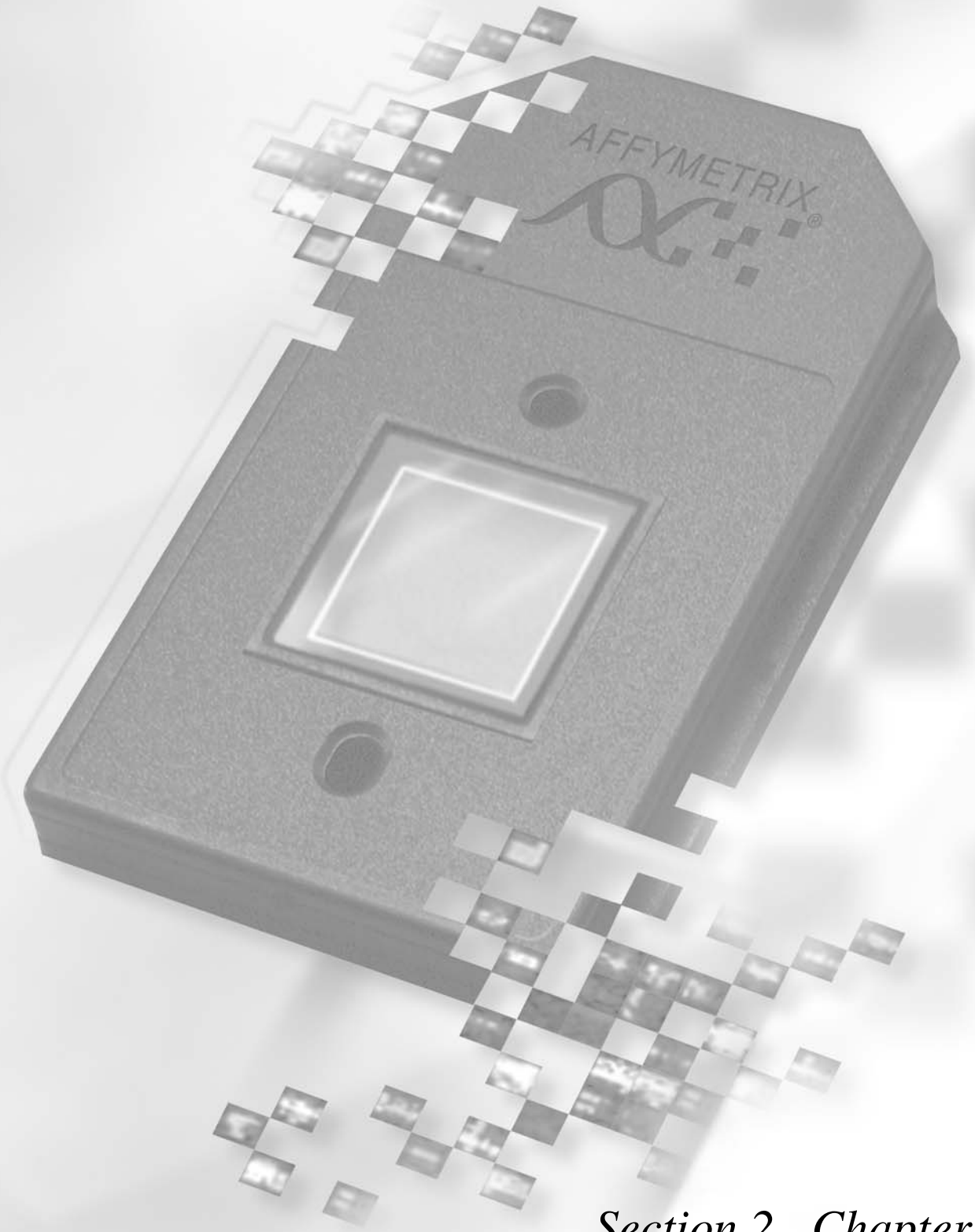


Figure 2.2.1
GeneChip® Probe Array



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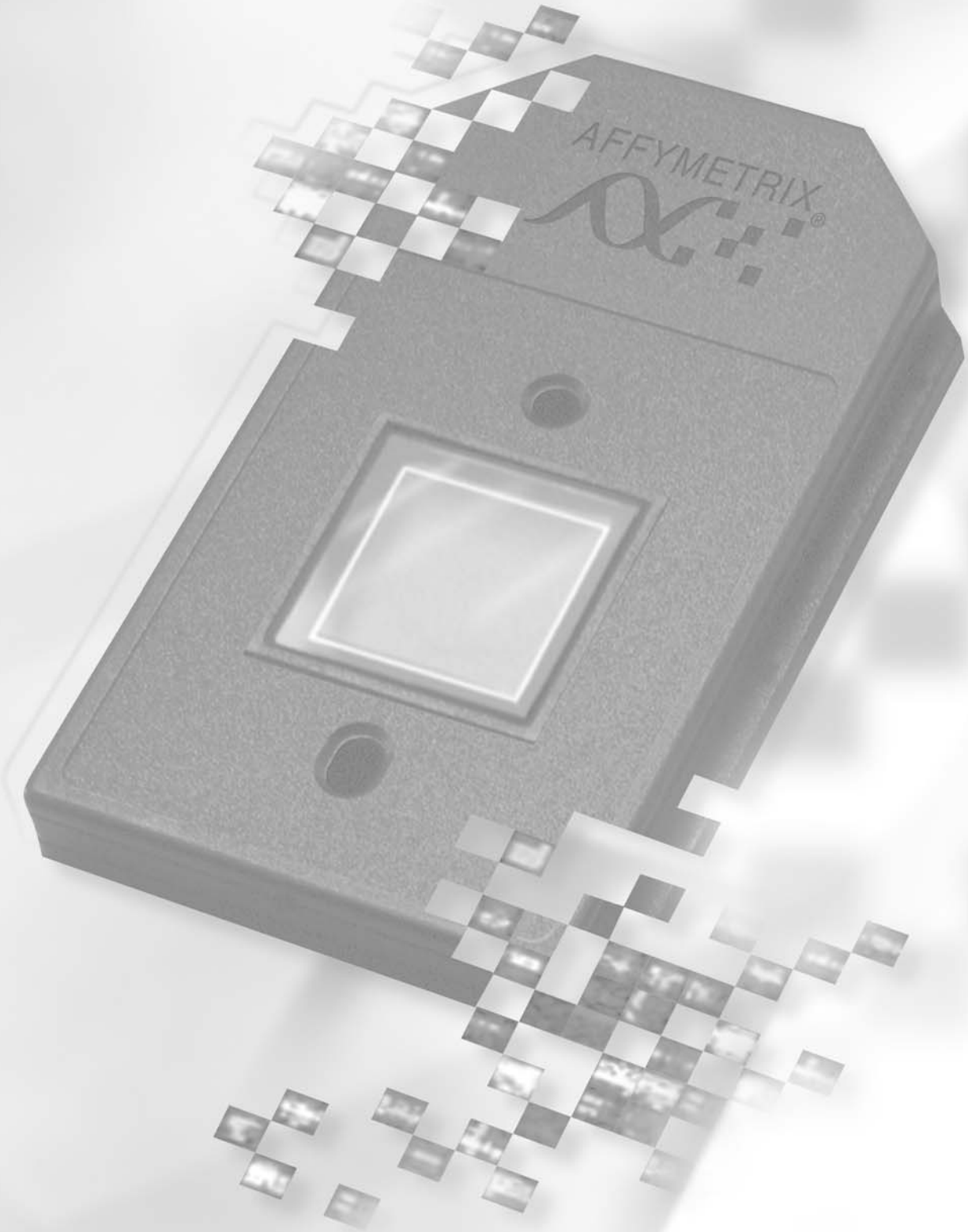


Eukaryotic Arrays: Washing, Staining, and Scanning

This Chapter Contains:

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

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

Reagents and Materials Required

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

- 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
- Bleach (5.25% Sodium Hypochlorite), VWR Scientific, P/N 37001-060 (or equivalent)

Miscellaneous Supplies

- Sterile, RNase-free, microcentrifuge vials, 1.5 mL, USA Scientific, P/N 1415-2600 (or equivalent)
- Micropipettors, (P-2, P-20, P-200, P-1000), Rainin Pipetman (or equivalent)
- Sterile-barrier pipette tips and non-barrier pipette tips
- Tygon Tubing, 0.04″ inner diameter, Cole-Parmer, P/N H-06418-04
- Tough-Spots, Label Dots, USA Scientific, P/N 9185-0000

Reagent Preparation

Wash Buffer A: Non-Stringent Wash Buffer

(6X SSPE, 0.01% Tween-20)

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

Wash Buffer B: Stringent Wash Buffer

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

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

2X Stain Buffer

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

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

10 mg/mL Goat IgG Stock

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

✔ Note

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

Experiment and Fluidics Station Setup

Step 1: Defining File Locations

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

✔ Note

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

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

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

Step 2: Entering Experiment Information

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

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

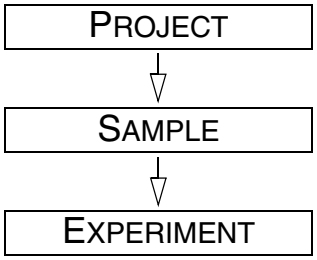
- Experiment Name
- Probe Array Type

In GCOS, three additional fields are required:

- Sample Name
- Sample Type
- Project

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

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



Step 3: Preparing the Fluidics Station

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

Setting Up the Fluidics Station

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

✔ Note

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

Priming the Fluidics Station

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

Priming should be done:

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

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

Probe Array Wash and Stain

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

✔ Note

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

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

Preparing the Staining Reagents

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

SAPE Stain Solution

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

Table 2.3.1
SAPE Solution Mix

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

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.
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.

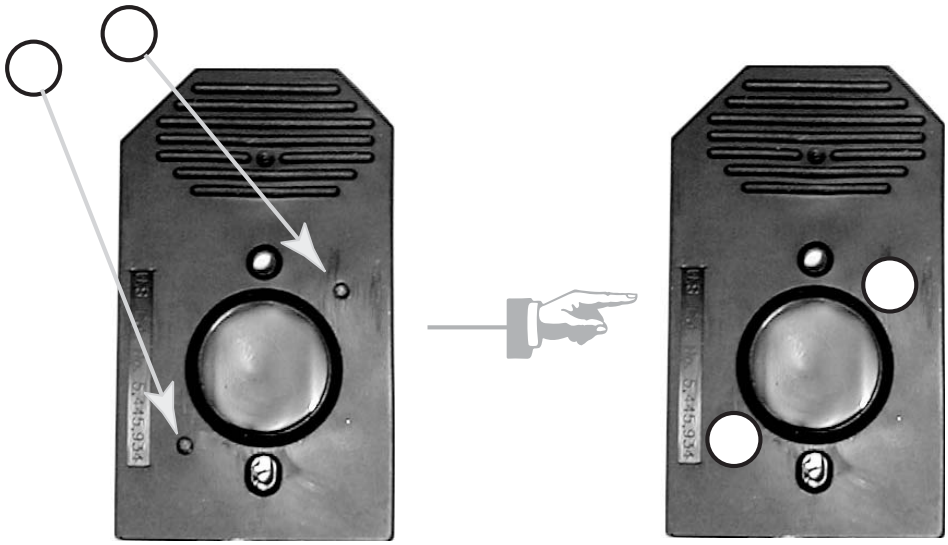


Figure 2.3.1
Applying Tough-Spots™ to the probe array cartridge

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.

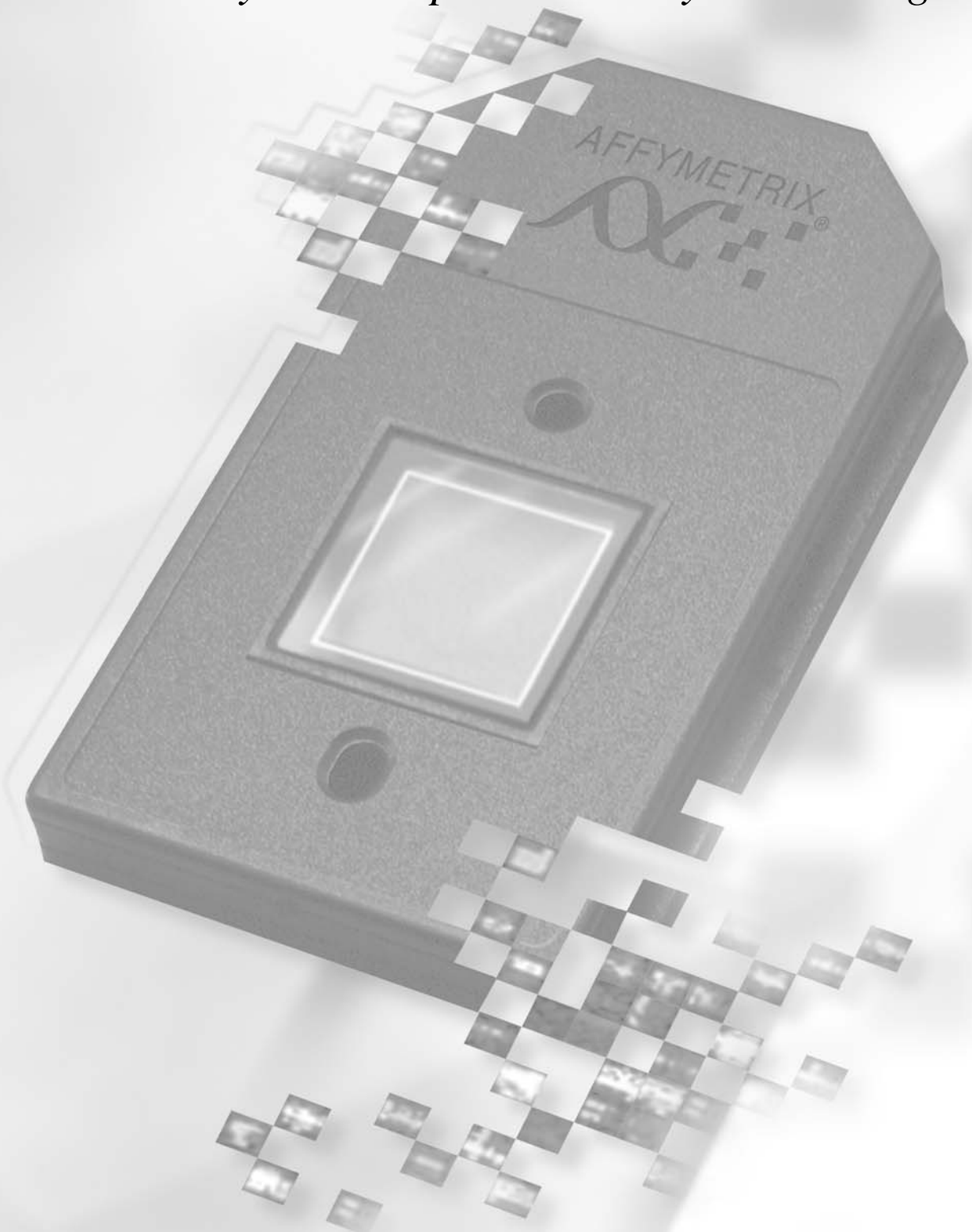
4. To return to the default values for the protocol selected, click the **Defaults** button.
5. After all the protocol conditions are modified as desired, change the name of the edited protocol in the **Protocol Name** box.

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

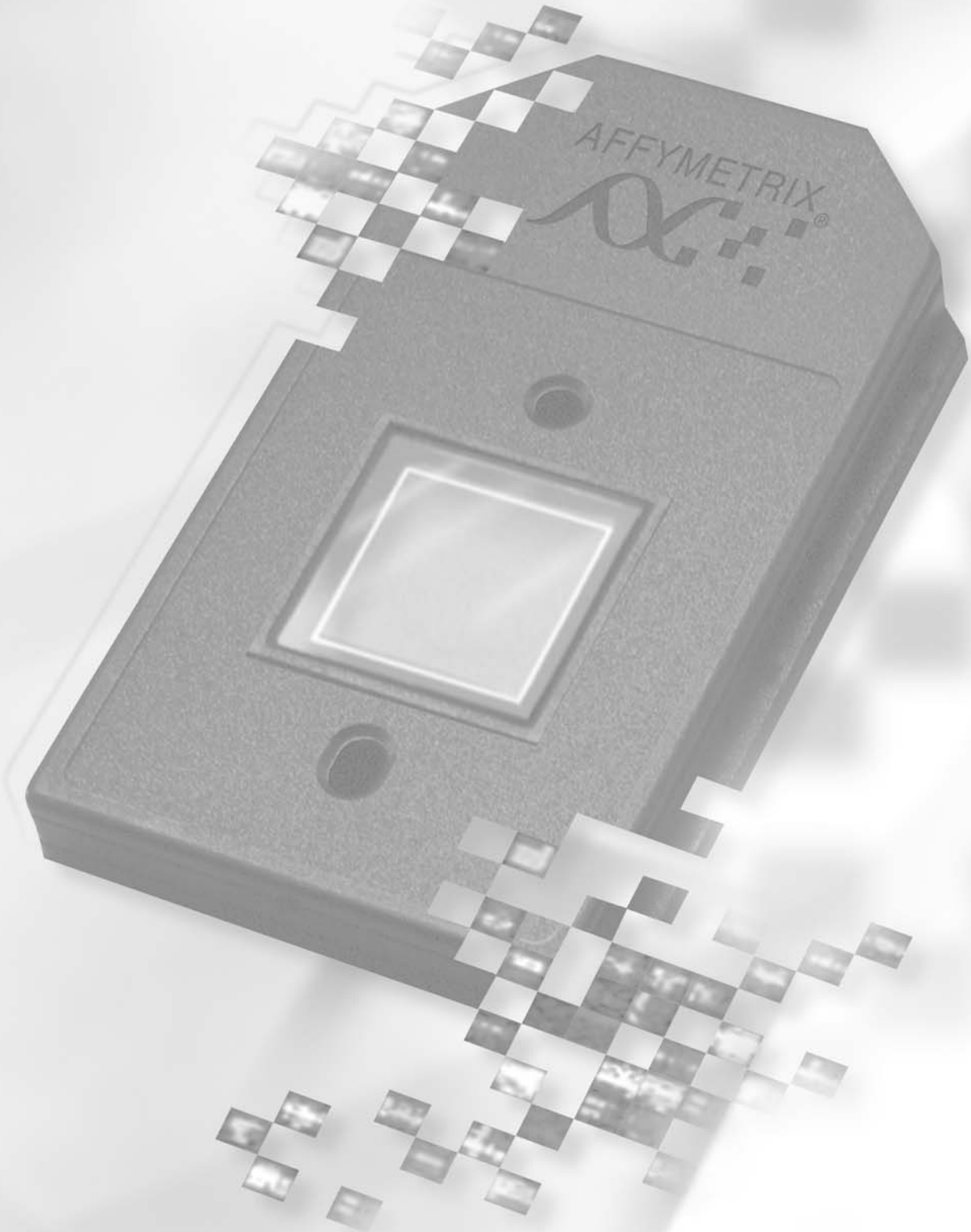
6. Click **Save**, then close the dialog box.
   Enter **0** (zero) for hybridization time if hybridization step is not required. Likewise, enter **0** (zero) for the stain time if staining is not required. Enter **0** (zero) for the number of wash cycles if a wash solution is not required.

