


**GeneChip®**  
**Expression Analysis Technical Manual**

**With Specific Protocols for Using the GeneChip®  
Hybridization, Wash, and Stain Kit**

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Chapter 1

**Overview**

Chapter 1



## Chapter Contents

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### **This Chapter Contains:**

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

## Introduction and Objectives

Welcome to the *Affymetrix GeneChip® Expression Analysis Technical Manual*. This manual is a technical guide for using GeneChip expression analysis probe arrays. All protocols included in this manual have been used successfully by scientists at Affymetrix, or have been recommended by our collaborators during the development of particular products. The field of mRNA gene expression monitoring is rapidly evolving and periodic technical updates to this manual will reflect the newest protocols and information for using GeneChip probe arrays. This manual applies to all GeneChip 3' eukaryotic arrays in cartridge format and GeneChip prokaryotic arrays in cartridge format.

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.<sup>1,2</sup> Up to 1.3 million different oligonucleotide probes are synthesized on each array. Each oligonucleotide is located in a specific area on the array called a probe cell. Each probe cell contains hundreds of thousands to millions of copies of a given oligonucleotide.

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

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

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<sup>1</sup> 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).

<sup>2</sup> See [www.affymetrix.com](http://www.affymetrix.com) for current GeneChip technology references.

light emitted at 570 nm is proportional to the bound target at each location on the probe array.

## GeneChip® Expression Analysis Overview

The following major steps outline GeneChip expression analysis:

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

Due to the differences in the RNA species between eukaryotic and prokaryotic organisms, different target labeling protocols have been optimized. Chapters 2 through 6 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 using GeneChip® reagent kits for preparing biotinylated target from purified eukaryotic and prokaryotic RNA samples suitable for hybridization to GeneChip expression probe arrays. For more information on these procedures, please contact Affymetrix Technical Support at 1-888-DNA-CHIP, +44 (0)1628 552550 in Europe, or +81-(0)3-5730-8200 in Japan.

For eukaryotic samples, using protocols referenced in [Chapter 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, [Chapter 4](#) 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, and probe array controls. 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. Refer to [Chapter 3](#) for hybridization of eukaryotic samples, and [Chapter 5](#) for prokaryotic samples.

## STEP 3: FLUIDICS STATION SETUP

Specific experimental information is defined using Affymetrix® Microarray Suite or GeneChip Operating Software (GCOS) on a PC-compatible workstation. The probe array type, sample description, and comments are entered and saved with a unique experiment name. The fluidics station is then prepared for use by priming with the appropriate buffers. Refer to the *GeneChip® Expression Wash, Stain and Scan User Manual*, P/N 702731 for information on fluidics station setup for eukaryotic samples, and [Chapter 6](#) for prokaryotic samples. For more information on the fluidics station, refer to the *GeneChip® 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. The *GeneChip® Expression Wash, Stain and Scan User Manual*, P/N 702731 provides information for eukaryotic samples, and [Chapter 6](#) provides information for prokaryotic samples.

## STEP 5: PROBE ARRAY SCAN

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

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

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

## STEP 6: DATA ANALYSIS

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

## Precautions

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

### WARNING

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**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.**

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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

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

## Interfering Conditions

**CAUTION** 

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**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).**

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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 *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](http://www.affymetrix.com) for current GeneChip technology references.



## Limitations

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

**IMPORTANT** 

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

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Chapter 2

**Eukaryotic Target Preparation**

Chapter 2



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### This Chapter Contains:

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

## Introduction

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

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

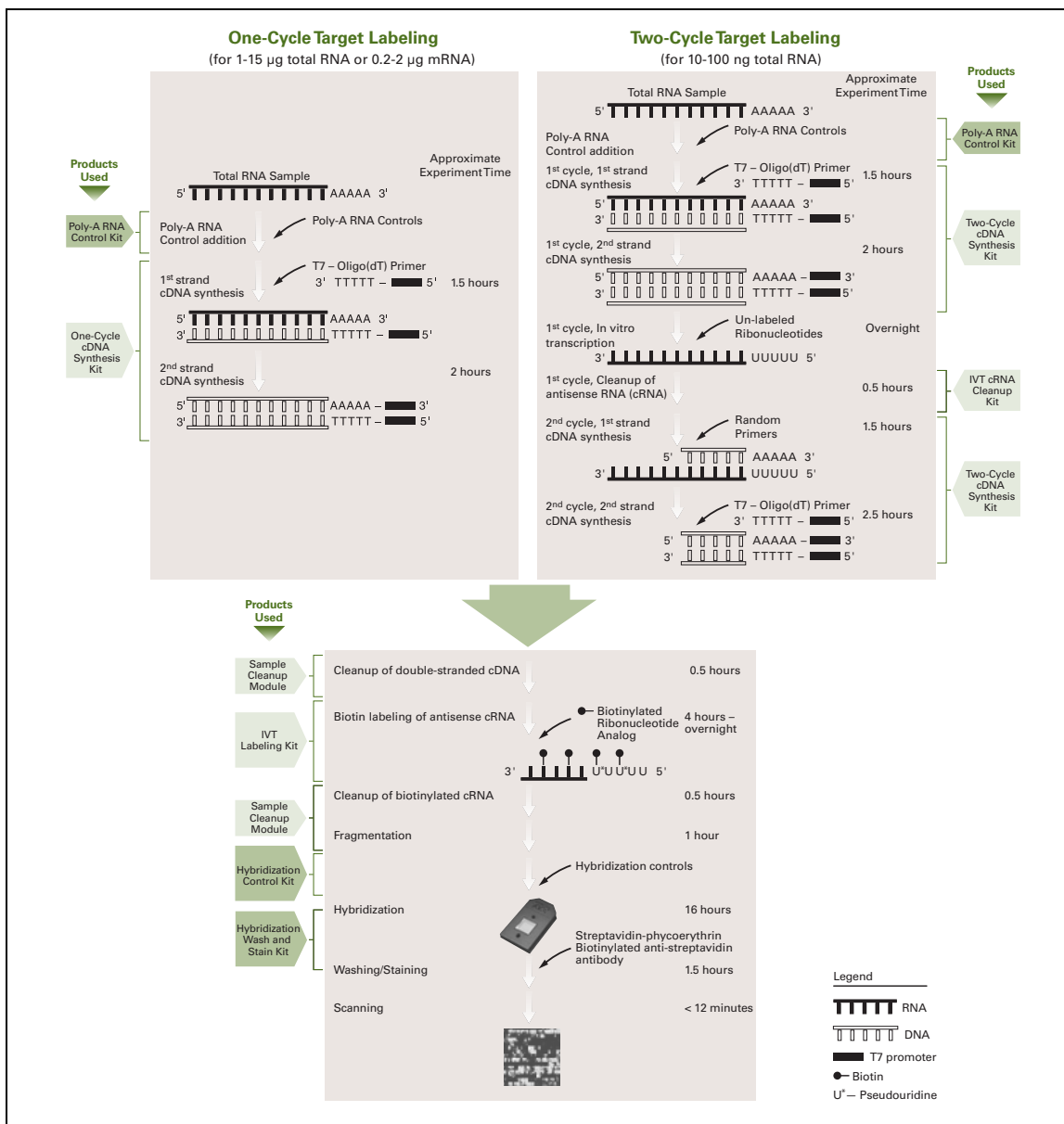
**Table 2.1**

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](#). 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](#). 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.



**Figure 2.1**  
GeneChip® Eukaryotic Labeling Assays for Expression Analysis



## Reagents and Materials Required

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

**IMPORTANT !**

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

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### Total RNA Isolation

- TRIzol Reagent: Invitrogen Life Technologies, P/N 15596-018, or QIAzol™ Lysis Reagent: QIAGEN, P/N 79306
- 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

- GeneChip® One-Cycle Target Labeling and Control Reagents: Affymetrix, P/N 900493. A convenient package containing all required labeling and control reagents to perform 30 one-cycle labeling reactions. Each of these components may be ordered individually (described below) as well as in this complete kit. Contains:
  - 1 GeneChip® IVT Labeling Kit (Affymetrix, P/N 900449)
  - 1 GeneChip® One-Cycle cDNA Synthesis Kit (Affymetrix, P/N 900431)
  - 1 GeneChip® Sample Cleanup Module (Affymetrix, P/N 900371)
  - 1 GeneChip® Poly-A RNA Control Kit (Affymetrix, P/N 900433)
  - 1 GeneChip® Hybridization Control Kit (Affymetrix, P/N 900454)

### Two-Cycle Target Labeling

- GeneChip® Two-Cycle Target Labeling and Control Reagents: Affymetrix, P/N 900494. A convenient package containing required labeling and control reagents to perform 30 two-cycle labeling reactions. Each of these components may be ordered individually (described below) as well as in this complete kit. Contains:
  - 1 GeneChip® IVT Labeling Kit (Affymetrix, P/N 900449)
  - 1 GeneChip® Two-Cycle cDNA Synthesis Kit (Affymetrix, P/N 900432)
  - 1 GeneChip® Sample Cleanup Module (Affymetrix, P/N 900371)
  - 1 GeneChip® IVT cRNA Cleanup Kit (Affymetrix, 900547)
  - 1 GeneChip® Poly-A RNA Control Kit (Affymetrix, P/N 900433)
  - 1 GeneChip® Hybridization Control Kit (Affymetrix, P/N 900454)
- MEGAscript® High Yield Transcription Kit: Ambion Inc, P/N 1334 Purchased separately from Ambion, Inc. (2 kits required to complete 30 reactions)

## Miscellaneous Reagents

- 10X TBE: Cambrex, P/N 50843
- Absolute ethanol (stored at  $-20^{\circ}\text{C}$  for RNA precipitation; store ethanol at room temperature for use with the GeneChip Sample Cleanup Module and IVT cRNA Kit)
- 80% ethanol (stored at  $-20^{\circ}\text{C}$  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

## 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 [Chapter 1](#).

### IMPORTANT !

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**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.**

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

## ISOLATION OF RNA FROM YEAST

### Total RNA

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

### Poly-A mRNA

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

## ISOLATION OF RNA FROM ARABIDOPSIS

### Total RNA

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

### 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 or QIAzol reagents.

### 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. QIAzol™ Lysis Reagent from QIAGEN can also be used.

## 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 or QIAzol reagent isolation and hot phenol extraction methods; see methods [on page 23](#) 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  $-20^{\circ}\text{C}$  for at least 1 hour.
3. Centrifuge at  $\geq 12,000 \times g$  in a microcentrifuge for 20 minutes at  $4^{\circ}\text{C}$ .
4. Wash pellet twice with 80% ethanol.
5. Air dry pellet. Check for dryness before proceeding.
6. Resuspend pellet in DEPC-treated  $\text{H}_2\text{O}$ . 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  $\mu\text{L}$  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.

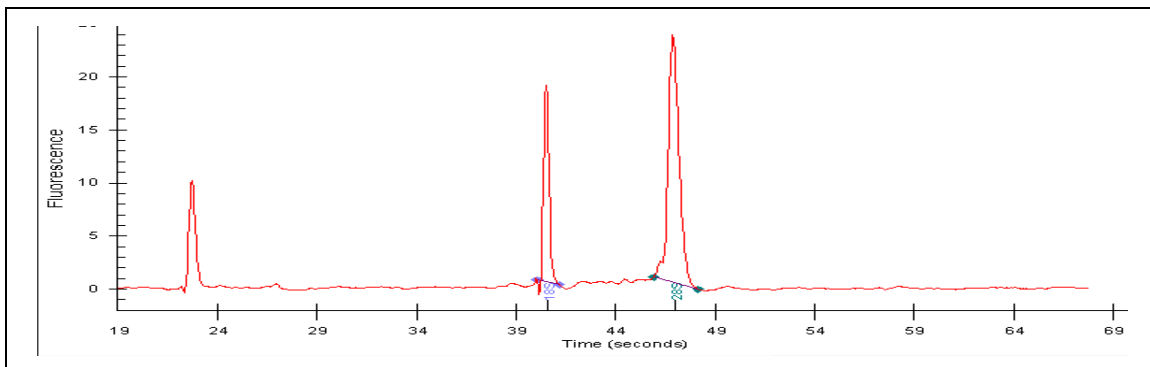
- **Glycogen**

Addition of 0.5 to 1  $\mu\text{L}$  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  $\mu\text{g/mL}$  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 2.2](#) for an example of good-quality total RNA sample.

**Figure 2.2**

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<sup>1</sup>

### 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 dilutions. 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 dilutions (referred to as a ratio of copy number) summarized in [Table 2.2](#).

**Table 2.2**  
Final Dilutions of Poly-A RNA Controls in Samples

Poly-A RNA Spike	Final Dilution (estimated ratio of copy number)
<i>lys</i>	1:100,000
<i>phe</i>	1:50,000
<i>thr</i>	1:25,000
<i>dap</i>	1:6,667

The controls are then amplified and labeled together with the samples. Examining the hybridization intensities of these controls on GeneChip arrays helps to monitor the labeling process independently from the quality of the starting RNA samples. Anticipated relative signal strength follows the order of *lys* < *phe* < *thr* < *dap*.