Section 3:

Prokaryotic Sample and Array Processing

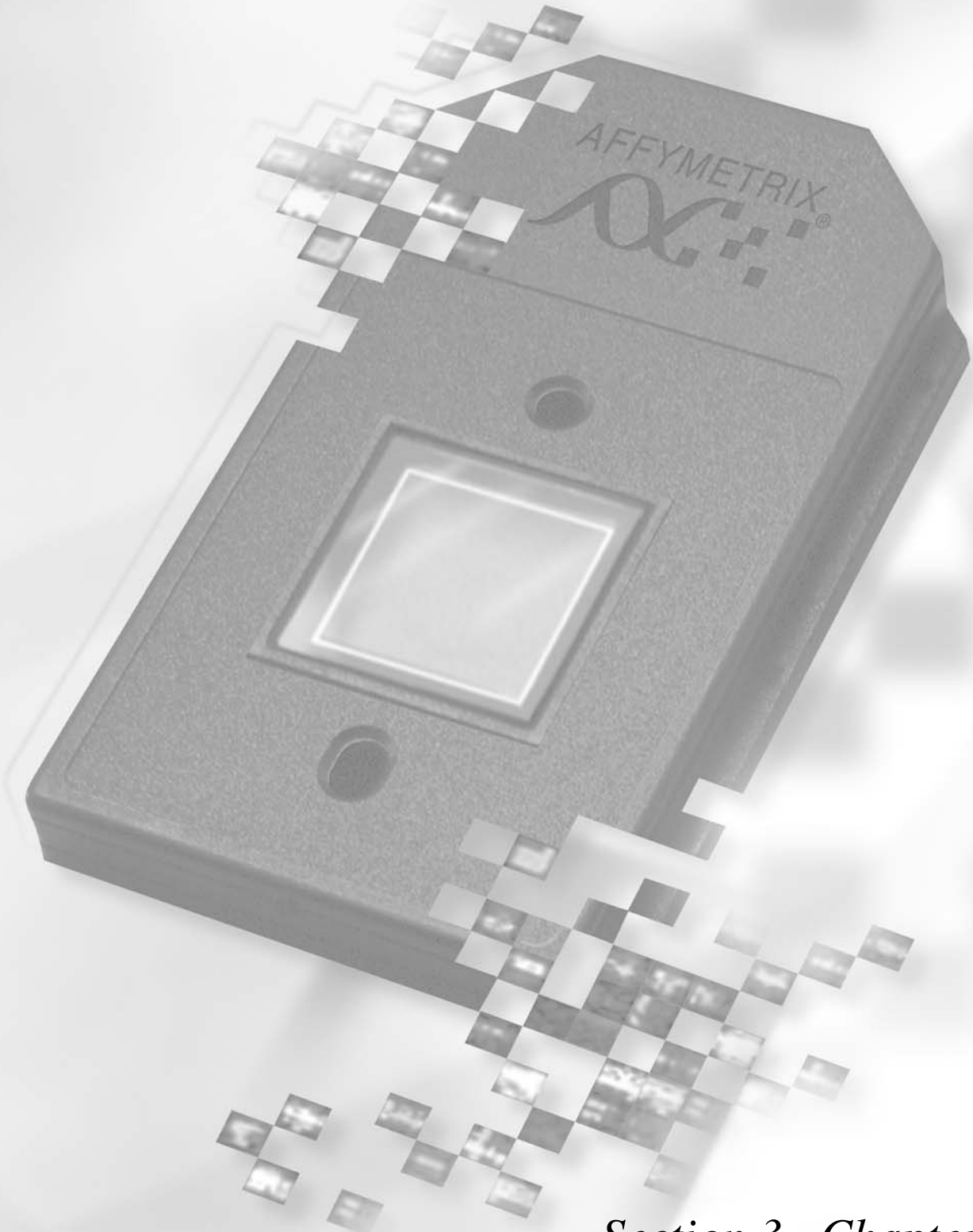


Section 3



Contents

Section 3 *Prokaryotic Sample and Array Processing*



Prokaryotic

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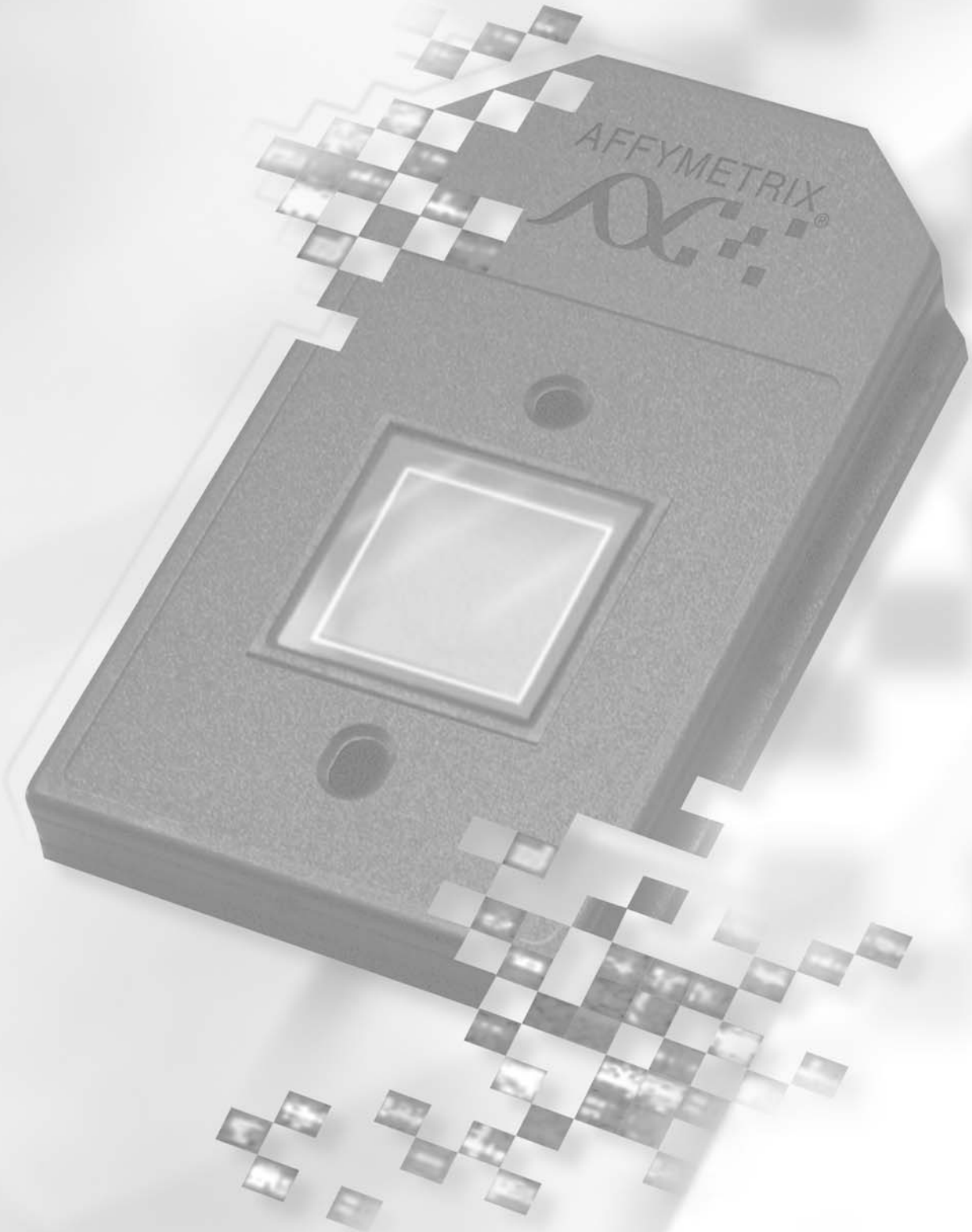


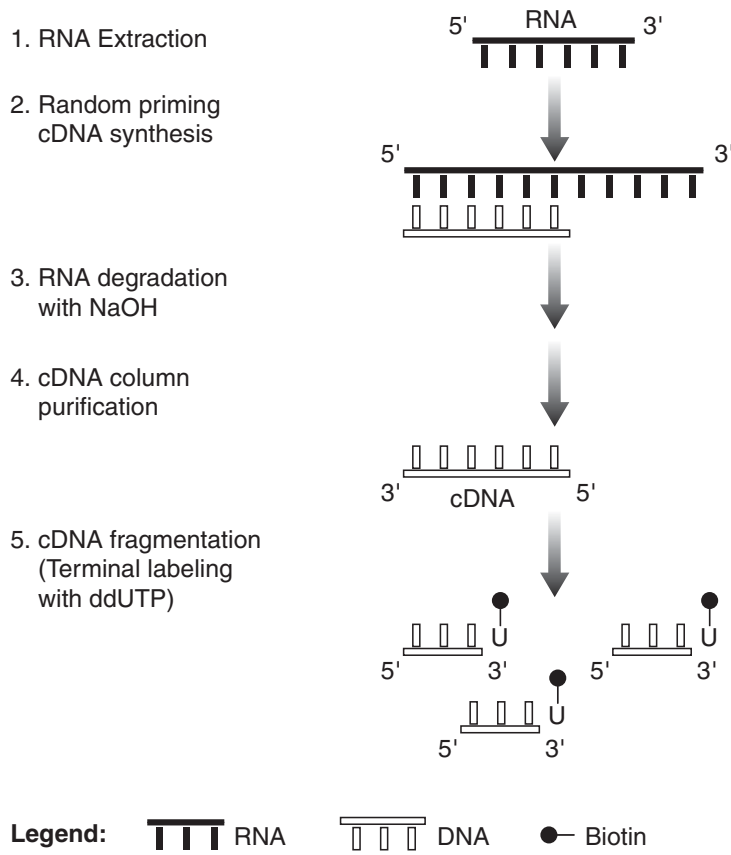
Prokaryotic Target Preparation

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 ddUTP 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. For supplier phone numbers in the U.S. and Europe, please refer to the Supplier and Reagent Reference List, Appendix A, of this manual. Information and part numbers listed are based on U.S. catalog information. Additional reagents needed for the complete analysis are listed in the appropriate chapters. Appendix A contains a master list of all reagents used in this manual.

Labeling

- dATP, dCTP, dGTP, dTTP, 100 mM, Amersham Pharmacia Biotech, P/N 27-2035-01

✔ Note

Prepare a mix of all 4 dNTPs at a final concentration of 10 mM each following the instructions below.

- Random Primers, 3 µg/µL, Invitrogen Life Technologies, P/N 48190-011
- 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
- QIAquick PCR Purification Kit, QIAGEN, P/N 28104
- 10X One-Phor-All Buffer, Amersham Pharmacia Biotech, P/N 27-0901-02
- Deoxyribonuclease I (DNase I), Amersham Pharmacia Biotech, P/N 27-0514-01
- Enzo® BioArray™ Terminal Labeling Kit, Affymetrix, P/N 900181
- EDTA, 0.5M, pH 8.0, Invitrogen Life Technologies, P/N 15575-020

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

10 mM dNTP mix

For 1000 µL:

100 µL 100 mM dATP

100 µL 100 mM dCTP

100 µL 100 mM dGTP

100 µL 100 mM dTTP

600 µL Nuclease-free H_2O

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

75 ng/µL Random Primers

For 1000 µL:

25 µL 3 µg/µL Random Primers

975 µL 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.

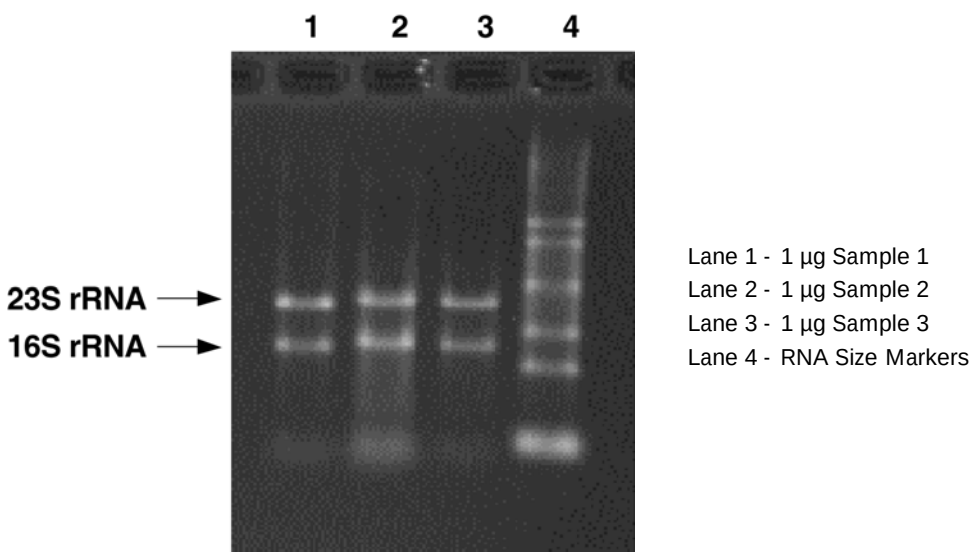


Figure 3.1.1
Typical RNA preparation from *E. coli*

cDNA Synthesis

cDNA Synthesis

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

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.

Step1: cDNA Synthesis

1. Prepare the following mixture for primer annealing:

Table 3.1.1
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
130 pM Spike-in-Control Transcripts (optional)	2 µL	8 pM
Nuclease-free DI H_2O	Up to 30.0 µL	—
Total Volume Added	*30 µL*	

Note

We strongly recommend using control transcripts to monitor the assay sensitivity and performance.

Probe sets for control genes from yeast, Arabidopsis and B. subtilis have been tiled on the GeneChip® P. aeruginosa Genome Array and E. coli Antisense Genome Array. To prepare spike controls containing RNA transcripts from B. subtilis genes, please refer to "Preparation of Control Spike-In Transcripts", Section 3, Chapter 2.

Assuming complete recovery of control transcripts in the labeling process, the addition of 2 µL of 130 pM controls results in a 2 pM final concentration in the hybridization cocktail.

Detection limit of the assay is estimated to be 1 pM, or better.
To monitor the assay sensitivity, it is recommended to use concentration of the individual spikes in a range of 0.1 to 2 pM.

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.

1. Incubate the RNA/Primer mix at the following temperatures:
   - 70°C for 10 minutes
   - 25°C for 10 minutes
   - Chill to 4°C

2. 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 3.1.2 to the RNA/primer hybridization mix.

Table 3.1.2
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*	

3. 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 2: 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 3: Purification and Quantitation of cDNA

1. Use QIAquick Column to clean up the cDNA synthesis product (for detailed protocol, see QIAquick PCR Purification Kit Protocols provided by the supplier). Elute the product with 40 µL of EB Buffer (supplied with QIAquick kit).
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 3.1.3
Fragmentation Reaction

Components	*Volume*	*Concentration*
10X One-Phor-All Buffer	5 µL	1X
cDNA	40 µL	3-7 µg
DNase I (see note below)	X µL	0.6 U/µg of cDNA
Nuclease-free H_2O	Up to 50 µL	-
Total Volume	*50 µL*	

✔ Note

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. 0.6 U 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 Enzo BioArray Terminal Labeling Kit with Biotin-ddUTP (Affymetrix, P/N 900181) to label the 3' termini of the fragmentation products.

IMPORTANT *Follow the volumes and amounts below rather than the package insert. The reaction volume has been modified to be compatible with that required for the subsequent hybridization.*

1. Prepare the following reaction mix:

Table 3.1.4
Terminal Label Reaction*

Components	*100 Format (Midi)*	*49 Format (Standard)*
5X Reaction Buffer	12 µL	20 µL
10X $CoCl_2$	6 µL	10 µL
Biotin-ddUTP	1 µL	1 µL
Terminal Deoxynucleotide Transferase	2 µL	2 µL
Fragmentation Product (1.5 - 6 µg)	39 µL	Up to 50 µL
Total Volume	*60 µL*	*100 µL*

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

2. Incubate the reaction at 37°C for 20 to 60 minutes.
3. Stop the reaction by adding 2 µL of 0.5M EDTA.

Note *When the amount of fragmentation product exceeds 3 µg, extend the reaction time to up to 60 minutes.*

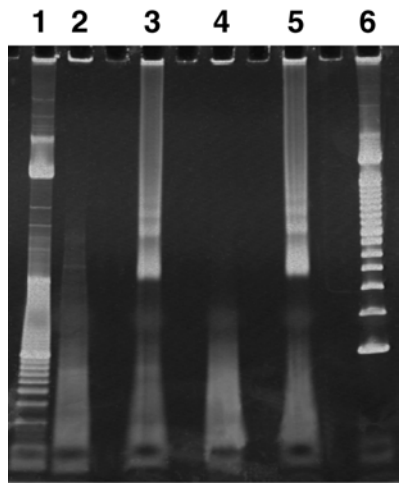
4. The target is ready to be hybridized onto probe arrays, as described in Section 3, Chapter 3, *Prokaryotic Target Hybridization*. Alternatively, it may be stored at -20°C for later use.