The Poly-A RNA Control Stock and Poly-A Control Dil Buffer are provided with the kit to prepare the appropriate serial dilutions based

<sup>1</sup> 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.

on [Table 2.3](#). 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 dilution of the spike-in controls in the samples.

**IMPORTANT** 

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

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**Table 2.3**  
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

**TIP** 

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

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**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** 

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

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## 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 2.4**

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 2.5**  
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 25).
  - 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 2.4](#) and [Table 2.5](#)).
  - 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 2.6](#), is for a single reaction.

**Table 2.6**  
Preparation of First-Strand Master Mix

Component	Volume
5X 1 <sup>st</sup> Strand Reaction Mix	4 $\mu$ L
DTT, 0.1M	2 $\mu$ L
dNTP, 10 mM	1 $\mu$ L
Total Volume	7 $\mu$ L

- 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.
- Transfer 7  $\mu$ L of **First-Strand Master Mix** to each RNA/T7-Oligo(dT) Primer mix for a final volume of 18 or 19  $\mu$ L. 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.
  - Incubate for 2 minutes at 42°C.
  - Add the appropriate amount of **SuperScript II** to each RNA sample for a final volume of 20  $\mu$ L.
    - For 1 to 8  $\mu$ g of total RNA: 1  $\mu$ L **SuperScript II**
    - For 8.1 to 15  $\mu$ g of total RNA: 2  $\mu$ L **SuperScript II**
    - For every  $\mu$ g of mRNA add 1  $\mu$ L **SuperScript II**.
    - For mRNA quantity less than 1  $\mu$ g, use 1  $\mu$ L **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.
  - 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 2.7](#), is for a single reaction.

**Table 2.7**

Preparation of Second-Strand Master Mix

Component	Volume
RNase-free Water	91 $\mu$ L
5X 2 <sup>nd</sup> Strand Reaction Mix	30 $\mu$ L
dNTP, 10 mM	3 $\mu$ L
<i>E. coli</i> DNA ligase	1 $\mu$ L
<i>E. coli</i> DNA Polymerase I	4 $\mu$ L
RNase H	1 $\mu$ L
Total Volume	130 $\mu$ L

- 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  $\mu\text{L}$  of Second-Strand Master Mix to each first-strand synthesis sample from *Step 2: First-Strand cDNA Synthesis* for a total volume of 150  $\mu\text{L}$ .

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  $\mu\text{L}$  of T4 DNA Polymerase to each sample and incubate for 5 minutes at 16°C.
5. After incubation with T4 DNA Polymerase add 10  $\mu\text{L}$  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 48.  
Do not leave the reactions at 4°C for long periods of time.



## Two-Cycle cDNA Synthesis<sup>1</sup>

### 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 dilutions. 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 dilutions (referred to as a ratio of copy number) summarized below:

**Table 2.8**  
Final Dilutions of Poly-A RNA Controls in Samples

Poly-A RNA Spike	Final Dilution (estimated ratio of copy number)
lys	1:100,000
phe	1:50,000
thr	1:25,000
dap	1:6,667

The controls are then amplified and labeled together with the samples. Examining the hybridization intensities of these controls on GeneChip arrays helps to monitor the labeling process independently from the quality of the starting RNA samples. Anticipated relative signal strength follows the order of *lys* < *phe* < *thr* < *dap*.

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 2.9](#). This is a guideline when 10, 50, or 100 ng of total RNA

<sup>1</sup> 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.

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 vll. 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 2.9**  
Serial Dilutions of Poly-A RNA Control Stock

Starting Amount of Total RNA	Serial Dilutions				Volume to Add into 50 $\mu$ M T7-Oligo(dT) Primer
	First	Second	Third	Fourth	
10 ng	1:20	1:50	1:50	1:10	2 $\mu$ L
50 ng	1:20	1:50	1:50	1:2	2 $\mu$ L
100 ng	1:20	1:50	1:50		2 $\mu$ L

**TIP** 

Avoid pipetting solutions less than 2  $\mu$ L 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  $\mu\text{L}$  of the Poly-A Control Stock to 38  $\mu\text{L}$  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  $\mu\text{L}$  of the First Dilution to 98  $\mu\text{L}$  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  $\mu\text{L}$  of the Second Dilution to 98  $\mu\text{L}$  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  $\mu\text{L}$  of the Third Dilution to 18  $\mu\text{L}$  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  $-20^{\circ}\text{C}$  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  $\mu\text{M}$  to 5  $\mu\text{M}$ . 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 2.10**  
Preparation of T7-Oligo(dT) Primer/Poly-A Controls Mix

Component	Volume
T7-Oligo(dT) Primer, 50 $\mu\text{M}$	2 $\mu\text{L}$
Diluted Poly-A RNA controls (Table 2.9)	2 $\mu\text{L}$
RNase-free Water	16 $\mu\text{L}$
Total Volume	20 $\mu\text{L}$

**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 2.11**  
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 33).
- 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 2.12](#), is for a single reaction.

**Table 2.12**  
Preparation of First-Cycle, First-Strand Master Mix

Component	Volume
5X 1 <sup>st</sup> Strand Reaction Mix	2.0 $\mu$ L
DTT, 0.1M	1.0 $\mu$ L
RNase Inhibitor	0.5 $\mu$ L
dNTP, 10 mM	0.5 $\mu$ L
SuperScript II	1.0 $\mu$ L
Total Volume	5.0 $\mu$ L

- 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  $\mu$ L of **First-Cycle, First-Strand Master Mix** to each total RNA sample/T7-Oligo(dT) Primer/Poly-A Controls Mix (as in [Table 2.11](#)) from the previous step for a final volume of 10  $\mu$ L. 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 38.

**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.

#### TIP

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 2.13](#), is for a single reaction.

**Table 2.13**  
Preparation of First-Cycle, Second-Strand Master Mix

Component	Volume
RNase-free Water	4.8 µL
Freshly diluted MgCl <sub>2</sub> , 17.5 mM*	4.0 µL
dNTP, 10 mM	0.4 µL
<i>E. coli</i> DNA Polymerase I	0.6 µL
RNase H	0.2 µL
Total Volume	10.0 µL

\* Make a fresh dilution of the MgCl<sub>2</sub> each time. Mix 2 µL of MgCl<sub>2</sub>, 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  $\mu\text{L}$  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  $\mu\text{L}$ .  
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 40.

**NOTE** 

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**No cDNA cleanup is required at this step.**

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**STEP 4: FIRST-CYCLE, IVT AMPLIFICATION OF cRNA**

MEGAscript® T7 Kit (purchased separately from 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 2.14](#), is for a single reaction.

**Table 2.14**  
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 38 for a final volume of 50  $\mu$ L.

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 42. Alternatively, samples may be stored at -20°C for later use.

## STEP 5: FIRST-CYCLE, CLEANUP OF cRNA

IVT cRNA Cleanup Kit 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® IVT cRNA Cleanup Kit.

### IMPORTANT !

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#### 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  $\geq 8,000 \times g$  ( $\geq 10,000$  rpm). Discard flow-through and Collection Tube.
5. Transfer the spin column into a new **2 mL Collection Tube** (supplied). Pipet 500 µL **IVT cRNA Wash Buffer** onto the spin column. Centrifuge for 15 seconds at  $\geq 8,000 \times g$  ( $\geq 10,000$  rpm) to wash. Discard flow-through.

**NOTE** 

---

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

---

6. Pipet 500  $\mu\text{L}$  80% (v/v) ethanol onto the spin column and centrifuge for 15 seconds at  $\geq 8,000 \times g$  ( $\geq 10,000$  rpm). Discard flow-through.
7. Open the cap of the spin column and centrifuge for 5 minutes at maximum speed ( $\leq 25,000 \times g$ ). Discard flow-through and Collection Tube.

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

**TIP** 

---

**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  $\mu\text{L}$  of RNase-free Water directly onto the spin column membrane. Ensure that the water is dispensed directly onto the membrane. Centrifuge 1 minute at maximum speed ( $\leq 25,000 \times g$ ) to elute. The average volume of eluate is 11  $\mu\text{L}$  from 13  $\mu\text{L}$  RNase-free Water.

9. To determine cRNA yield for samples starting with 50 ng or higher, remove 2  $\mu\text{L}$  of the cRNA, and add 78  $\mu\text{L}$  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*.

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  $-20^{\circ}\text{C}$  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 43 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 2.15](#), is for a single reaction.

**Table 2.15**  
Preparation of Second-Cycle, First-Strand Master Mix

Component	Volume
5X 1 <sup>st</sup> Strand Reaction Mix	4 $\mu$ L
DTT, 0.1M	2 $\mu$ L
RNase Inhibitor	1 $\mu$ L
dNTP, 10 mM	1 $\mu$ L
SuperScript II	1 $\mu$ L
Total Volume	9 $\mu$ L

- 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.
- Transfer 9  $\mu$ L of **Second-Cycle, First-Strand Master Mix** to each cRNA/random primer sample from [Step 6: Second-Cycle, First-Strand CDNA Synthesis, substep 1 on page 44](#), for a final volume of 20  $\mu$ L.  
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.
  - 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.
  - Add 1  $\mu$ L of **RNase H** to each sample for a final volume of 21  $\mu$ L.  
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.
  - 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 46](#).

## 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 45](#) 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.

---

**TIP** 

---

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 2.16](#), is for a single reaction.

**Table 2.16**  
Preparation of Second-Cycle, Second-Strand Master Mix

Component	Volume
RNase-free Water	88 $\mu$ L
5X 2 <sup>nd</sup> Strand Reaction Mix	30 $\mu$ L
dNTP, 10 mM	3 $\mu$ L
<i>E. coli</i> DNA Polymerase I	4 $\mu$ L
Total Volume	125 $\mu$ L

- 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  $\mu$ L of the **Second-Cycle, Second-Strand Master Mix** to each sample from [Step 7: Second-Cycle, Second-Strand cDNA Synthesis, substep 1 on page 46](#), for a total volume of 150  $\mu$ L. 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  $\mu$ L of **T4 DNA Polymerase** to the samples for a final volume of 152  $\mu$ L. 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 48](#). 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  $\mu$ L 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

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

---

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



(supplied). Pipet 750  $\mu\text{L}$  of the **cDNA Wash Buffer** onto the spin column. Centrifuge for 1 minute at  $\geq 8,000 \times g$  ( $\geq 10,000$  rpm). Discard flow-through.

**NOTE** 

**cDNA Wash Buffer is supplied as a concentrate. Ensure that ethanol is added to the cDNA Wash Buffer before use (see IMPORTANT note on page 48 before starting).**

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

**TIP** 

**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  $\mu\text{L}$  of **cDNA Elution Buffer** directly onto the spin column membrane. Incubate for 1 minute at room temperature and centrifuge 1 minute at maximum speed ( $\leq 25,000 \times g$ ) to elute. Ensure that the cDNA Elution Buffer is dispensed directly onto the membrane. The average volume of eluate is 12  $\mu\text{L}$  recovered from the 14  $\mu\text{L}$  of Elution Buffer.

**NOTE** 

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

**We do not recommend gel analysis or spectrophotometric quantitation for cDNA prepared from total RNA. This is due to the presence of other nucleic acid species in the sample that can interfere with the results.**

8. After cleanup, please proceed to *Synthesis of Biotin-Labeled cRNA for Both the One-Cycle and Two-Cycle Target Labeling Assays* on page 50.

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

GeneChip IVT Labeling Kit is used for this step.

### NOTE

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

### IMPORTANT

Store all reagents in a  $-20^{\circ}\text{C}$  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 2.17**  
IVT Reaction Set Up

Starting Material	Volume of cDNA to use in IVT
<b>Total RNA</b>	
10 to 100 ng	all (~12 $\mu\text{L}$ )
1.0 to 8.0 $\mu\text{g}$	all (~12 $\mu\text{L}$ )
8.1 to 15 $\mu\text{g}$	6 $\mu\text{L}$
<b>mRNA</b>	
0.2 to 0.5 $\mu\text{g}$	all (~12 $\mu\text{L}$ )
0.6 to 1.0 $\mu\text{g}$	9 $\mu\text{L}$
1 to 2.0 $\mu\text{g}$	6 $\mu\text{L}$

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 2.18**

Component	Volume
Template cDNA*	variable (see <a href="#">Table 2.17</a> )
RNase-free Water	variable (to give a final reaction volume of 40 $\mu$ L)
10X IVT Labeling Buffer	4 $\mu$ L
IVT Labeling NTP Mix	12 $\mu$ L
IVT Labeling Enzyme Mix	4 $\mu$ L
Total Volume	40 $\mu$ L

\*0.5 to 1  $\mu$ g 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.