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

After purification of the target, 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 DNA Ladder
Lane 2 - Fragmented and labeled enriched *E. coli* RNA
Lane 3 - Fragmented and labeled enriched *E. coli* RNA with avidin
Lane 4 - Fragmented and labeled total *E. coli* RNA
Lane 5 - Fragmented and labeled total *E. coli* RNA with avidin
Lane 6 - 100 bp DNA Ladder

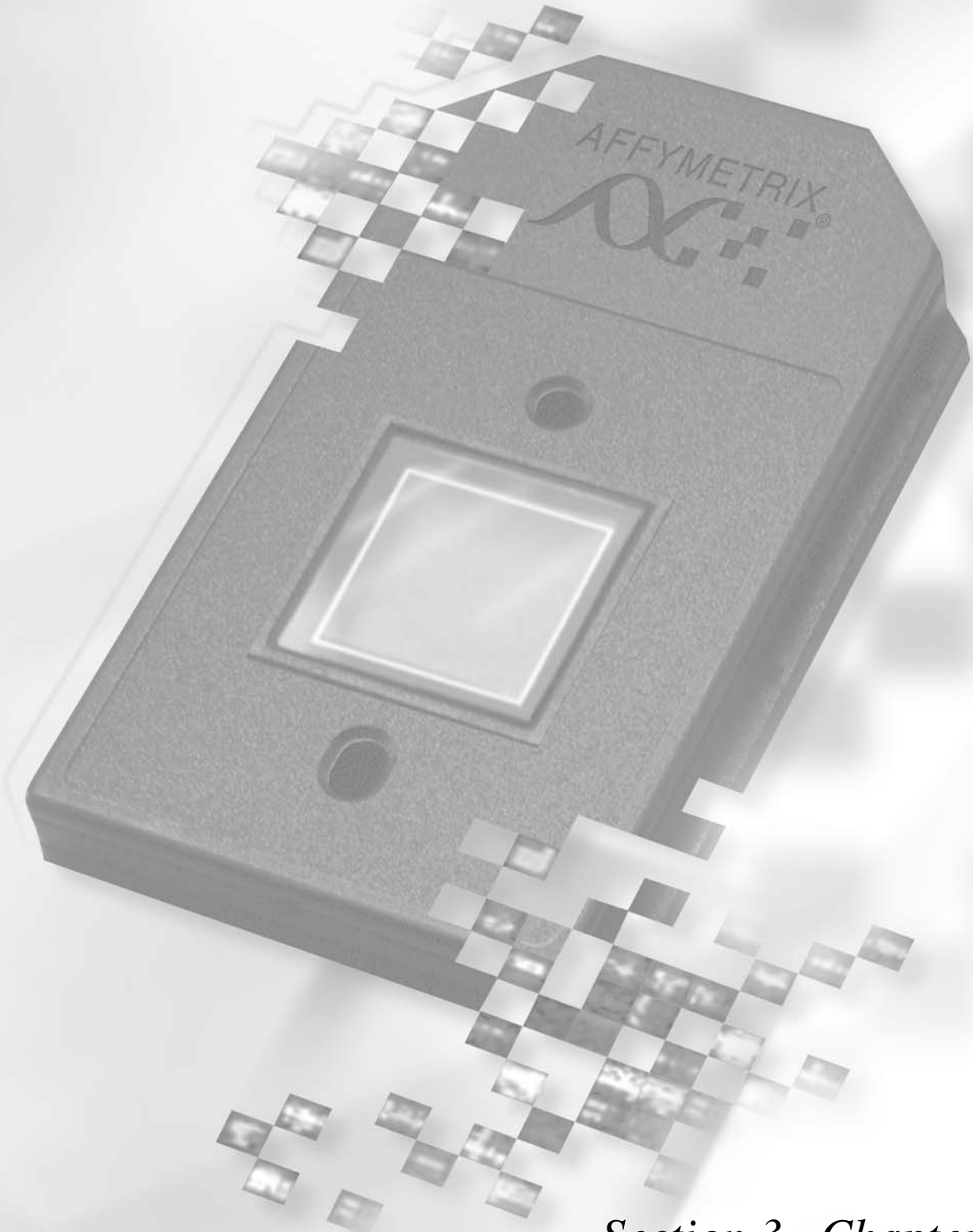
Figure 3.1.2
Gel-shift assay for monitoring *E. coli* target labeling efficiency

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 each tube.
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 are 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.



Prokaryotic

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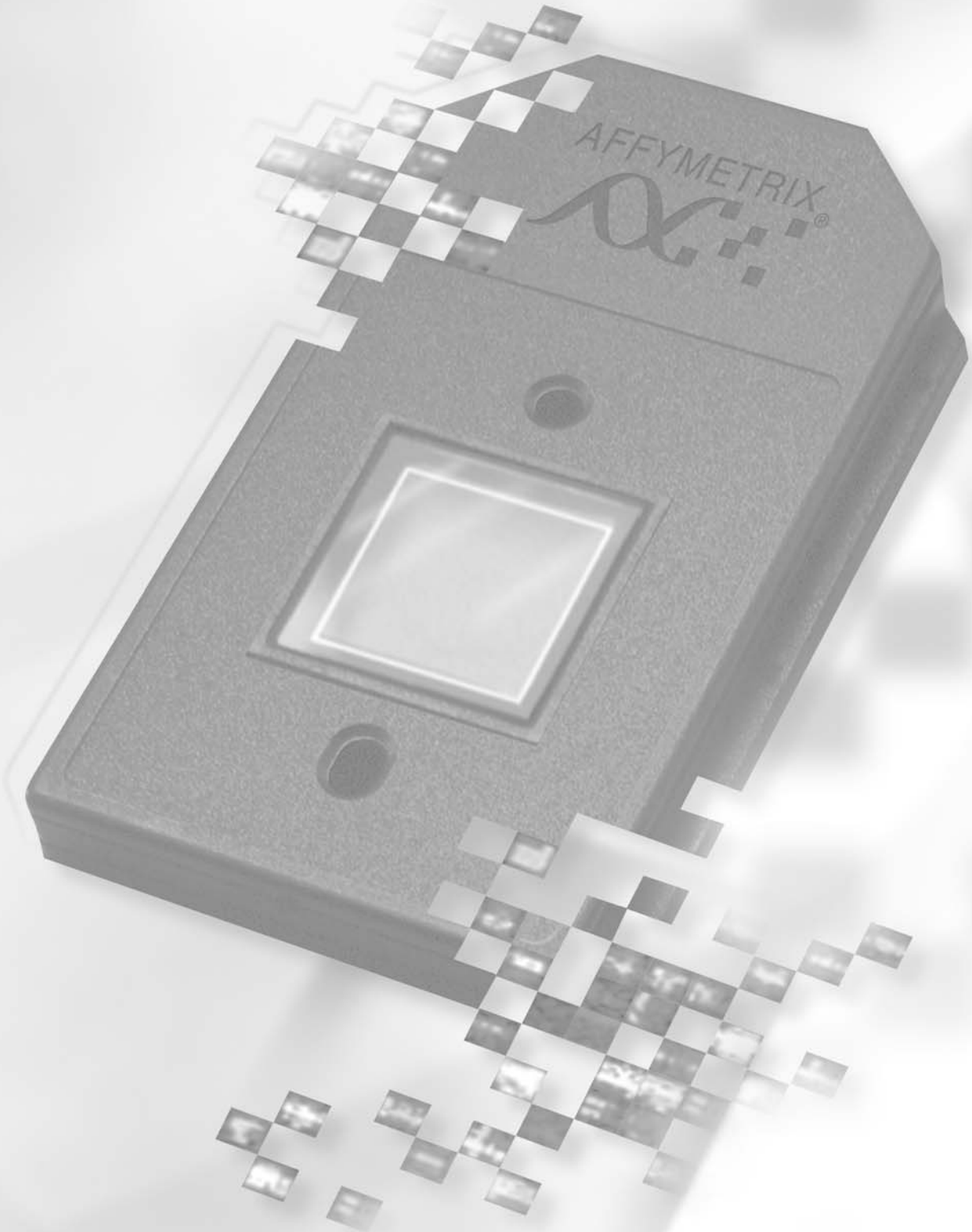


Preparation of Control Spike-In Transcripts

This Chapter Contains:

- Detailed steps for producing full-length control spike sense RNA.

After completing the procedures described in this chapter, the control sense transcripts can be added to purified prokaryotic RNA samples prior to enrichment and labeling procedure as described in Section 3, Chapter 1.

Overview

This chapter describes protocols used to generate sense RNA controls from *B. subtilis* genes. These control transcripts can be spiked into *P. aeruginosa* or *E.coli* total RNA used for target preparation at a predetermined concentration to monitor labeling, hybridization, and staining efficiency.

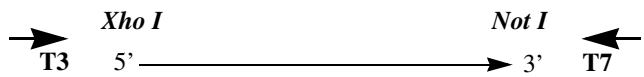
To be used as control for assay performance, GeneChip® *P. aeruginosa* and *E. coli* Antisense Genome Arrays contain probe sets with sequences of *dap, thr, phe,* and *lys* genes from *B. subtilis.* These genes have been cloned into Stratagene pBluescript as an *Xho I* to *Not I* insert, 5´ to 3´ respectively (see Section 2, Chapter 2, *Controls for Eukaryotic Arrays*).

pGIBS-lysATCC 87482

pGIBS-pheATCC 87483

pGIBS-thrATCC 87484

pGIBS-dapATCC 87486



These clones can be digested with the *Not I* restriction enzyme to produce linear template DNA for the subsequent *in vitro* transcription (IVT) to produce sense RNA by T3 RNA polymerase as control molecules.

Bacteria containing these recombinant plasmids can be obtained from the American Type Culture Collection (ATCC).

Reagents and Materials Required

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

- Expression Control Clones, American Type Culture Collection (ATCC)
  - pGIBS-lys ATCC 87482
  - pGIBS-phe ATCC 87483
  - pGIBS-thr ATCC 87484
  - pGIBS-dap ATCC 87486
- *Not* I restriction Endonuclease, New England BioLabs, P/N R0189S
- Phase Lock Gel, Brinkmann Instruments, P/N 955 15 415
- Phenol/chloroform/isoamyl alcohol, Ambion, P/N 9732
- MEGAscript™ T3 Kit, Ambion, P/N 1338

Miscellaneous Reagents

- NaAcetate (NaOAc), 3M
- Absolute Ethanol
- 80% Ethanol
- RNeasy Mini Kit, QIAGEN, P/N 74104
- TE, 1X, BioWhittaker Molecular Applications / Cambrex, P/N 16-013B

Bacterial Plasmid DNA Preparation

1. Grow *E. coli* bacterial cultures containing recombinant plasmids according to established protocols (a minimum 50 mL of culture volume is recommended).
2. Prepare plasmid DNA from overnight cultures using standard procedures or commercial kits.

   We have obtained reliable results using QIAGEN Plasmid Kits for plasmid DNA isolation.

Linearization of Plasmid DNA Preparation

1. In a 50 µL reaction volume, digest 10 µg of plasmid with the restriction enzyme, *Not I*, according to the enzyme manufacturer's recommendations.
2. Analyze 50 ng of the uncut and linearized plasmid by gel electrophoresis on a 1% agarose gel. Complete digestion of the plasmid is required for IVT. Repeat restriction enzyme digestion, if necessary.

Purification of Linearized Plasmid DNA

Purify the linearized plasmid from restriction enzymes and potential RNase contaminants before proceeding to IVT using the following Phase Lock Gel (PLG)-phenol/chloroform extraction procedure.

Phase Lock Gels form an inert sealed barrier between the aqueous and organic phases of phenol/chloroform extractions. The solid barrier allows more complete recovery of the sample (aqueous phase) and minimizes interface contamination of the sample. PLG's are sold as pre-measured aliquots in 1.5 mL tubes to which sample and phenol/chloroform are directly added.

1. Pellet the Phase Lock Gel (1.5 mL tube with PLG I-heavy) in a microcentrifuge at ≥ 12,000 x g for 20 seconds.
2. Dilute the linearized plasmid to final volume of 150 µL with TE and add equal volume of (25:24:1) Phenol:chloroform:isoamyl alcohol (saturated with 10 µM tris-HCl, pH 8.01 / 1 mM EDTA). Vortex.
3. Transfer the mix to the PLG tube and microcentrifuge at ≥ 12,000 x g for 2 minutes.
4. Transfer the top aqueous phase to a new 1.5 mL tube.
5. Add 0.1 volumes (15 µL) of 3M NaOAc and 2.5 volumes (375 µL) of absolute ethanol to the samples. Vortex.
6. Immediately centrifuge at ≥ 12,000 x g in a microcentrifuge at room temperature for 20 minutes.
7. Carefully remove supernatant.
8. Wash pellet with 0.5 mL of 80% ethanol, then centrifuge at ≥ 12,000 x g at room temperature for 5 minutes.

9. Remove the supernatant very carefully and air dry the pellet.
10. Resuspend DNA pellet in 15 µL of RNase-free water.
11. Quantify the DNA by absorbance at 260 nm (50 µg/mL of DNA for 1 absorbance unit at 260 nm).

✔ Note *The quality of DNA template can be monitored by the A_{260}/A_{280} ratio, which should be between 1.8 and 2.0 for pure DNA.*

In vitro Transcription (IVT) to Produce Control Sense Transcripts

Use MEGAscript™ T3 Kit for the IVT reaction.

1. To make up the reaction mix, follow the procedures in the instruction manual provided by Ambion.

✔ Note *No tracer is involved in this assay.*

2. Incubate the reaction for 4 hours at 37°C.
3. Clean up the reaction product with RNeasy mini column.
4. Quantify the transcript by absorbance at 260 nm (40 µg/mL RNA = 1 absorbance unit at 260 nm).

✔ Note *It is recommended to examine the quality and integrity of the IVT product on an agarose gel. The expected IVT product sizes are shown in Table 3.2.1.*

IMPORTANT *Aliquot and freeze the IVT transcripts at -80°C. Avoid repeated freeze / thaw cycles.*

Preparing the Control Transcript Mix

1. Prepare stock solutions for each of the five transcripts at 650 pM for each transcript. Use Table 3.2.1 to calculate the amount of transcript needed.

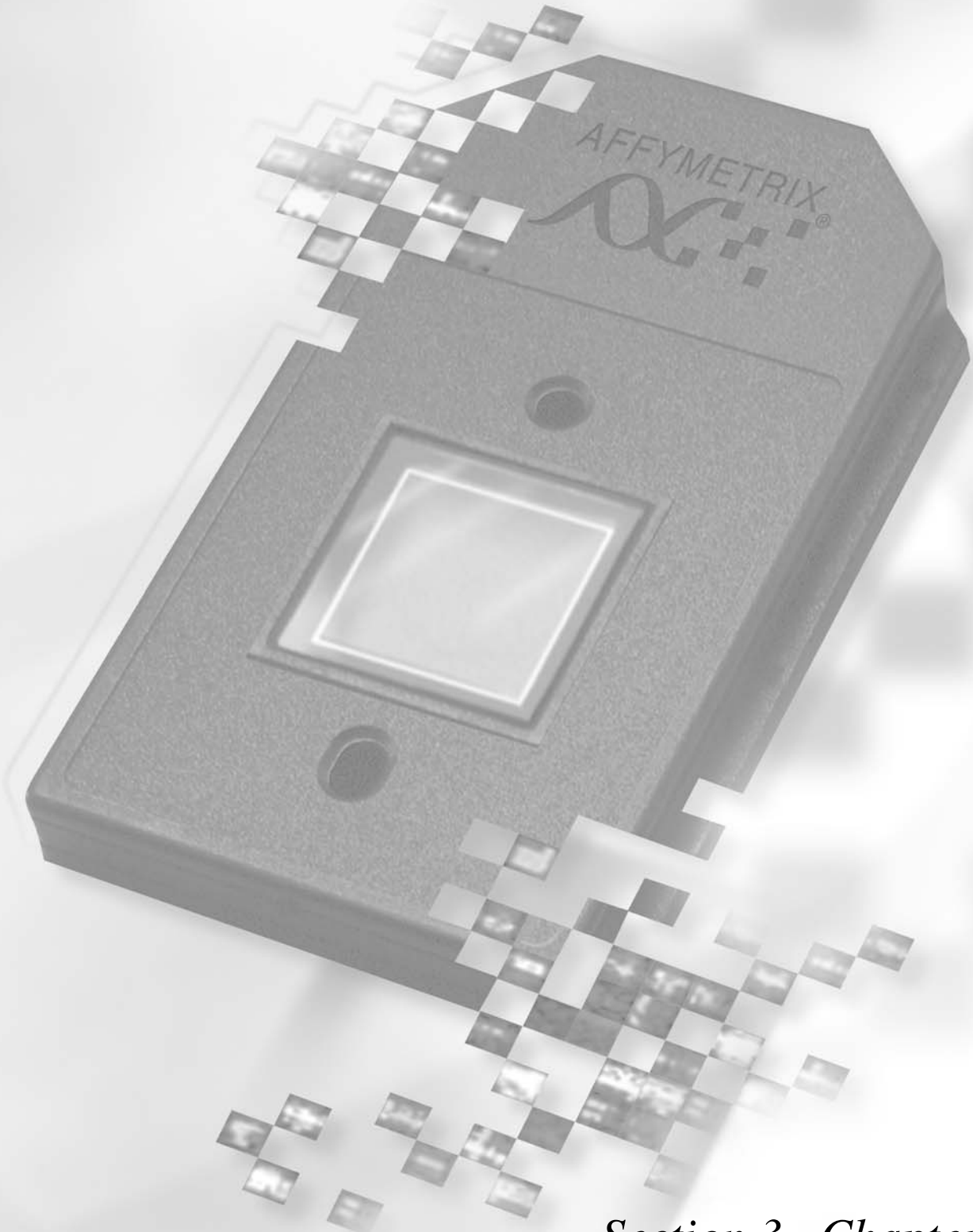
Table 3.2.1
Conversions for Preparing Control Transcript Mix

Control RNA	*Size (kb)*	*Molecular Weight*	*pmoles / µg*
lys	1	330,000	3.03
phe	1.32	435,600	2.30
dap	1.84	607,200	1.65
thr	1.98	653,400	1.53

2. Mix equal volumes of the 650 pM stocks for all four transcripts for a final concentration of 130 pM for each transcript.
3. Apply 2 µL of the transcript mix with each 5-10 µg of total RNA prior to the cDNA synthesis procedure (as described in Section 3, Chapter 1, *Prokaryotic Target Preparation*). Final concentration applied on the array for the control transcripts would be 2 pM, assuming 100% recovery during the cDNA synthesis and labeling process.

Note *Different concentrations of transcript stock can be prepared to generate "staggered" concentrations for different transcripts to monitor the dynamic range of the assay.*

IMPORTANT *Aliquot and freeze the IVT transcripts at -80°C. Avoid repeated freeze / thaw cycles.*



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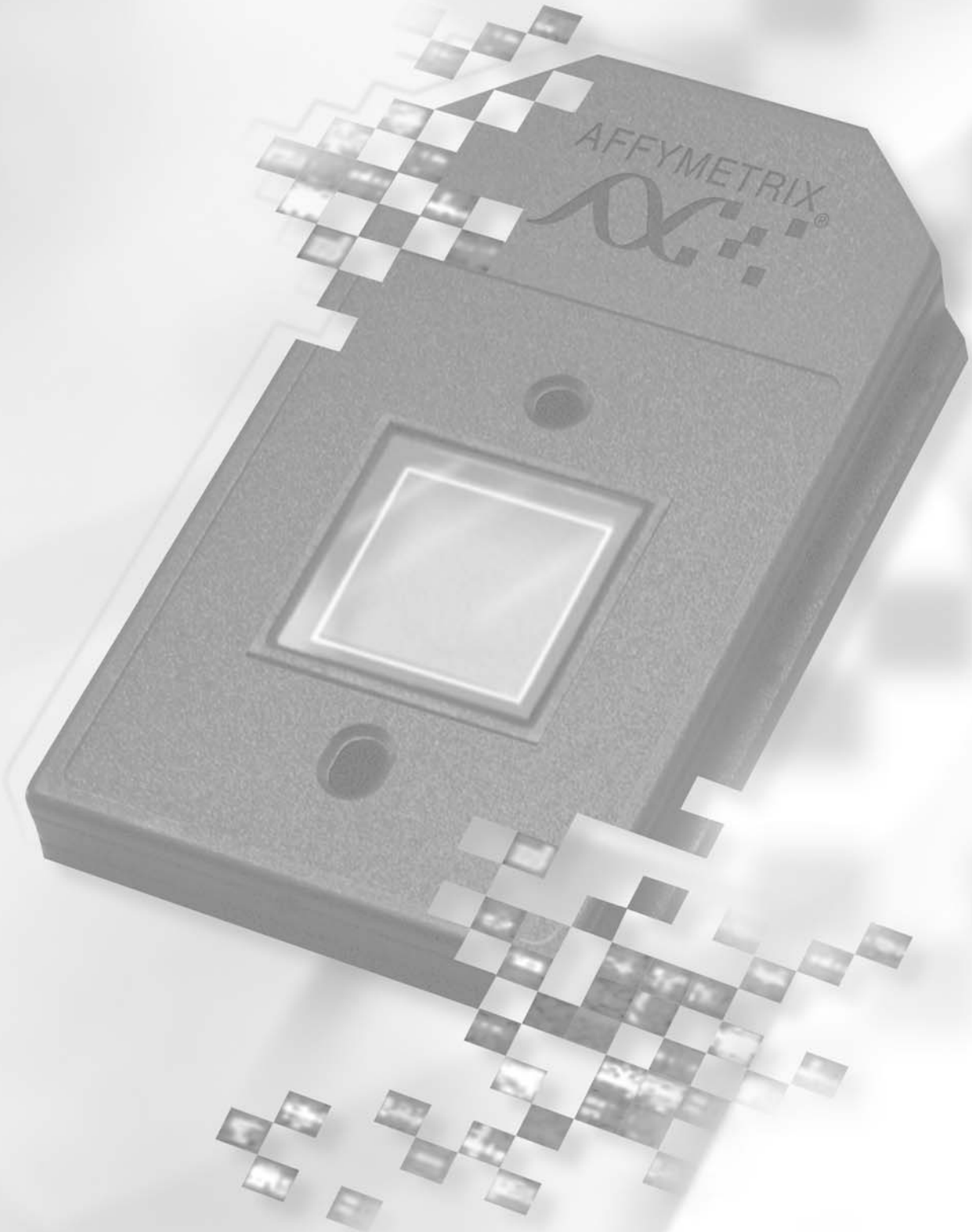


Prokaryotic Target Hybridization

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 on page 3.4.3.

Reagents and Materials Required

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

- Water, Molecular Biology Grade, BioWhittaker Molecular Applications / Cambrex, P/N 51200
- Acetylated Bovine Serum Albumin (BSA) solution, 50 mg/mL, Invitrogen Life Technologies, P/N 15561-020
- Herring Sperm DNA, Promega Corporation, P/N D1811
- Micropure Separator, Millipore, P/N 42512 (optional)
- Control Oligo B2, 3 nM, Affymetrix, P/N 900301 (can be ordered separately)
- NaCl, 5M, RNase-free, DNase-free, Ambion, P/N 9760G
- MES Free Acid Monohydrate 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 X-100 (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 (1.22M MES, 0.89M [Na^+])

For 1,000 mL:

70.4g MES free acid monohydrate

193.3g 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

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 3.3.1
Hybridization Cocktail for Single Probe Array*3

Components	*100 Format (Midi)*	*49 Format (Standard)*	*Final Concentration*
2X MES Hybridization Buffer	65 µL	100.0 µL	1X
3 nM B2 Control Oligo	2.2 µL	3.3 µL	50 pM
10 mg/mL Herring Sperm DNA	1.3 µL	2.0 µL	0.1 mg/mL
50 mg/mL BSA	1.3 µL	2.0 µL	0.5 mg/mL
100% DMSO	9.2 µL	-	7%
Fragmented and Labeled cDNA	51 µL	Up to 92.7 µL	1.5 – 6.0 µg
Molecular Biology Grade Water	To a final volume of 130 µL	To a final volume of 200 µL	
Total Volume	*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.*

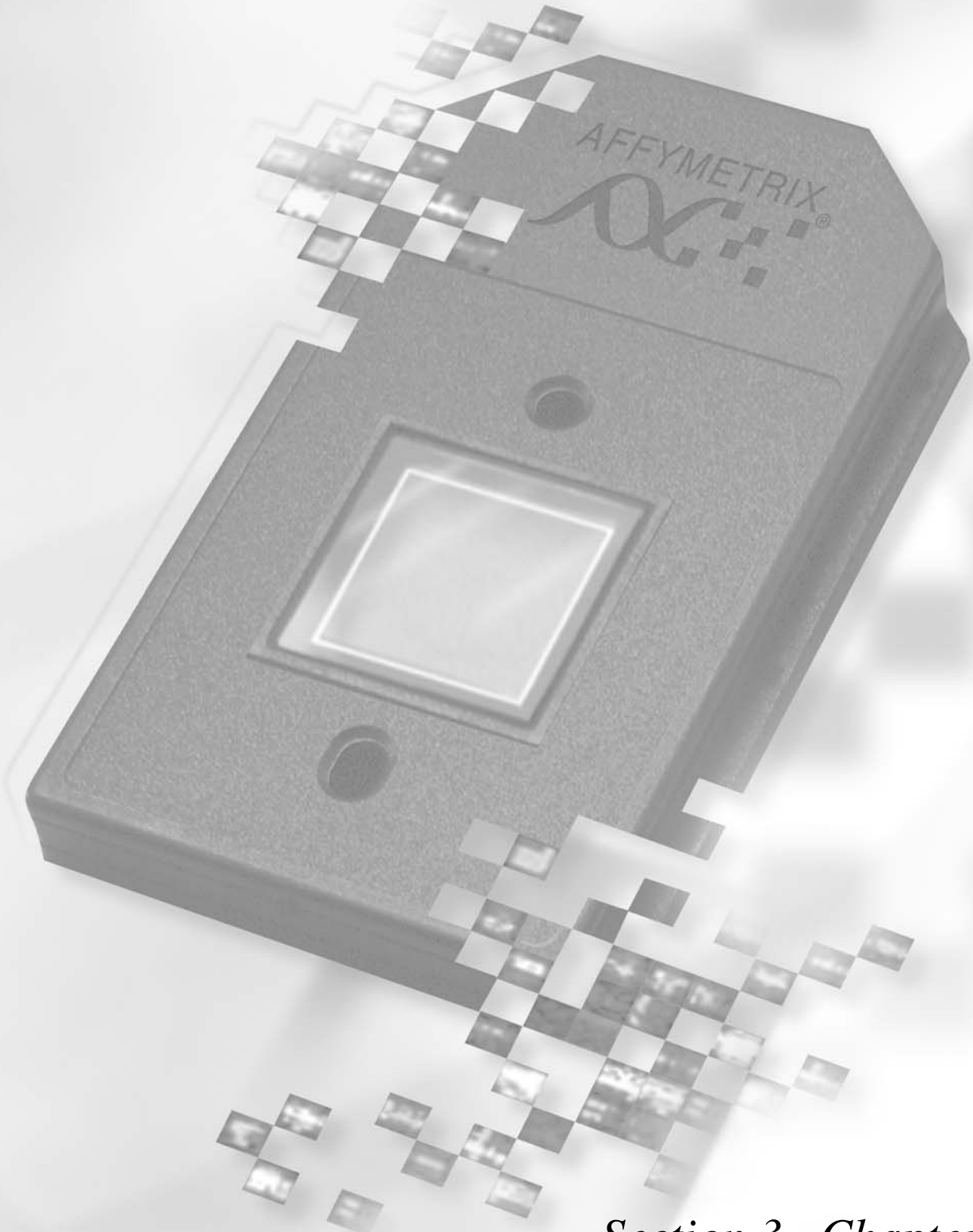
4. Place probe array in the hybridization oven set at the temperatures indicated below.
   - *P. aeruginosa* 50°C
   - *E. coli Antisense* 45°C

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 4, *Prokaryotic Arrays: Washing, Staining, and Scanning* to prepare reagents required immediately after completion of hybridization.



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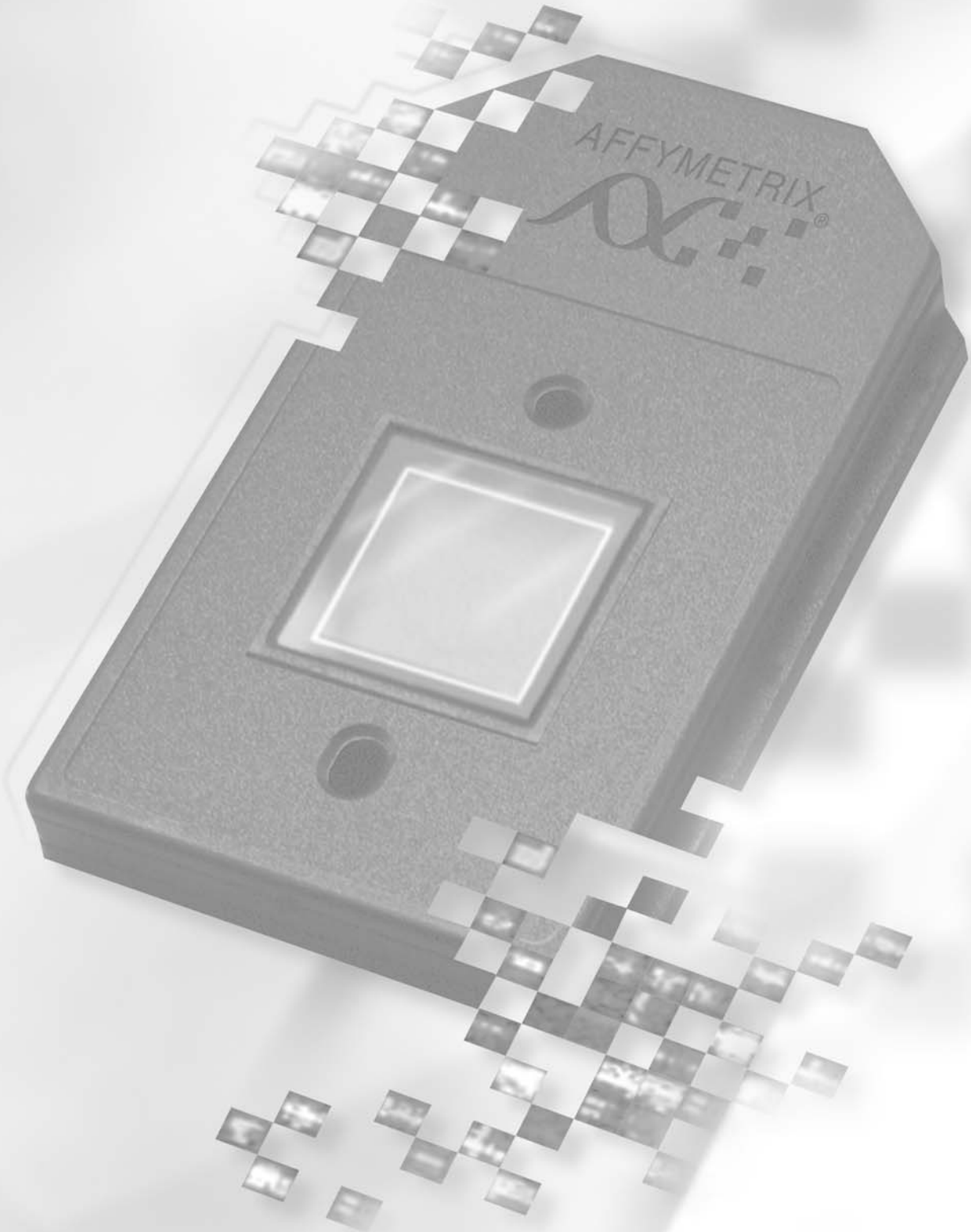


Prokaryotic Arrays: Washing, Staining, and Scanning

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. For supplier phone numbers in the U.S. and Europe, please refer to the Supplier and Reagent Reference List, Appendix A, of this manual. Information and part numbers listed are based on U.S. catalog information. Additional reagents needed for the complete analysis are listed in the appropriate sections. Appendix A contains a master list of all reagents used in this manual.

- Water, Molecular Biology Grade, BioWhittaker Molecular Applications / Cambrex, P/N 51200
- Distilled water, Invitrogen Life Technologies, P/N 15230-147
- Acetylated 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
- 10% surfact-Amps20 (Tween-20), Pierce Chemical, P/N 28320
- Bleach (5.25% Sodium Hypochlorite), VWR Scientific, P/N 21899-504 (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

Reagent Preparation

Wash 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 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 3 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 12X MES Stock Buffer (see Section 3, Chapter 3)

92.5 mL 5M NaCl

2.5 mL 10% Tween 20

113.3 mL 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.

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.

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 defined in Microarray Suite.

1. Select **Run** → **Experiment Info** from the menu bar. Alternatively, click the **New Experiment** icon on the tool bar.

   ⇒ The Experiment Information dialog box appears, allowing the experiment name to be defined along with several other parameters, such as probe array type, sample description, and comments.

2. Type in the **Experiment Name**.

3. In the **Probe Array Type** box, click the arrow and select the experiment name as indicated below:

   - P.aeruginosa — select *Pae_G1a* from the drop-down list
   - E.coli — select *Ecoli_Antisense* from the drop-down list.

   Experiment name and probe array type are required. Complete as much of the other information as desired. The protocol information at the bottom of the dialog box is imported to the experiment information dialog box after the hybridization and scan have been completed.

4. Save the experiment by selecting **Save**.

   The name of the experiment is used by Microarray Suite to access the probe array type and data for the sample while it is being processed. Data files generated for the sample are automatically labeled to correspond to the experiment name. Microarray Suite automatically fills in the Protocol section of this dialog box with information on array processing from the fluidics station.

5. Close the Experiment Information dialog box.

Step 3: Preparing the Fluidics Station

The Fluidics Station 400, 450, or 250 is used to wash and stain the probe arrays. It is operated using 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. Click **Run** for each module to begin priming.

Probe Array Wash and Stain

Following hybridization, the wash and stain procedures are carried out by the Fluidics Station. A modified FlexMidi_euk2v3 fluidic script (FlexMidi_euk2v3_450, if you are using the FS-450) is used for the GeneChip *P. aeruginosa* Genome Array, and the ProkGE-WS2 fluidic 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.

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 refrigerator and tap the tube to mix well before preparing stain solution.

   Do not freeze SAPE. Always prepare the SAPE stain solution immediately before use.

Table 3.4.1
Streptavidin Solution Mix - Vial 1

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	6.0 µL	10 µg/mL
DI H_20	270.0 µL	—
Total Volume	*600 µL*	

Table 3.4.2
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 Biotin Anti-streptavidin	6.0 µL	5 µg/mL
DI H_20	264.0 µL	—
Total Volume	*600 µL*	

Table 3.4.3
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
DI 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* Antisense Genome Array	ProkGE-WS2 * (*if using FS-450, ProkGE-WS_450)
■ GeneChip *P. aeruginosa* Genome Array	Modified FlexMidi_euk2v3** (**See *Table 3.4.4*. 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, 450 or 250.

Table 3.4.4
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* fluidic protocol under a different name to avoid confusion.

1. Modify and save the fluidic protocol for the assay:
   a. Modify the fluidic 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. 2nd Stain Time (seconds) from 300 to 600; and
      iii. 3rd Stain Time (seconds) from 300 to 600.
   c. Save the modified fluidic 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, 450, or 250.

Table 3.4.5
Fluidics Protocols

	FlexMidi_euk2v3[a]	*Modified FlexMidi_euk2v3 for **P. aeruginosa** Array*	*Standard Format for **E. coli** Antisense Array ProkGE-WS-2*[b]
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 *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	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
Stain	Stain the probe array for *600 seconds* in SAPE Solution Mix at 25°C	Stain the probe array for *600 seconds* in Streptavidin Solution Mix at 25°C	Stain the probe array for 10 minutes 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
2nd Stain	Stain the probe array for *300 seconds* in antibody solution mix at 25°C	Stain the probe array for *600 seconds* in antibody solution mix at 25°C	Stain the probe array for 10 minutes in antibody solution. Mix at 25°C.
3rd Stain	Stain the probe array for *300 seconds* in SAPE Solution at 25°C	Stain the probe array for *600 seconds* in SAPE Solution at 25°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 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. FlexMidi_euk2v_450 for the FS-450/250
b. prokGE_ws2_450 for the FS-450/250

If you are using the Fluidics Station 450/250:

Washing and Staining the Probe Array

1. 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.
2. Remove any microcentrifuge vial(s) remaining in the sample holder of the fluidics station module(s) being used.
3. 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 phycoerythrin (SAPE) solution mix in sample holder 1.
   b. Place one vial containing the anti-streptavidin biotinylated antibody 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.

4. When the protocol is complete, the LCD window displays the message **EJECT CARTRIDGE**.
5. Remove the probe arrays from the fluidics station modules by first pressing down the cartridge lever to the eject position.
6. Lift up on the needle lever to disengage the needles from the microcentrifuge vials. Remove the three empty microcentrifuge vials from the needle holders.
7. Check the probe array window for large bubbles or air pockets.
   - If bubbles are present, refer to *Table 3.4.6*.
   - 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 close the washblock and proceed to *Probe Array Scan* on page 3.4.15.
8. If there are no more samples to hybridize, shut down the fluidics station following the procedure in *Shutting Down the Fluidics Station* on page 3.4.17.
9. Keep the probe arrays at 4°C and in the dark until ready for scanning.
10. 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 3.4.6
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 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 3.4.15. If several attempts to fill the probe array without bubbles are unsuccessful, the array should be filled with *Wash 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. 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.
2. Remove any microcentrifuge tube remaining in the sample holder of the fluidics station module(s) being used.
3. 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.