- Carefully mix the reagents and collect the mixture at the bottom of the tube by brief (~5 seconds) microcentrifugation.
- 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  $\mu$ L (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.

- 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 52.

## 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 mixture, and mix well by pipetting. Do not centrifuge.

4. Apply sample (700  $\mu$ L) to the **IVT cRNA Cleanup Spin Column** sitting in a **2 mL Collection Tube**. Centrifuge for 15 seconds at  $\geq 8,000 \times g$  ( $\geq 10,000$  rpm). Discard flow-through and Collection Tube.
5. Transfer the spin column into a new **2 mL Collection Tube** (supplied). Pipet 500  $\mu$ L **IVT cRNA Wash Buffer** onto the spin column. Centrifuge for 15 seconds at  $\geq 8,000 \times g$  ( $\geq 10,000$  rpm) to wash. Discard flow-through.

**NOTE** 

**IVT cRNA Wash Buffer is supplied as a concentrate. Ensure that ethanol is added to the IVT cRNA Wash Buffer before use (see IMPORTANT note on page 52 before starting).**

6. Pipet 500  $\mu$ L 80% (v/v) ethanol onto the spin column and centrifuge for 15 seconds at  $\geq 8,000 \times g$  ( $\geq 10,000$  rpm). Discard flow-through.
7. Open the cap of the spin column and centrifuge for 5 minutes at maximum speed ( $\leq 25,000 \times g$ ). Discard flow-through and Collection Tube.

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

**TIP** 

**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  $\mu$ L of **RNase-free Water** directly onto the spin column membrane. Ensure that the water is dispensed directly onto the membrane. Centrifuge 1 minute at maximum speed ( $\leq 25,000 \times g$ ) to elute.
9. Pipet 10  $\mu$ L of **RNase-free Water** directly onto the spin column membrane. Ensure that the water is dispensed directly onto the membrane. Centrifuge 1 minute at maximum speed ( $\leq 25,000 \times g$ ) to elute.

10. For subsequent photometric quantification of the purified cRNA, we recommend dilution of the eluate between 1:100 fold and 1:200 fold.
11. Store cRNA at  $-20^{\circ}\text{C}$ , or  $-70^{\circ}\text{C}$  if not quantitated immediately. Alternatively, proceed to [Step 2: Quantification of the cRNA](#).

## STEP 2: QUANTIFICATION OF THE cRNA

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

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

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

$$\begin{aligned}\text{adjusted cRNA yield} &= \text{RNA}_{\text{m}} - (\text{total RNA}_{\text{i}}) (y) \\ \text{RNA}_{\text{m}} &= \text{amount of cRNA measured after IVT } (\mu\text{g}) \\ \text{total RNA}_{\text{i}} &= \text{starting amount of total RNA } (\mu\text{g}) \\ y &= \text{fraction of cDNA reaction used in IVT}\end{aligned}$$

**Example:** Starting with 10  $\mu\text{g}$  total RNA, 50% of the cDNA reaction is added to the IVT, giving a yield of 50  $\mu\text{g}$  cRNA. Therefore, adjusted cRNA yield = 50  $\mu\text{g}$  cRNA - (10  $\mu\text{g}$  total RNA) (0.5 cDNA reaction) = 45.0  $\mu\text{g}$ .

Use adjusted yield in [Fragmenting the cRNA for Target Preparation on page 57](#).

---

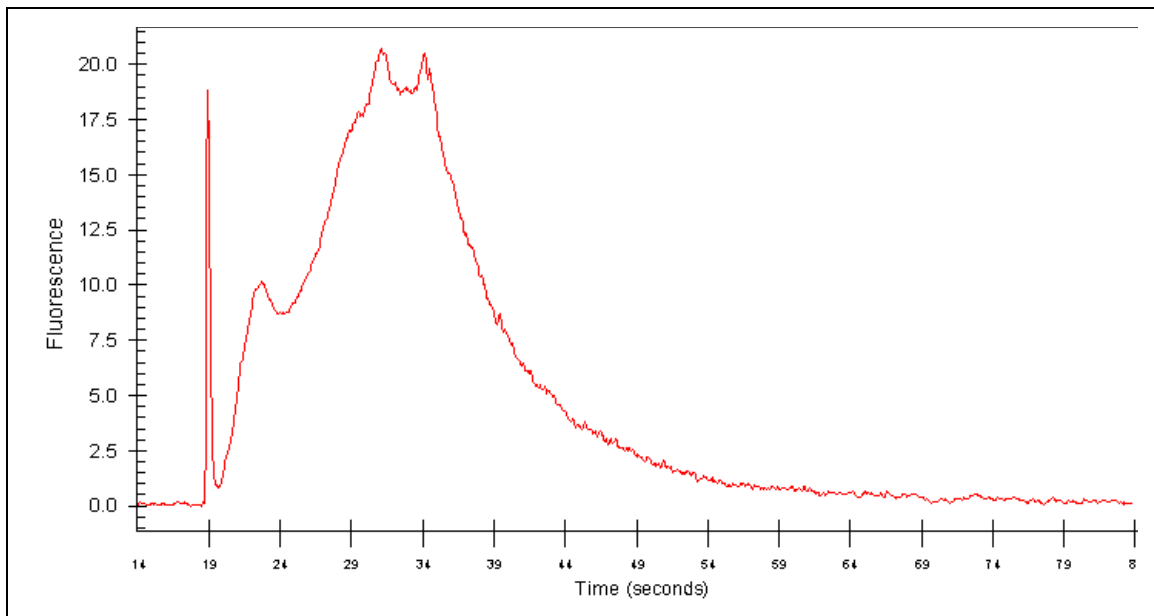
### NOTE

Please refer to [Chapter 3 Eukaryotic Target Hybridization](#) 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.