4. When the LCD window indicates, replace the microcentrifuge tube containing the streptavidin stain with a microcentrifuge tube containing antibody stain solution into the sample holder, making sure that the metal sampling needle is in the tube with its tip near the bottom.
5. When the LCD window indicates, replace the microcentrifuge tube containing antibody solution with the microcentrifuge tube containing the SAPE solution.
6. When the protocol is complete, the LCD window displays the message **EJECT CARTRIDGE**.
7. Remove microcentrifuge tube containing stain and replace with an empty microcentrifuge tube.
8. Remove the probe arrays from the fluidics station modules by first moving the cartridge lever to the **EJECT** position.
9. Check the probe array window for large bubbles or air pockets.
   - If bubbles are present, refer to *Table 3.4.7*.
   - 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 3.4.15.

   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 4.17.

✔ Note

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

Table 3.4.7
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 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 3.4.15. If several attempts to fill the probe array without bubbles are unsuccessful, the array should be filled with *Wash 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. The probe array is scanned after the wash protocols are complete. Make sure laser is warmed up prior to scanning by turning the laser 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 probe array was stored at 4°C, warm to room temperature before scanning. Refer to the Microarray Suite 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 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 3.4.1*.

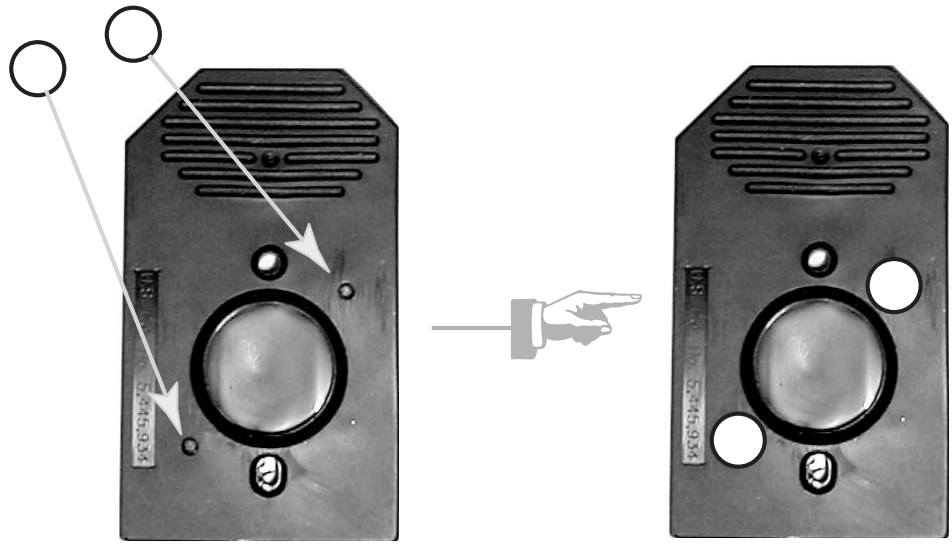


Figure 3.4.1
Applying Tough-Spots™ to the probe array cartridge

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, 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 fluidic 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 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 item to be changed and input the new parameters as needed, keeping parameters within the ranges shown below in *Table 3.4.8*.

Table 3.4.8
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	15 to 50
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 3.4.6.
- Wash B corresponds to Post Hyb Wash #2 in Table 3.4.6.
- Wash A2 corresponds to Post Stain Wash in Table 3.4.6.
- Wash A3 corresponds to Final Wash in Table 3.4.6.

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.

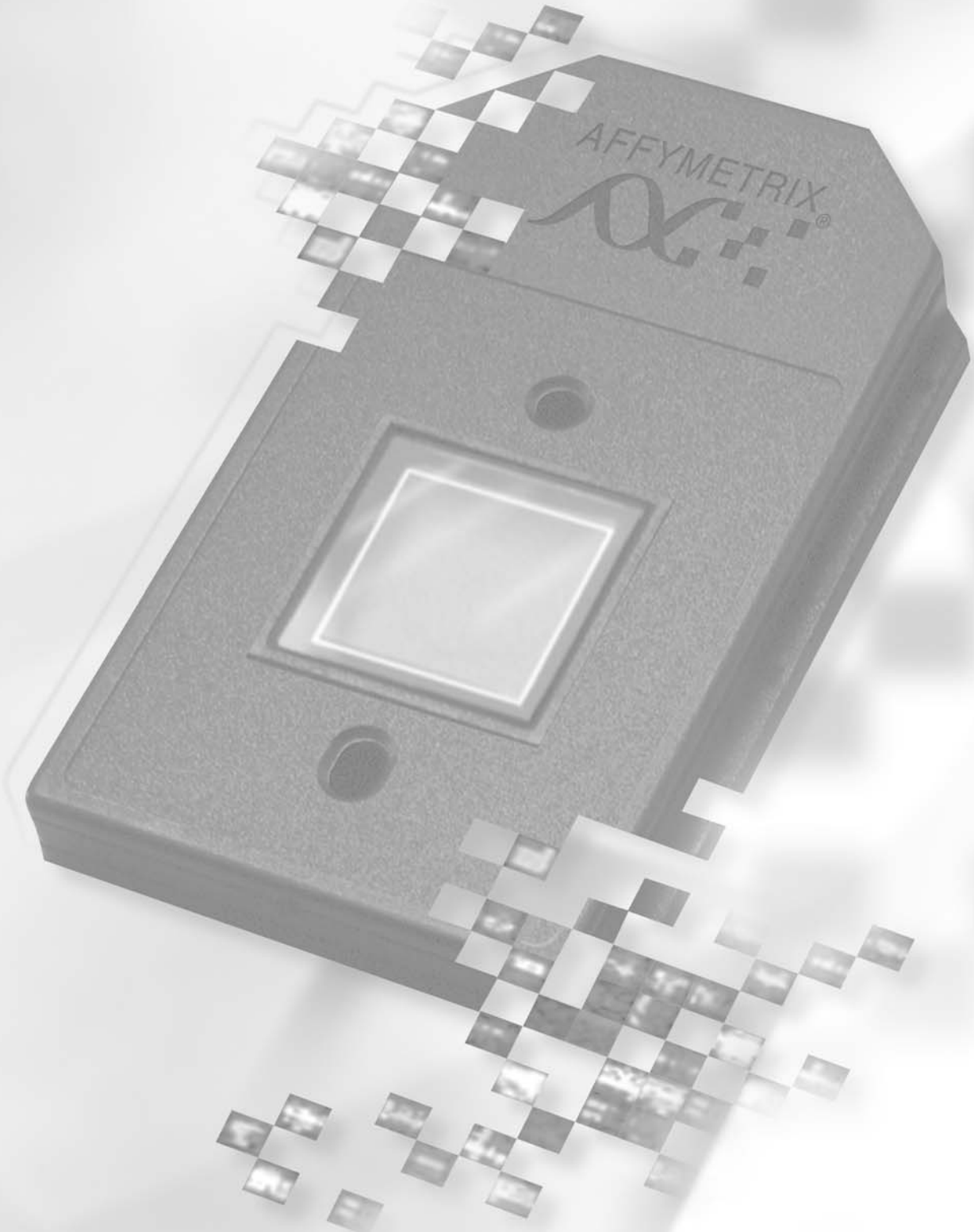
! CAUTION *If the protocol is saved without entering a new **Protocol Name**, the original protocol parameters will be overwritten.*

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

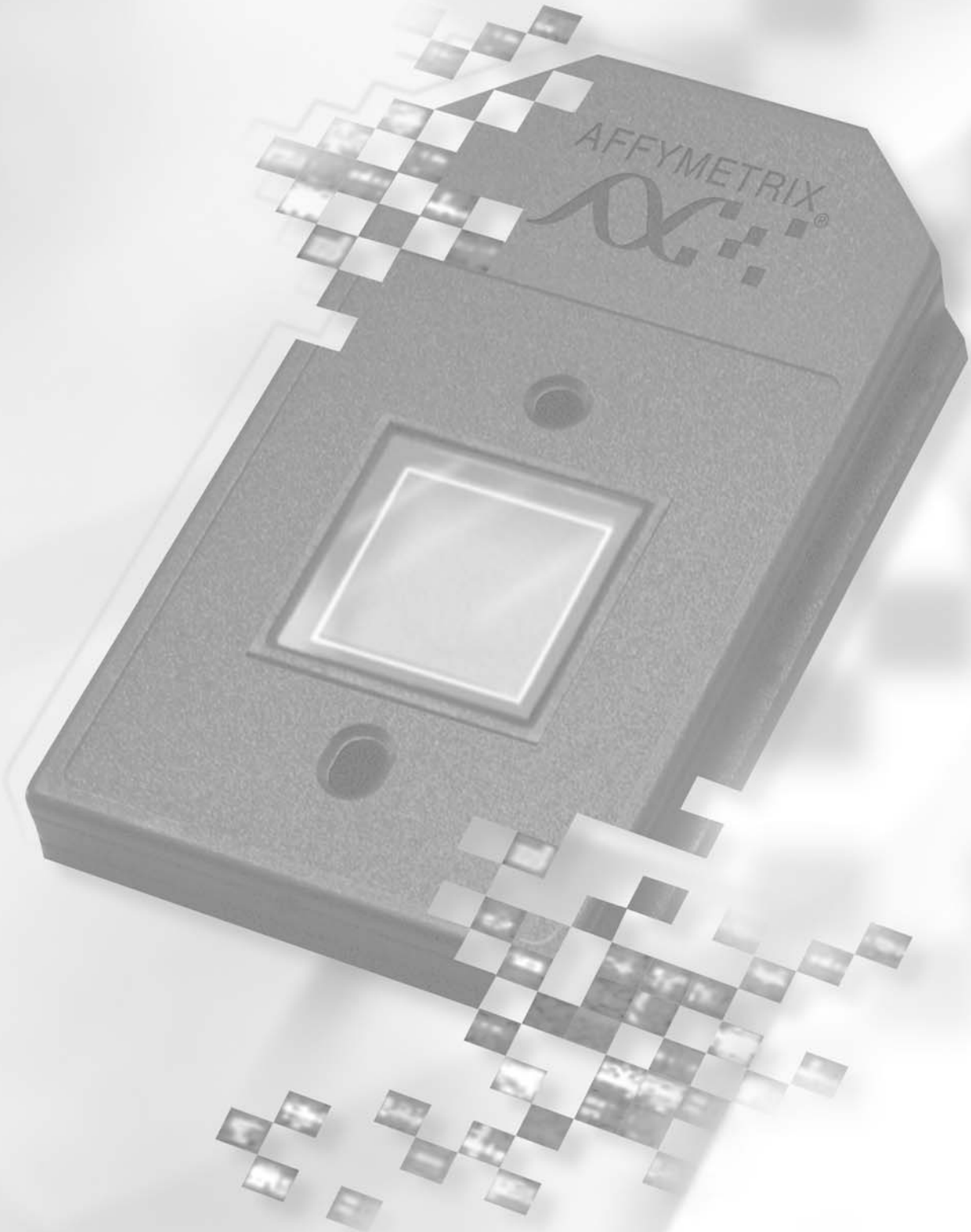
   Enter **0** (zero) for hybridization time if hybridization step is not required. Likewise, enter **0** (zero) for the stain time if staining is not required. Enter **0** (zero) for the number of wash cycles if a wash solution is not required.

Section 4:

Fluidics Station Maintenance Procedures



Section 4



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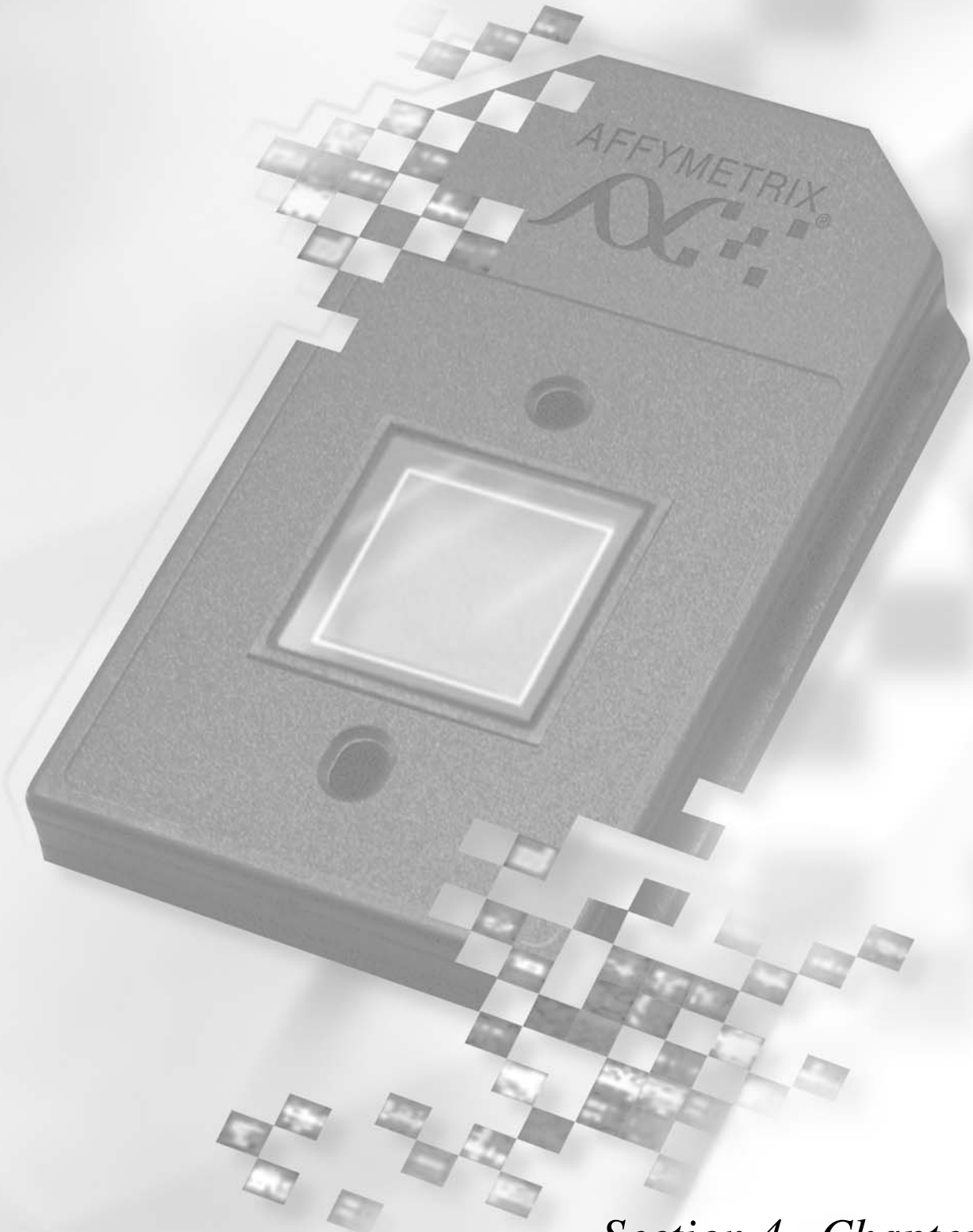
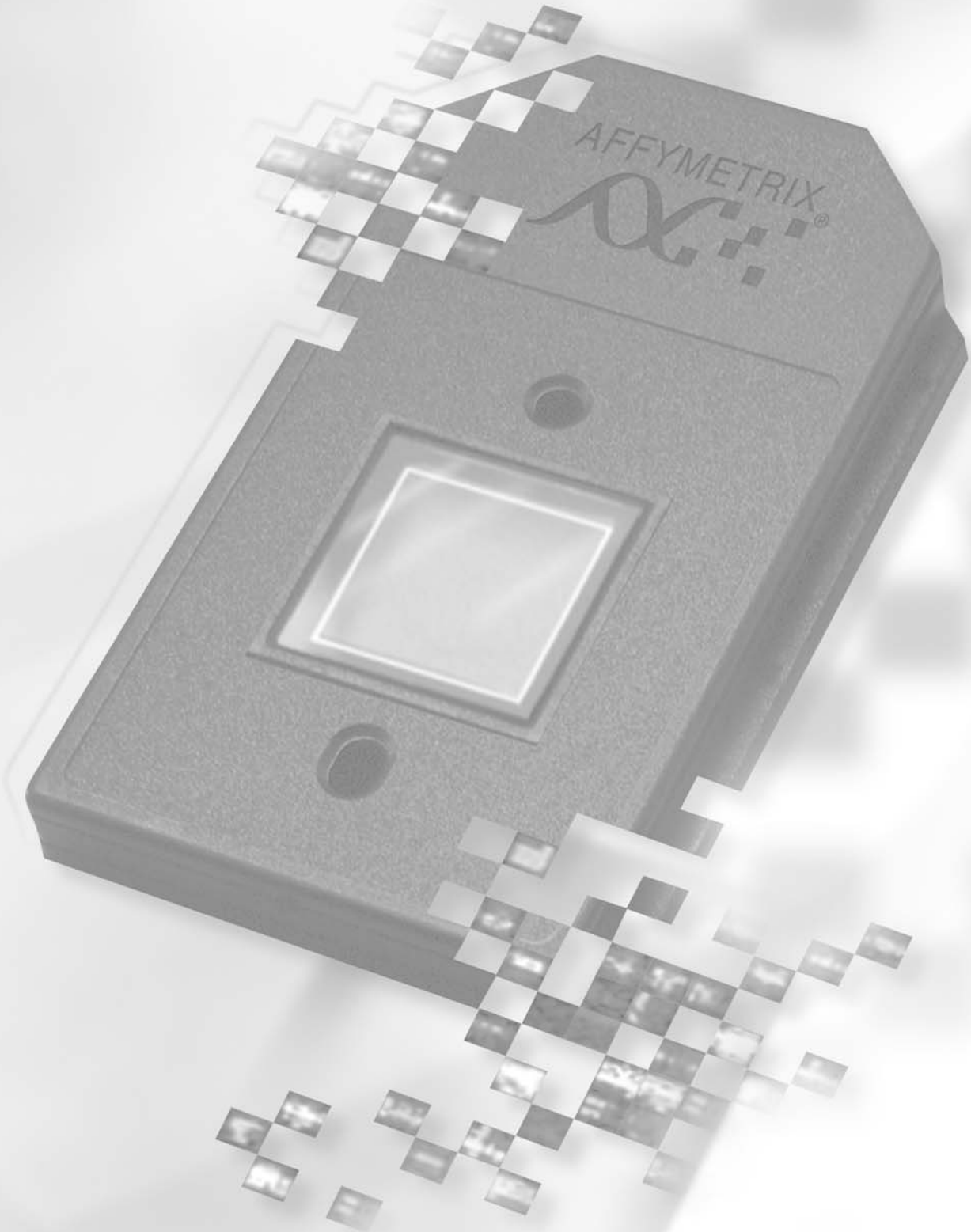


F.S. Maintenance

Section 4, Chapter 1

Section 4, Chapter 1



Fluidics Station Maintenance Procedures

This Section Contains:

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

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
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.