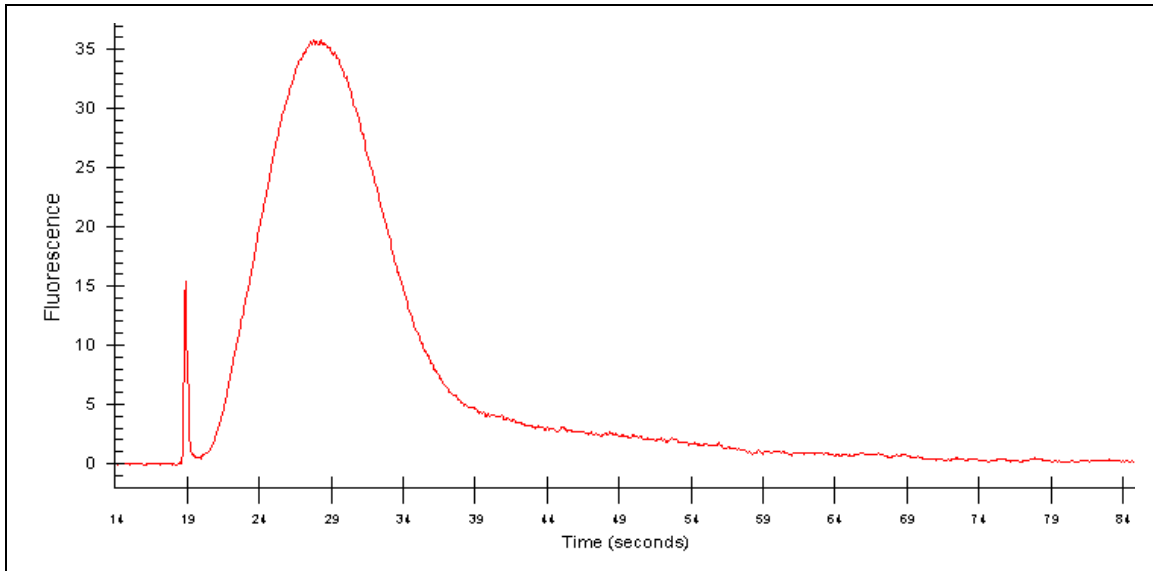
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### STEP 3: CHECKING UNFRAGMENTED SAMPLES BY GEL ELECTROPHORESIS

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 2.3** 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 2.4**

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



## Fragmenting the cRNA for Target Preparation

Sample Cleanup Module is used for this step.

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

Affymetrix recommends that the cRNA used in the fragmentation procedure be sufficiently concentrated to maintain a small volume during the procedure. This will minimize the amount of magnesium in the final hybridization cocktail. Fragment an appropriate amount of cRNA for hybridization cocktail preparation and gel analysis (refer to [Chapter 3, Eukaryotic Target Hybridization](#)).

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 54](#). The total volume of the reaction may be scaled up or down dependent on the amount of cRNA to be fragmented.

**Table 2.19**  
Sample Fragmentation Reaction by Array Format\*

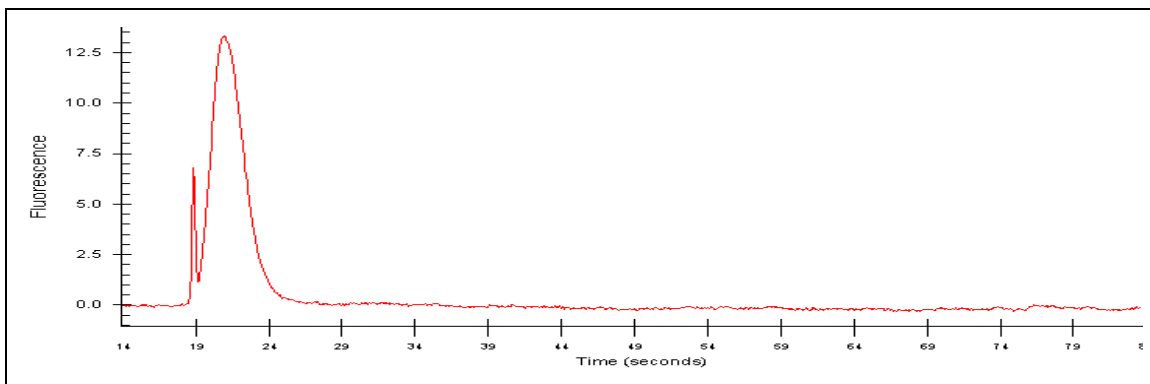
Component	49/64 Format	100 Format
cRNA	20 µg (1 to 21 µL)	15 µg (1 to 21 µL)
5X Fragmentation Buffer	8 µL	6 µL
RNase-free Water (variable)	to 40 µL final volume	to 30 µL final volume
Total Volume	40 µL	30 µL

\*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 2.5](#).

The standard fragmentation procedure should produce a distribution of RNA fragment sizes from approximately 35 to 200 bases.

4. Store undiluted, fragmented sample cRNA at  $-20^{\circ}\text{C}$  (or  $-70^{\circ}\text{C}$  for longer-term storage) until ready to perform the hybridization, as described in [Chapter 3, \*Eukaryotic Target Hybridization\*](#).



**Figure 2.5**

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.

Chapter 3

**Eukaryotic Target Hybridization**

Chapter 3



## Chapter Contents

Reagents and Materials Required . . . . .	62
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### **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 the *GeneChip® Expression Wash, Stain and Scan User Manual*, P/N 702731.

## Reagents and Materials Required

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

- GeneChip® Hybridization, Wash and Stain Kit: Affymetrix, P/N 900720 (30 reactions)
  - Hybridization Module from Box 1:
    - Pre-Hybridization Mix
    - 2X Hybridization Mix
    - DMSO
    - Nuclease-free Water
- GeneChip Eukaryotic Hybridization Control Kit: [Affymetrix](#), P/N 900454 (30 reactions) or P/N 900457 (150 reactions), both contain Control cRNA and Control Oligo B2
- Control Oligo B2, 3 nM: [Affymetrix](#), P/N 900301

### 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

## Eukaryotic Target Hybridization

Please refer to the table below for the necessary amount of cRNA required for the specific probe array format used. These preparations take into account that it is necessary to make extra hybridization cocktail due to a small loss of volume (10-20  $\mu\text{L}$ ) during each hybridization.

1. Mix the following for each target, scaling up volumes if necessary 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.

### NOTE S

DMSO will solidify when stored at 4°C. Please ensure that the reagent is completely thawed prior to use. After the first use, it is recommended to store DMSO at room temperature.

**Table 3.1**  
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 Dilution
Fragmented and Labeled cRNA <sup>†</sup>	15 $\mu\text{g}$	10 $\mu\text{g}$	5 $\mu\text{g}$	0.05 $\mu\text{g}/\mu\text{L}$
Control Oligonucleotide B2 (3 nM)	5 $\mu\text{L}$	3.3 $\mu\text{L}$	1.7 $\mu\text{L}$	50 pM
20X Eukaryotic Hybridization Controls ( <i>bioB</i> , <i>bioC</i> , <i>bioD</i> , <i>cre</i> )	15 $\mu\text{L}$	10 $\mu\text{L}$	5 $\mu\text{L}$	1.5, 5, 25, and 100 pM respectively
2X Hybridization Mix	150 $\mu\text{L}$	100 $\mu\text{L}$	50 $\mu\text{L}$	1X
DMSO	30 $\mu\text{L}$	20 $\mu\text{L}$	10 $\mu\text{L}$	10%
Nuclease-free Water	to final volume of 300 $\mu\text{L}$	to final volume of 200 $\mu\text{L}$	to final volume of 100 $\mu\text{L}$	
Total Volume	300 $\mu\text{L}$	200 $\mu\text{L}$	100 $\mu\text{L}$	

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

<sup>†</sup>Please see [Chapter 2](#) for the amount of adjusted fragmented cRNA to use when starting from total RNA.

### 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 aliquoting.

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 with an appropriate volume of Pre-Hybridization Mix (see [Table 3.2](#)) by filling it through one of the septa.

**Table 3.2**

Probe Array Cartridge Volumes for Pre-Hybridization Mix and Hybridization Cocktail

Array	Volume
49 Format (Standard)	200 µL
64 Format	200 µL
100 Format (Midi)	130 µL
169 Format (Mini)	80 µL
400 Format (Micro)	80 µL

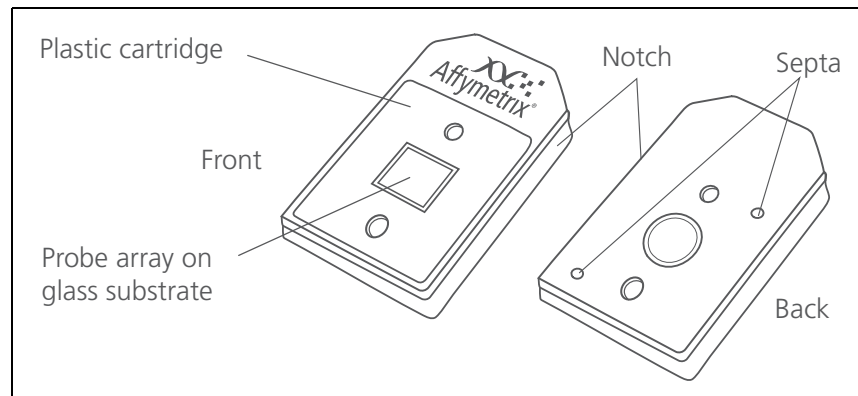
**NOTE** 

---

Each array has two septa (see [Figure 3.1](#) for location of the probe array septa). In order to fill the array, first vent the array chamber by inserting a clean, unused pipette tip into one of the septa; then insert the pipette tip of a micropipettor into the remaining septum to fill.

---





**Figure 3.1**  
GeneChip® Probe Array

5. Incubate the probe array filled with Pre-Hybridization Mix at 45°C for 10 minutes with rotation.
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 the hybridization cocktail at maximum speed in a microcentrifuge for 5 minutes to collect any insoluble material from the hybridization mixture.
8. Remove the array from the hybridization oven. Vent the array with a clean pipette tip and extract the Pre-Hybridization Mix from the array with a micropipettor. Refill the array with the appropriate volume of the clarified hybridization cocktail, avoiding any insoluble matter at the bottom of the tube (see [Table 3.2](#)).
9. Place probe array into the hybridization oven, set to 45°C.
10. To avoid stress to the motor, load probe arrays in a balanced configuration around the axis. Rotate at 60 rpm.
11. Hybridize for 16 hours.

During the latter part of the 16-hour hybridization, proceed to the *GeneChip® Expression Wash, Stain and Scan User Manual*, P/N 702731, to prepare reagents for the washing and staining steps required immediately after completion of hybridization.



Chapter 4

## Prokaryotic Target Preparation

Chapter 4



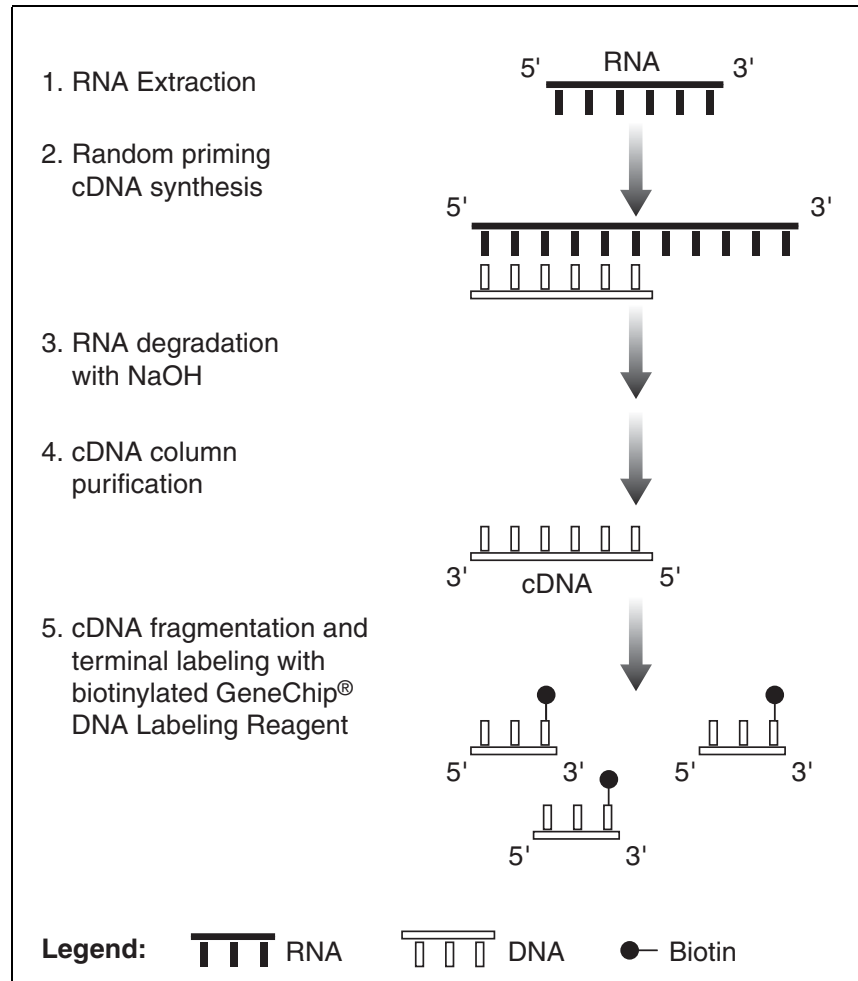
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### **This Chapter Contains:**