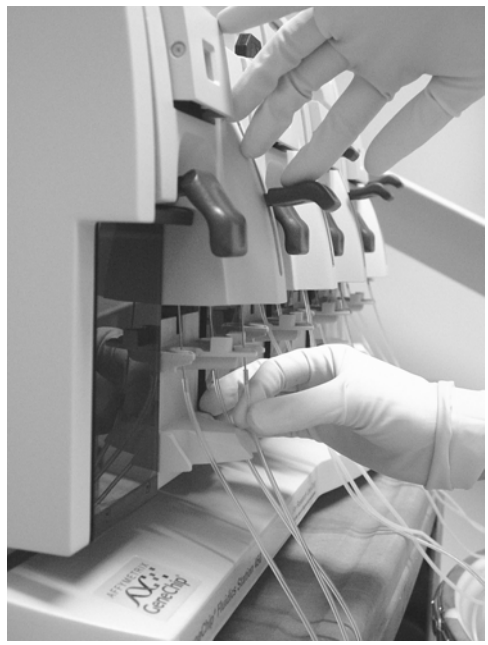


Figure 4.1.2
Inserting tubes on the needles. Take care not to break or bend the needles.

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.

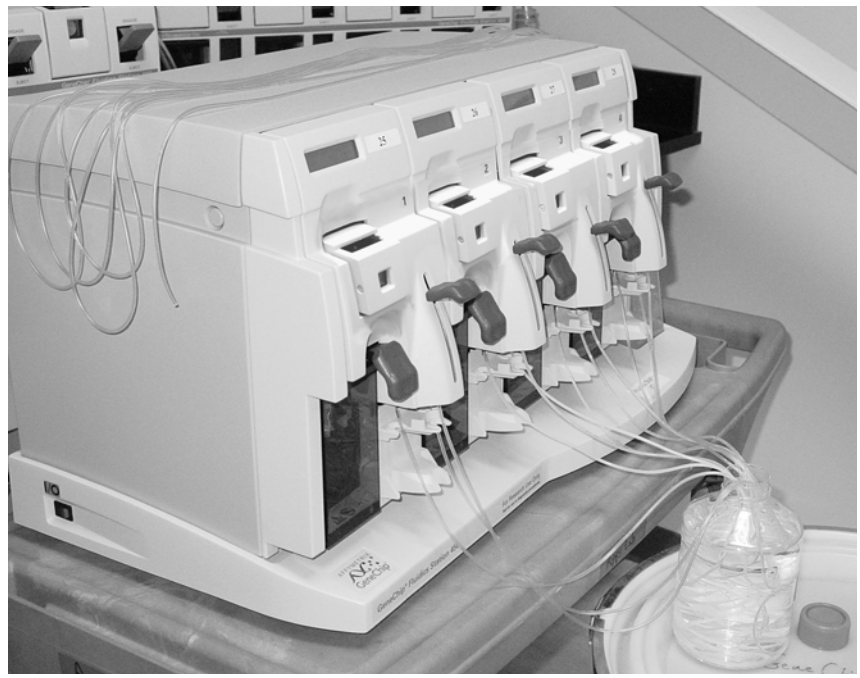


Figure 4.1.3
The tubes extending from the modules to the bleach bottle. Note that the probe array cartridges must be removed before the protocol can begin.

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.

9. 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.

10. 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 out, as this may damage the needle in the process.*

11. 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.

12. 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.

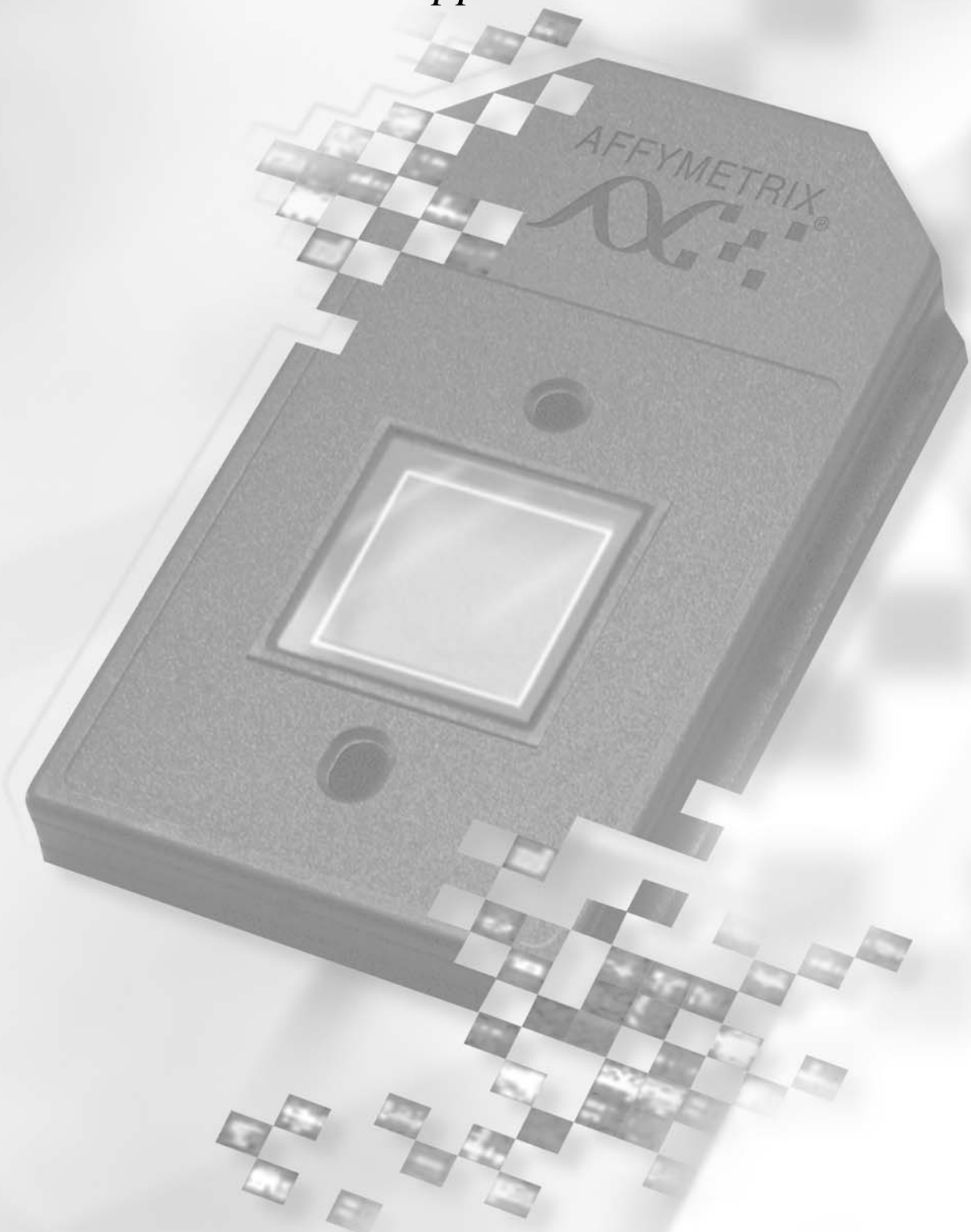
1. Prepare 2 liters of 0.525% sodium hypochlorite solution using distilled water. Mix well.
2. Place all three wash lines of the fluidics station in 1 liter of 0.525% sodium hypochlorite solution.
3. Run the Prime protocol (page 2.4.8) on all four modules with wash lines in 0.525% sodium hypochlorite solution (instead of wash buffers A and B).
4. Run the Shutdown protocol (page 2.4.20) 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 4.1.4) 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) 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) 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) with wash lines in distilled water.
10. Run the **Prime** protocol (Fluidics Station 400) or the **Prime_450** protocol (Fluidics Station 450) 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) with distilled water.

✔ Note

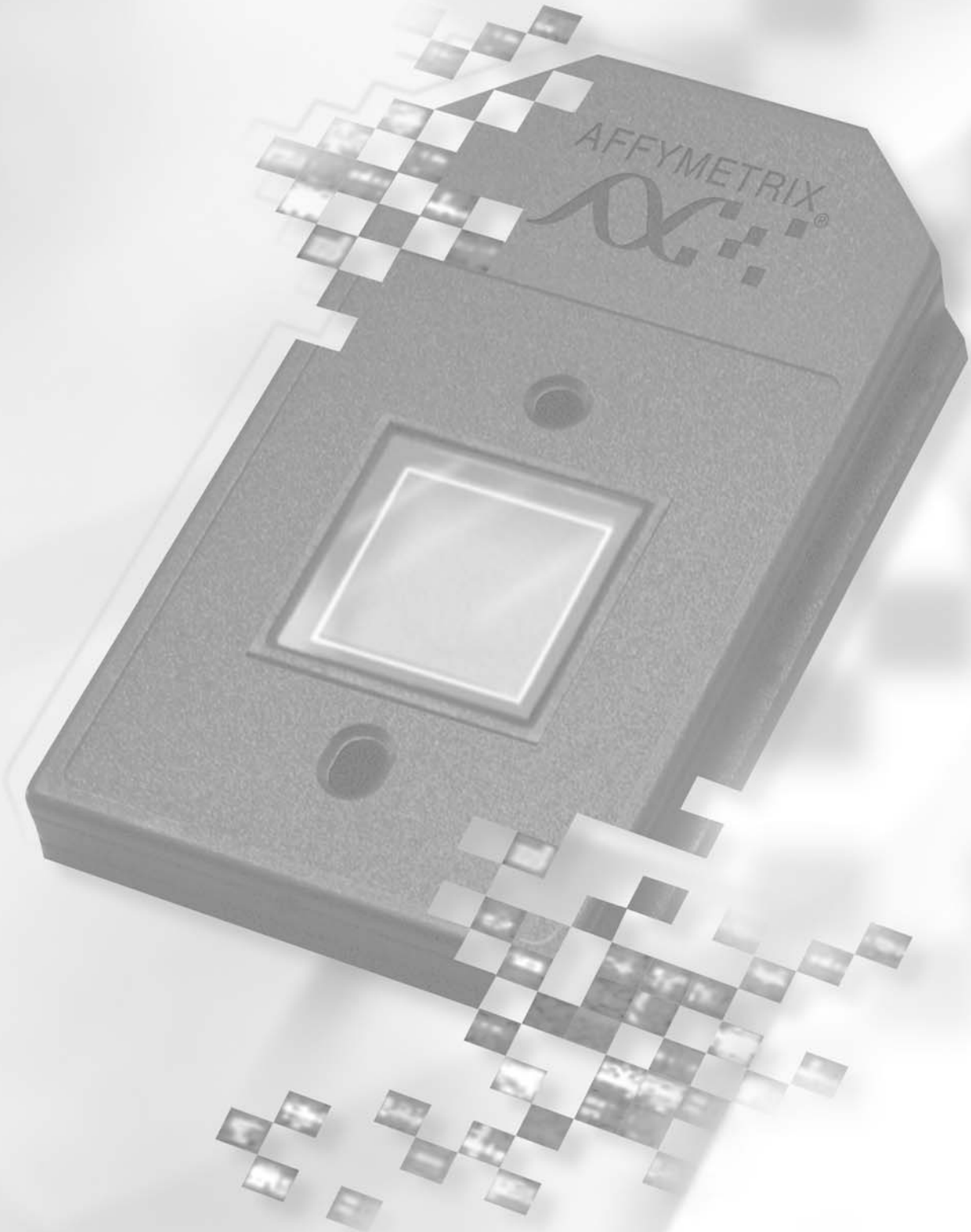
At the end of each step, the fluidics station will indicate a 'ready' status. The fluidics station should not be used until this entire procedure (steps 1-11) is complete.

Section 5:

Appendices



Section 5



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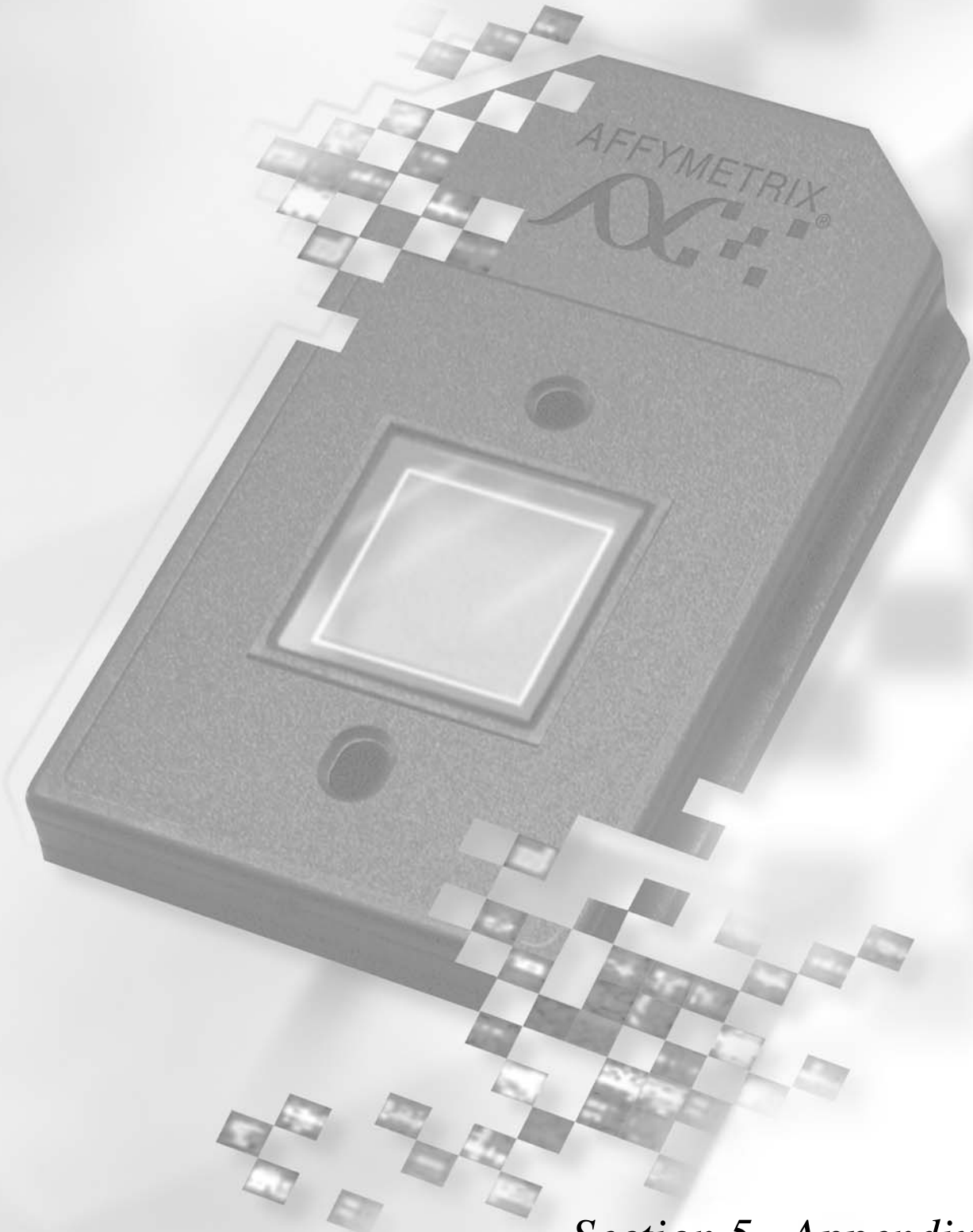
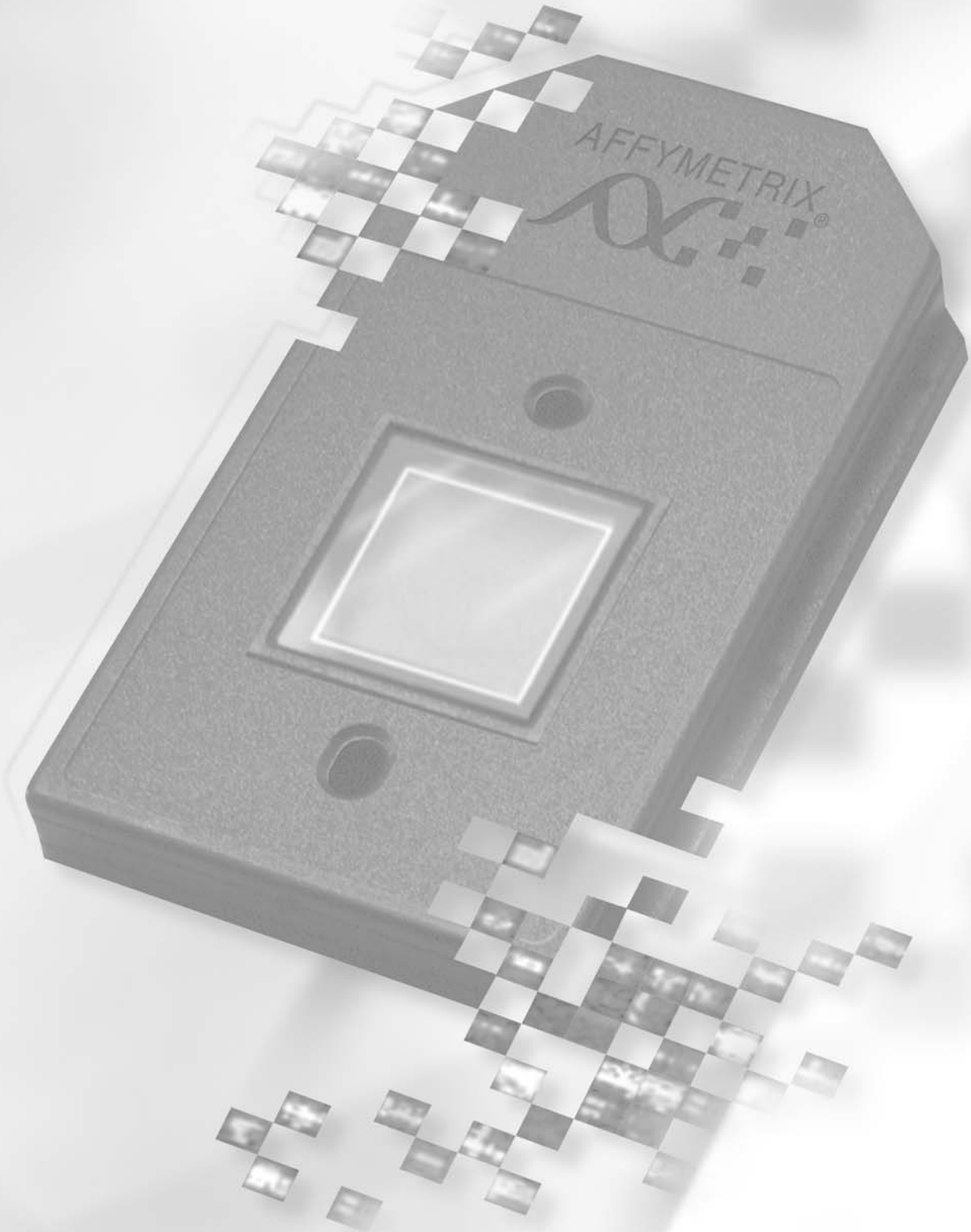


Section 5, Appendix A

Section 5, Appendix A



Supplier and Reagent Reference List

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
USA

Tel: 1-888-362-2447 (1-888-DNA-CHIP)
Fax: 1-408-731-5441
E-mail: support@affymetrix.com

Affymetrix UK Ltd
Voyager, Mercury Park,
Wycombe Lane, Wooburn Green,
High Wycombe HP10 0HH
United Kingdom

Tel: +44 (0)1628 552550
Fax: +44 (0)1628 552585
E-mail: supporteurope@affymetrix.com

Affymetrix Japan, K.K.
Mita NN Bldg
16 Floor, 4-1-23 Shiba,
Minato-ku, Tokyo 108-0014
Japan

Tel: +81-(0)3-5730-8200
Fax: +81-(0)3-5730-8201
E-mail: supportjapan@affymetrix.com

www.affymetrix.com

Supplier Contact Information

Supplier	*U.S.*	*United Kingdom*	*France*	*Germany*
Ambion www.ambion.com	(800) 888-8804	+44 (0)1993 706 500	+33 (0)1 42 53 14 53	+49 (0)692 88082
Amersham/Pharmacia Biotech www.apbiotech.com	(800) 323-9750	+44 (0)800 515 313	+33 (0)1 69 35 67 00	+49 (0)761 49030
Amresco www.amresco-inc.com	(800) 448-4442	+44 (0)1582 745 000	+33 (0)4 70 03 88 55	+49 (0) 551 506860
ATCC www.atcc.org	(800) 638-6597		U.S. (703) 365-2700	
BioWhittaker Molecular Applications / Cambrex www.cambrex.com	(800) 341-1574	+44 (0)1189 795 234	(Belgium) +32 8-732-1611	
Brinkmann Instruments www.brinkmann.com	(800) 421-9988	see web site for nearest distributor		
Cambrex www.cambrex.com	(800) 341-1574	see web site for nearest distributor		
Cole-Parmer www.coleparmer.com	(800) 323-4340	+44 (0)1815 747 556	+33 (0)3 88 67 14 14	+49 (0)785 17069
CLONTECH www.clontech.com	(800) 662-CLON	+44 (0)1256 476 500	+33 (0)1 34 60 24 24	+49 (0)622 134170
Epicentre Technologies www.epicentre.com	(800) 284-8474	+44 (0)1223 366 500	+33 (0)1 30 46 39 00	+49 (0)515 29020
Eppendorf - 5 Prime www.5prime.com	(800) 533-5703 (303) 440-3705			
Invitrogen Life Technologies www.invitrogen.com	(800) 955-6288	00 800 5345 5345 (Toll-free for Europe)		
Millipore Corp www.millipore.com	(800) 645-5476	+44 (0)1923 816 375	+33 (0)1 30 12 70 00	+49 (0)619 64940
Molecular Probes www.probes.com	(541) 465-8300	+44 (0)1223 316 855	+33 (0)4 70 03 88 55	+49 (0)551 371062
New England Biolabs www.neb.com	(800) 632-5227	+44 (0)800 318 846	+33 (0)1 34 60 24 24	+49 (0)800 2465 227
Novagen www.novagen.com	(800) 207-0144	+44 (0)800 622 935	+33 (0)1 30 46 39 00	+49 (0)800 6931 000
Operon Technologies oligos.qiagen.com	(800) 688-2248		00800-67 673377	
Pierce Chemical www.piercenet.com	(800) 874-3723	+44 (0)1244 382 525	+33 (0)4 70 03 88 55	+49 2241 96850
Promega Corporation www.promega.com	(800) 356-9526	+44 (0)800 378 994	+33 (0)8 00 48 79 99	+49 (0)621 85010
Proligo www.gensetoligos.com	(800) 995-0308		+33 (0)1 43 56 59 00	
QIAGEN www.qiagen.com	(800) 426-8157	+44 (0)1293 422 911	+33 (0)1 60 92 09 20	+49 (0)210 3892 230

Supplier	*U.S.*	*United Kingdom*	*France*	*Germany*
Rainin www.rainin.com	(800) 472-4646	+44 (0)1582 456 666	see web site for nearest distributor	
Roche Molecular Biochemical biochem.roche.com	(800) 262-1640	+44 (0)1273 480 444	+33 (0)4 76 76 30 87	+49 (0)621 75985 68
Sigma-Aldrich www.sigma-aldrich.com	(800) 325-3010	+44 (0)1202 733 114	+33 (0)4 74 82 28 88	+49 (0)896 5131130
USA Scientific www.usascientific.com	(800) 522-8477	U.S. (352)-237-6288		
Vector Laboratories www.vectorlabs.com	(800) 227-6666	+44 (0)1733 237 999	44 86 22 75	+49 (0)9342 39499 or 0800 253 9472
VWR Scientific Products www.vwrsp.com	(800) 932-5000 (908) 757-4045			

Reagents and Materials Quick List

	Reagent	Vendor	P/N	Volume per Rxn	Volume in Kit	Rxns per Kit
First-Strand cDNA Synthesis						
☐	T7-Oligo(dT) Primer, 50 µM	Affymetrix	900375	2 µL	300 uL	150
☐	DEPC-Water	Ambion	9920	Variable	500 µL	---
☐	5x First-Strand cDNA Buffer	Invitrogen	Supplied with SS II RT	4 µL	1 mL	250
☐	0.1M DTT	Invitrogen	Supplied with SS II RT	2 µL	250 µL	125
☐	10 mM dNTP Mix[1]	Invitrogen	18427-013	1 µL	100 µL	100
☐	SuperScript II RT	Invitrogen	18064-014	1, 2, or 3 µL	50 µL (10,000 U)	50, 25, or 16
Second-Strand cDNA Synthesis						
☐	DEPC-Water	Ambion	9920	91 µL	500 mL	5,494
☐	5x Second-Strand cDNA Buffer	Invitrogen	10812-014	30 µL	500 µL	16
☐	10 mM dNTP Mix[1]	Invitrogen	18427-013	3 µL	100 µL	33
☐	***E. coli*** DNA Ligase	Invitrogen	18052-019	1 µL	10 µL (100 U)	10
☐	***E. coli*** DNA Polymerase I	Invitrogen	18010-025	4 µL	100 µL (1,000 U)	25
☐	***E. coli*** RNase H	Invitrogen	18021-071	1 µL	60 µL (120 U)	60
☐	T4 DNA Polymerase	Invitrogen	18005-025	2 µL	50 µL (250 U)	25
☐	0.5M EDTA	Invitrogen	15575-020	10 µL	400 mL (4 X 100)	40,000
cDNA Cleanup						
☐	GeneChip® Sample Cleanup Module[2]	Affymetrix	900371	---	---	30
IVT Reaction						
☐	Enzo® BioArrayô RNA Labeling Kit	Affymetrix	900182	---	---	10
☐	Distilled or Deionized Water	---	---	---	---	---
cRNA Cleanup						
☐	GeneChip Sample Cleanup Module[2]	Affymetrix	900371	---	---	30
cRNA Fragmentation						
☐	GeneChip Sample Cleanup Module[3]	Affymetrix	900371	---	---	>30

[1] 10 mM dNTP Mix used for both 1st and 2nd Strand reactions, for a total of 4 µL per reaction. Each tube of dNTP Mix contains 100 µL, so each tube is sufficient for 25 reactions.

[2] Note: The GeneChip Sample Cleanup Module contains reagents and columns for both the cDNA and the IVT cRNA cleanup.

[3] Note: 5x Fragmentation Buffer is included in the GeneChip Sample Cleanup Module.

	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 2.3.1* for details on other array formats).)						
	GeneChip® Eukaryotic Hybridization Control Kit[4]	Affymetrix	900299 or 900362	---	---	30 150
	Herring Sperm DNA	Promega	D1811	3 µL	1 mL	333
	Acetylated BSA[5]	Invitrogen	15561-020	3 µL	3 mL (3 X 1)	1,000
	2x MES Hybridization Buffer[6]	---	---	150 µL	50 mL	333
	Mol. Bio. or DEPC-Water	---	---	---	---	---
Stain Reagents (Following quantities are for the Antibody Amplification Staining Protocol)						
	2x MES Stain Buffer[7]	---	---	900 µL	250 mL	277
	DI water	---	---	806.4 µL	---	---
	Acetylated BSA[5]	Invitrogen	15561-020	72 µL	3 mL (3 X 1)	41
	Streptavidin Phycoerythrin (SAPE)	Molecular Probes	S-866	12 µL	1 mL	83
	Normal Goat IgG	Sigma	I5256	6 µL	1 mL	166
	Biotinylated Antibody	Vector Labs	BA-0500	3.6 µL	1 mL	277

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

[5] Total Acetylated 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.

[6] See page 2.3.6, for 50 mL. preparation.

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

Reagent List

A

Acetic Acid, Glacial, Sigma-Aldrich, P/N A6283
Acetylated Bovine Serum Albumin (BSA) solution, 50 mg/mL, Invitrogen Life Technologies, P/N 15561020
Ammonium Acetate, 7.5 M, Sigma-Aldrich, P/N A2706
Anti-streptavidin antibody (goat), biotinylated, Vector Laboratories, P/N BA-0500
Antibody (goat), Anti-streptavidin, biotinylated, 0.5 mg, Vector Laboratories, P/N BA-0500
Antibody, IgG, Goat, Reagent Grade, 50 mg, Sigma-Aldrich, P/N I5256
γ-S-ATP, 20 μmoles, Roche Molecular Biochemical, P/N 1162306

B

Bleach (5.25% Sodium Hypochlorite), VWR Scientific Products, P/N 21899-504
Bovine Serum Albumin (BSA) solution, 50 mg/mL, Acetylated, Invitrogen Life Technologies, P/N 15561020

C

CHROMA SPIN-100 Columns in Swing Bucket Format, CLONTECH, P/N K1302-1
Control Oligo B2, 30 nM, Affymetrix, P/N 900301

D

dATP, dCTP, dGTP, dTTP, Amersham Pharmacia Biotech, P/N 27-2035-01
Deoxyribonuclease I (DNase I), Amersham Pharmacia Biotech, P/N 27-0514-01
DMSO, Hybrid-Max®, Sigma-Aldrich, P/N D2650
DNA, Herring Sperm, Promega Corporation, P/N D1811
DNA Ligase, *E. coli*, Invitrogen Life Technologies, P/N 18052-019
DNA Polymerase, *E. coli*, Invitrogen Life Technologies, P/N 18010-025
DNA Polymerase, T4, Invitrogen Life Technologies, P/N 18005-025
dNTP, 10 mM, Invitrogen Life Technologies, P/N 18427-013
DTT, 100 mM, Epicentre Technologies, P/N M4410K (supplied with MMLV Reverse Transcriptase)

E

EDTA Disodium Salt, 0.5 M solution, 100 mL, Sigma-Aldrich, P/N E7889
EDTA, 0.5 M, pH 8.0, Invitrogen Life Technologies, P/N 15575-020
Enzo® BioArray™ HighYield™ RNA Transcript Labeling Kit, Affymetrix, P/N 900182
Ethidium Bromide, Sigma-Aldrich, P/N E8751
Expression Control Clones, American Type Culture Collection (ATCC)

pGIKS-bioB	ATCC 87487
pGIKS-bioC	ATCC 87488
pGIKS-bioD	ATCC 87489
pGIKS-cre	ATCC 87490
pGIBS-lys	ATCC 87482
pGIBS-phe	ATCC 87483

pGIBS-thr	ATCC 87484
pGIBS-trp	ATCC 87485
pGIBS-dap	ATCC 87486

G

GeneChip® Eukaryotic Hybridization Control Kit, Affymetrix, P/N 900299
Glycogen, Ambion, P/N 9510
Glycogen, 20 mg/mL, Roche Molecular Biochemical, P/N 901393
Goat IgG, Reagent Grade, Sigma-Aldrich, P/N I5256

H

HCl, 1N solution, VWR Scientific Products, P/N MK638860
Herring Sperm DNA, Promega Corporation, P/N D1811
Hybridization Oven 640, Affymetrix, P/N 800139

I

ImmunoPure® NeutrAvidin, Pierce Chemical, P/N 31000
ImmunoPure® Streptavidin, Pierce Chemical, P/N 21125

L

10 bp and 100 bp ladder, Invitrogen Life Technologies, P/N 10821-015 and15628-019, respectively

M

Magnesium acetate (MgOAc), Sigma-Aldrich, P/N M2545
MasterPure™ RNA Purification Kit, Epicentre Technologies, P/N MCR85102
MEGAscript T3 Kit, Ambion, P/N 1338
MEGAscript T7 Kit, Ambion, P/N 1334
MES Free Acid Monohydrate SigmaUltra, Sigma-Aldrich, P/N M5287
MES Sodium Salt, Sigma-Aldrich, P/N 5057
MMLV Reverse Transcriptase, New England BioLabs, P/N M0253L
MMLV Reverse Transcriptase Buffer, 10X, New England BioLabs, P/N M0253L
MOPS, Sigma-Aldrich, P/N M3183

N

NaCl, 5 M, RNase-free, DNase-free, Ambion, P/N 9760G
NaOH, 1N Solution, VWR Scientific Products, P/N MK469360
Novex XCell SureLock™ Mini-Cell, Invitrogen Life Technologies, P/N EI0001
Nuclease-free Water, Ambion, P/N 9930
Nucleotides, labeled, Biotin-11-CTP and Biotin-16-UTP, Enzo, P/N 42818 (CTP) and P/N 42814 (UTP)
Nucleotides, Biotin-11-CTP, Sigma-Aldrich, P/N B7048
Nucleotides, Biotin-16-UTP, Roche Molecular Biochemicals, P/N 1388908
Nucleotides, Biotin-16-UTP, Sigma-Aldrich, P/N B6923

O

Oligo B2, Control, Control Oligo for the antisense probe array, HPLC purified
5´-bio GTCGTCAAGATGCTACCGTTCAGGA- 3´
Oligotex Direct mRNA Kit, QIAGEN, P/N 72012, 72022, or 72041
Oligotex mRNA Kit, QIAGEN, P/N 70022, 70042, or 70061

P

PBS, pH 7.2, Invitrogen Life Technologies, P/N 20012-027
Pellet Paint, Novagen, P/N 69049-3
PEO-Iodoacetyl-Biotin, 50 mg, Pierce Chemical, P/N 21334ZZ
Phase Lock Gel, Brinkmann Instruments, P/N 955 15 415
Phenol/chloroform/isoamyl alcohol, Ambion, P/N 9732
Phycoerythrin-Streptavidin, Molecular Probes, P/N S-866
Polynucleotide Kinase, T4, New England BioLabs, P/N 201L
Potassium acetate (KOAc), Sigma-Aldrich, P/N P5708
Primer, T7– $(dT)_{24}$, (Genset Corp), HPLC purified
5´- GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-(T)24 - 3´

Q

Qiashredder, QIAGEN, P/N 79654

R

R-Phycoerythrin Streptavidin, Molecular Probes, P/N S-866
Ribonuclease H (RNase H), *E. coli*, Epicentre Technologies, P/N R0601K
RNA/DNA Mini Column Kit, QIAGEN, P/N 14123
RNase H, *E. coli*, Invitrogen Life Technologies, P/N 18021-021, or Epicentre Technologies, P/N R0601K
RNeasy Mini Kit, QIAGEN, P/N 74104, 74106

S

Second-strand buffer, 5X, Invitrogen Life Technologies, P/N 10812-014
Sodium Acetate, 3 M, pH 5.2, Sigma-Aldrich, P/N S7899
Sodium Hypochlorite, Sigma-Aldrich, P/N 7681-52-9
SSPE, 20X, BioWhittaker Molecular Applications / Cambrex, P/N 51214
Streptavidin, ImmunoPure®, Pierce Chemical, P/N 21125
Sucrose Gel Loading Dye, 5X, Amresco, P/N E-274
SUPERase•In™, Ambion, P/N 2696
SuperScript II RT, Invitrogen Life Technologies, P/N 18064-071
SuperScript Choice system, Invitrogen Life Technologies, P/N 18090-019
SYBR Gold, Molecular Probes, P/N S-11494
SYBR Green II, Cambrex, P/N 50523, or Molecular Probes, P/N S7586

T

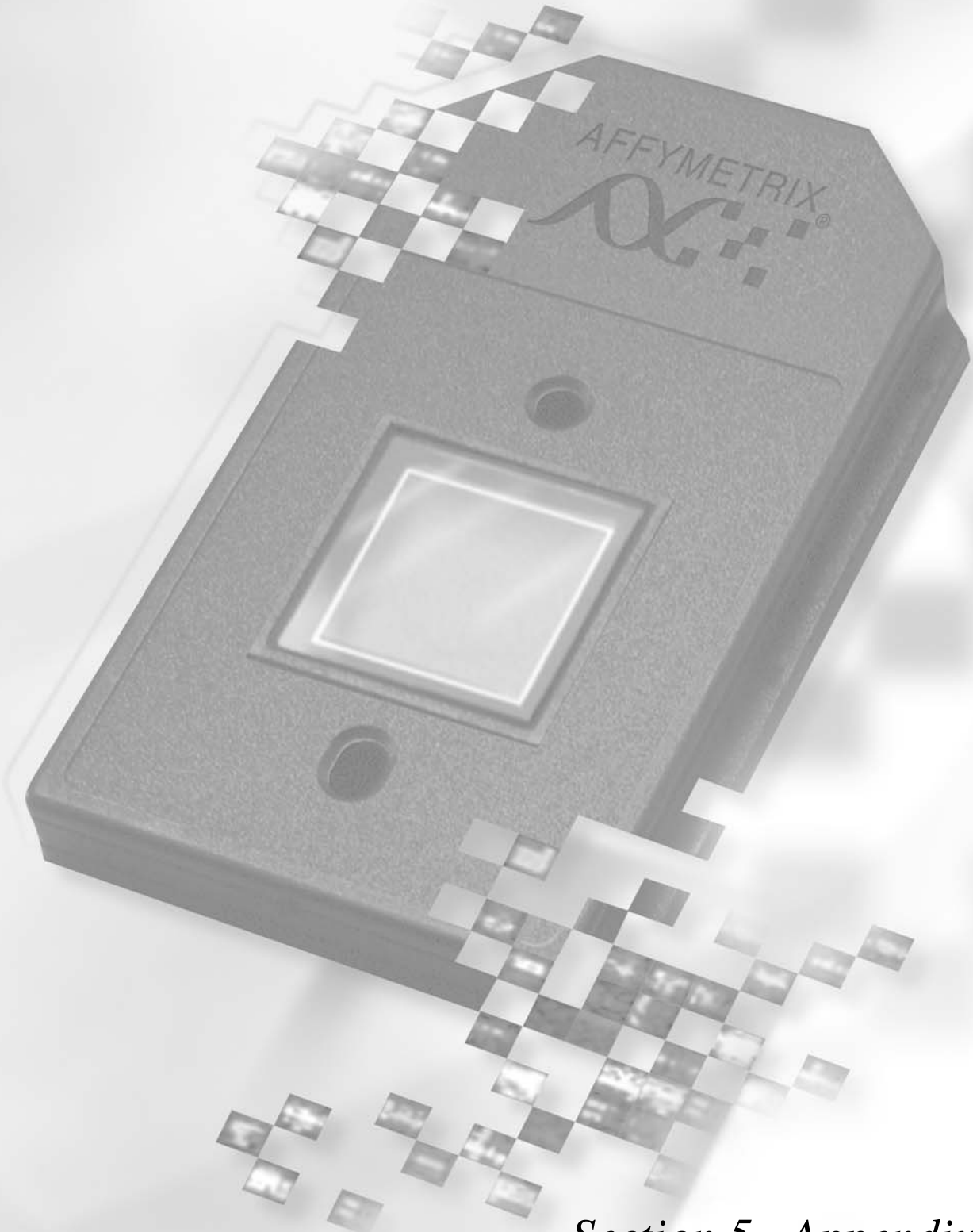
TBE, 10X, Cambrex, P/N 50843
TBE Gel (4-20%), 1.0 mm, 12 well, Invitrogen Life Technologies, P/N EC62252
TE, 1X, BioWhittaker Molecular Applications / Cambrex, P/N 51235
Tough-Spots, Label Dots, USA Scientific, P/N 9185
Tris pH 7.0, 1M, Ambion, P/N 9850G
Trizma Base, Sigma-Aldrich, P/N T1503
TRIzol Reagent, Invitrogen Life Technologies, P/N 15596-018
Tubes, Sterile, RNase-free, microcentrifuge, 1.5 mL, USA Scientific, P/N 1415-2600
Tubing, Tygon, 0.04" inner diameter, Cole Parmer, P/N H-06418-04
Tween-20, 10%, Pierce Chemical, P/N 28320

V

Vacuum filter units 1 liter capacity, 0.20 µm or 0.45 µm, Corning, P/N 25988-1L

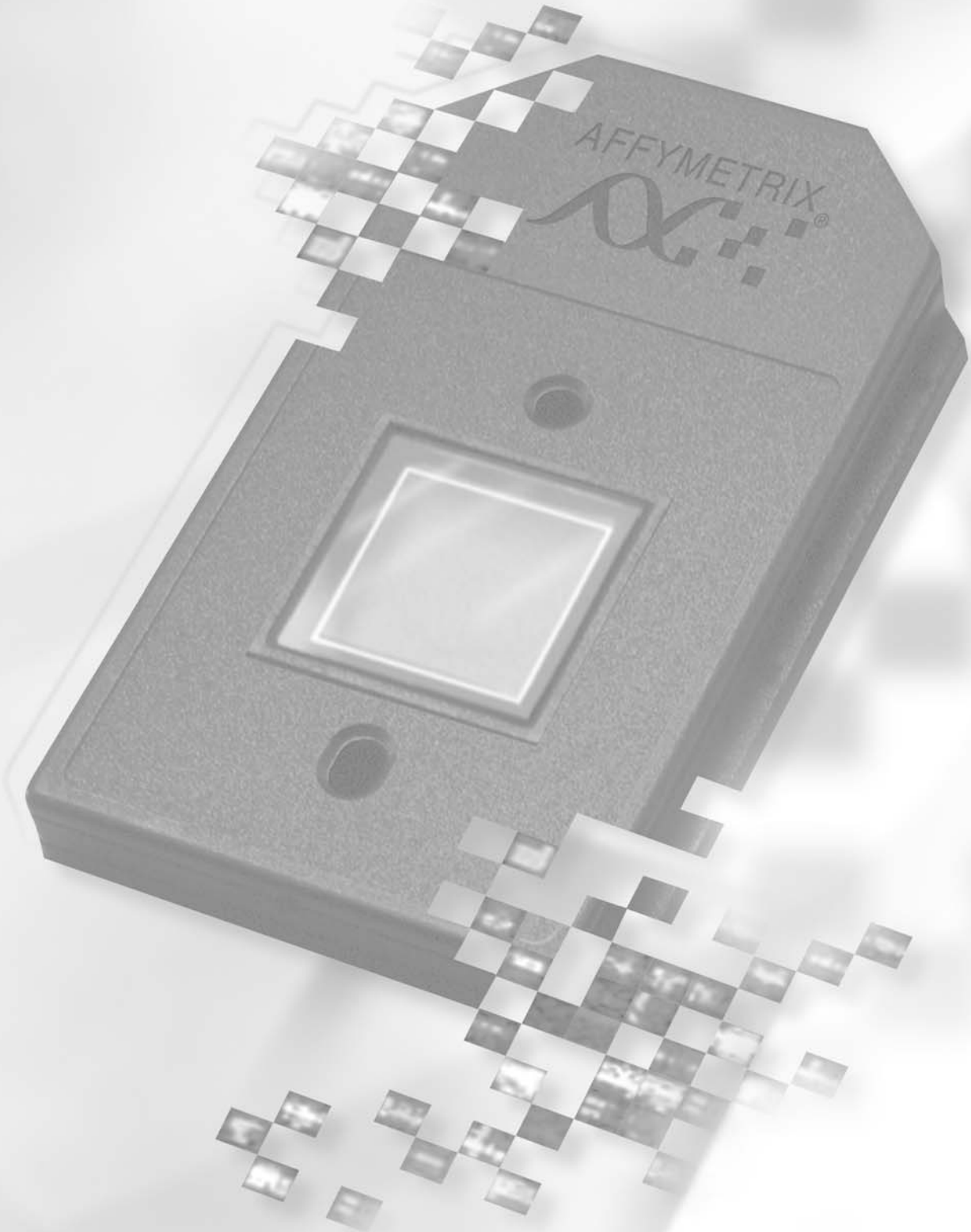
W

Water, DEPC-Treated, Ambion, P/N 9902
Water, Distilled, Invitrogen Life Technologies, P/N 15230147
Water, Molecular Biology Grade, BioWhittaker Molecular Applications / Cambrex, P/N 51200



Section 5, Appendix B

Section 5, Appendix B



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?

We currently recommend 5 µg of total RNA for our standard eukaryotic expression arrays. Reducing the amount of starting material used in the standard assay may result in a subsequent decrease in sensitivity. Additionally, a *Technical Note: GeneChip Eukaryotic Small Sample Target Labeling Assay Version II* is available on www.affymetrix.com describing a small-sample target-labeling protocol and reporting results from Affymetrix using this protocol on reduced starting material. Contact Affymetrix Technical Support for any questions concerning this alternative protocol.

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 2.3.1* 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 after its first hybridization?

Assuming no RNase contamination, cRNA targets can be stored for at least one year 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 this manual, the quality of starting RNA is very important. Ratio of 260/280 absorbance values, as well as appearance of samples by gel electrophoresis, are suggested methods to detect any degradation of your RNA samples.
2. Target labeling: Various QC protocols described in this 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 house-keeping 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, background, scaling factor, noise, 3'/5' GAPDH and Actin ratios, and % Present calls.

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 (www.qiagen.com/literature/handbooks/rna/rnamini/1016272HBRNY_062001WW.pdf).

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

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.

Hybridization, Washing, and Staining

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

The standard gene expression hybridization time is 14-16 hours at 45°C. At high temperatures or with longer incubation times, the sample will evaporate. Loss of sample is undesirable for several reasons:

1. 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.
2. Sample loss compromises the possibility of repeating the experiment with the identical sample.
3. 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” fluidic 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 fluidic script do I use?

The appropriate fluidic script is specific to the array format and the organism (eukaryotic or prokaryotic)), 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 hybridization protocols in the respective sections of this manual for more detail.

Is there a possibility of contaminating the fluidics station with RNase when gene expression, genotyping, and health management 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: first-strand cDNA synthesis, second-strand 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 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 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 house-keeping 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:

1. Experimental design strategy should be the same at various sites.
2. 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.
3. Scanners are adjusted to the same PMT setting.
4. Same algorithm parameters are used.
5. Similar results from 3'/5' ratios, background, noise, and scaling factors. Check arrays for scratches and even hybridization/staining.
6. 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.

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 Fluidic 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 scanning 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. For a detailed description, please refer to *Affymetrix Microarray Suite User's Guide* (P/N 701099).

A second type of probe mask defines a select group of probe sets that can be used in normalization or scaling. Please refer to *Affymetrix Microarray Suite User's Guide* where this type of probe set mask file is described.

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 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 short cut menu in Microarray Suite 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 that this attribute be removed. To do this, open NT 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**.

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.

Experimental Design

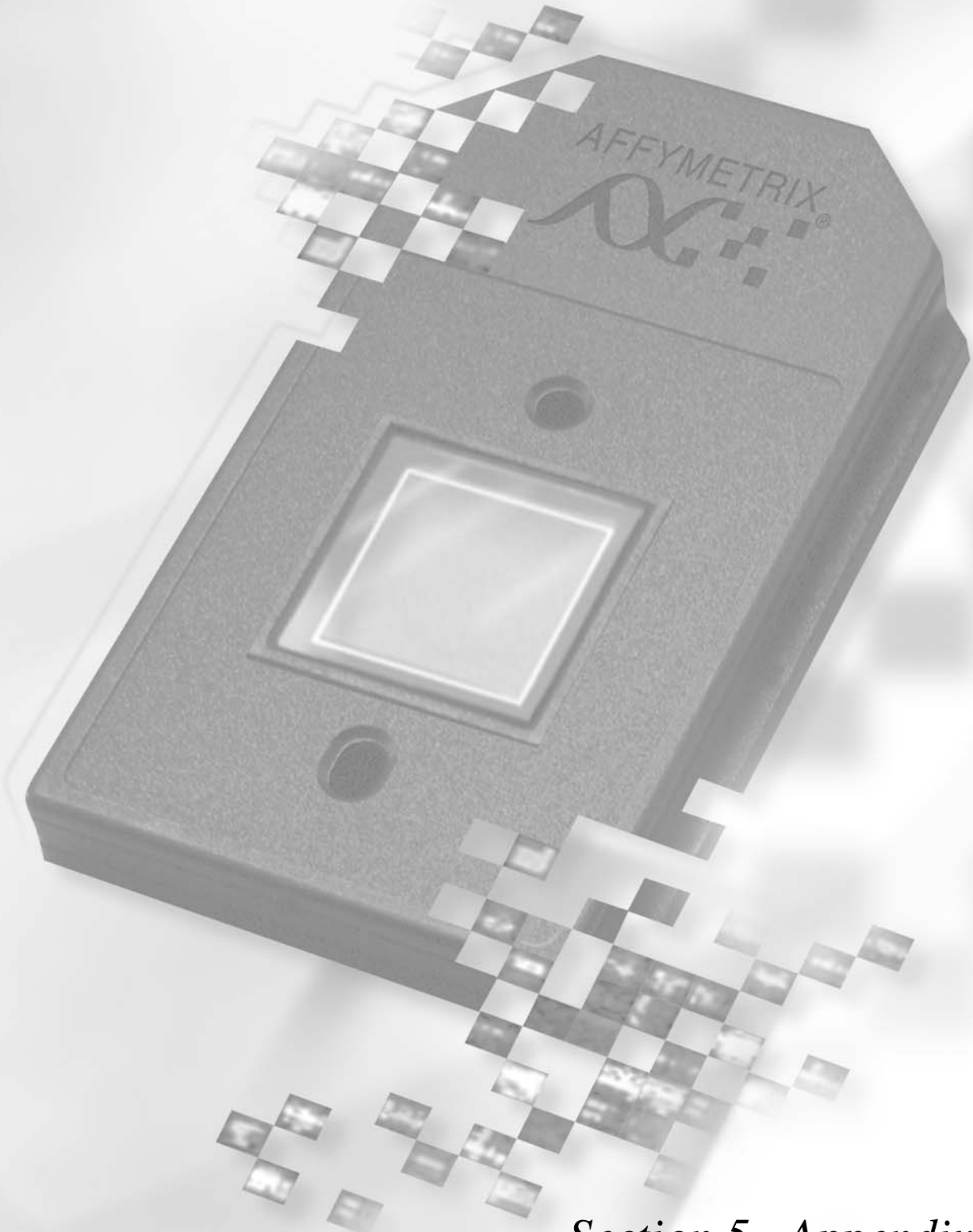
Which is greater, sample or assay variability?

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.
Enzo® BioArray™ HighYield™ RNA Transcript Labeling Kit		
Apparent insufficient volume in reagent tubes	The reagent tubes are opened before centrifugation.	The small volume may be expelled by opening. The tubes should be centrifuged briefly before use to ensure that reagents remain at the bottom of the tube.
Precipitation in the reaction buffer	After many freeze-thaw cycles, a precipitate may form.	Centrifuge briefly to remove precipitate before use. The precipitate formation does not interfere with the reaction.
Low yield	Poor quality template.	Check starting material quality.
	Loss in enzyme activity.	Repeat IVT.
	Incorrect DTT concentration due to DTT precipitation prior to addition to the IVT reaction.	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".
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.

Problem	*Likely Cause*	*Solution*
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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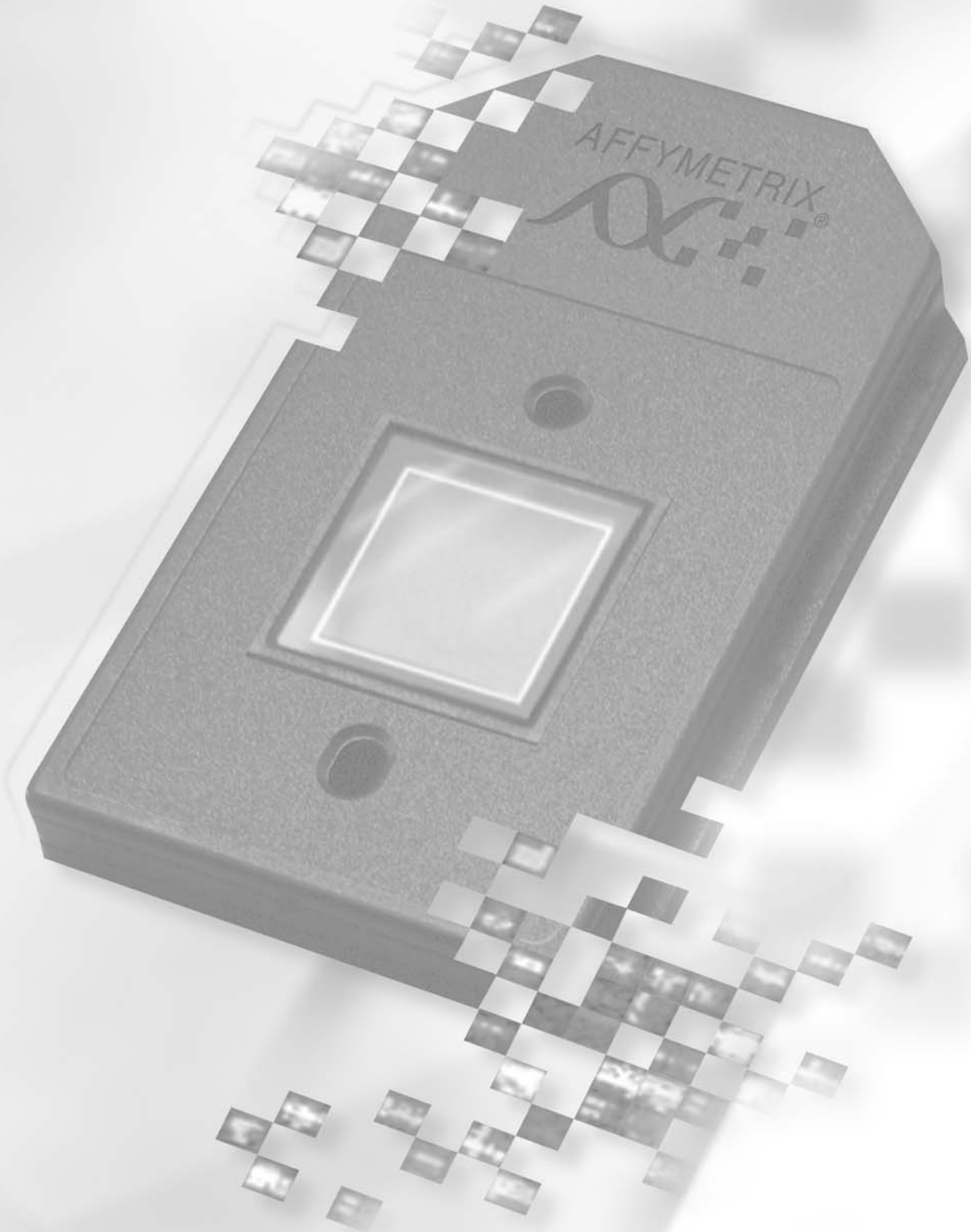

List of Controls on GeneChip Probe Arrays

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 Section 2, Chapter 2 and Chapter 3
	E. coli P1 Bacteriophage	*bioB* *bioC* *bioD* *cre*	Antisense biotinylated cRNA are used as hybridization controls.	GeneChip Eukaryotic Hybridization Control Kit, P/N 900299 Section 2, Chapter 3
	B. subtilis	*dap* *thr* *trp* *phe* *lys*	Poly-A-tailed sense RNA can be produced by IVT and spiked into isolated RNA samples as controls for the labeling and hybridization process. The spikes can also be used to estimate assay sensitivity.	N/A Section 2, Chapter 2
Prokaryotic Arrays	synthetic	B2 Oligo	Grid alignment.	Control Oligo B2, P/N 900301 Section 3, Chapter 3
	B. subtilis	*dap* *thr* *trp* *phe* *lys*	Sense RNA can be produced by IVT and spiked into purified sample RNA as control for the labeling and hybridization process. The spikes can also be used to estimate assay sensitivity.	N/A Section 3, Chapter 2 and Chapter 3

Registration

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www.affymetrix.com/support/technical/expression_registration.affx