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

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



**Figure 4.1**  
Target Labeling for Prokaryotic GeneChip® Antisense Arrays

## Reagents and Materials Required

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

### Labeling

- dNTP: Invitrogen Life Technologies, P/N 18427-013
- Random Primers, 3 µg/µL: Invitrogen Life Technologies, P/N 48190-011
- GeneChip® Eukaryotic Poly-A RNA Control Kit: Affymetrix, P/N 900433
- SuperScript II™ Reverse Transcriptase: Invitrogen Life Technologies, P/N 18064-071
- SUPERase•In™: Ambion, P/N 2696
- Nuclease-free Water: Ambion, P/N 9930
- NaOH, 1N solution: VWR Scientific Products, P/N MK469360
- HCl, 1N solution: VWR Scientific Products, P/N MK638860
- MinElute PCR Purification Kit: QIAGEN, P/N 28004
- 10X DNase I Buffer: USB, P/N 78331
- Deoxyribonuclease I (DNase I): Pierce, P/N 89835
- GeneChip® DNA Labeling Reagent: Affymetrix, P/N 900542
- Terminal Deoxynucleotidyl Transferase: Promega, P/N M1875
- EDTA, 0.5M, pH 8.0: Invitrogen Life Technologies, P/N 15575-020
- Non-stick RNase-free microfuge tubes, 0.5 mL and 1.5 mL: Ambion, P/N 12350 and P/N 12450, respectively

### Gel-Shift Assay

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

## Reagent Preparation

### 75 ng/μL Random Primers

For 1000 μL:

25 μL of 3 μg/μL Random Primers

975 μL of Nuclease-free H<sub>2</sub>O

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

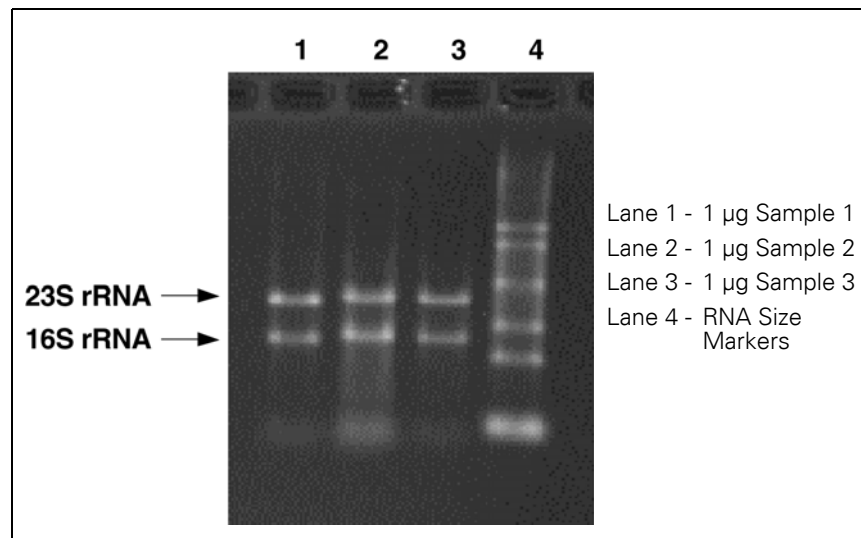
As starting material for the cDNA synthesis procedure, total RNA can be isolated by using standard procedures for bacterial RNA isolation or various commercial RNA isolation kits.

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



$1 \times 10^9$  cells are applied to a single purification column. Also, use the lysozyme at a concentration of 1 mg/mL, and not the recommended 400  $\mu\text{g/mL}$ . 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  $\mu\text{g/mL}$  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 4.2**  
Typical RNA preparation from *E. coli*

## cDNA Synthesis

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

### NOTE

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

### STEP 1: PREPARATION OF POLY-A RNA CONTROLS

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

**Table 4.1**  
Serial Dilutions of Poly-A RNA Control Stock

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

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

### TIP

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

The Poly-A RNA Control Stock contains *in vitro* synthesized, polyadenylated transcripts for *B. subtilis* genes that are pre-mixed at staggered concentrations. The concentrations of the spikes in the stock solution are: *lys* 7.6 nM, *phe* 15.2 nM, *thr* 30.4 nM, and *dap* 114.0 nM. Following the recommended dilutions as shown above, the final concentrations of the spikes in the hybridization cocktail (Table 5.1 on page 86) are *lys* 0.256 pM, *phe* 0.511 pM, *thr* 1.022 pM, and *dap* 3.833 pM.

### NOTE

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

---

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

**IMPORTANT** 

---

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

---

- A.** Add 2  $\mu\text{L}$  of the Poly-A RNA Control Stock to 38  $\mu\text{L}$  of Poly-A Control Dil Buffer for the First Dilution (1:20).
- B.** Mix thoroughly and spin down to collect the liquid at the bottom of the tube.
- C.** Add 2  $\mu\text{L}$  of the First Dilution to 38  $\mu\text{L}$  of Poly-A Control Dil Buffer to prepare the Second Dilution (1:20).
- D.** Mix thoroughly and spin down to collect the liquid at the bottom of the tube.
- E.** Add 2  $\mu\text{L}$  of this Second Dilution to the total RNA as indicated in [Table 4.2](#).

**NOTE** 

---

**The First Dilution of the poly-A RNA controls can be stored up to six weeks in a non-frost-free freezer at  $-20^{\circ}\text{C}$  and frozen-thawed up to eight times.**

---

## STEP 2: cDNA SYNTHESIS

1. Prepare the following mixture for primer annealing:

**Table 4.2**  
RNA/Primer Hybridization Mix

Component	Volume	Final Dilution
Total RNA	10 µg	0.33 µg/µL
75 ng/µL Random Primers	10 µL	25 ng/µL
Diluted poly-A RNA controls	2 µL	Variable
Nuclease-free H <sub>2</sub> O	Up to 30.0 µL	—
Total Volume	30 µL	

**NOTE** 

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

2. Incubate the RNA/Primer mix at the following temperatures:
  - 70°C for 10 minutes
  - 25°C for 10 minutes
  - Chill to 4°C
3. Prepare the reaction mix for cDNA synthesis. Briefly centrifuge the reaction tube to collect sample at the bottom and add the cDNA synthesis mix from [Table 4.3](#) to the RNA/primer hybridization mix.

**Table 4.3**  
cDNA Synthesis Components

Component	Volume	Final Dilution
RNA/Primer hybridization mix (from previous step)	30 $\mu$ L	
5X 1 <sup>st</sup> Strand Buffer	12 $\mu$ L	1X
100 mM DTT	6 $\mu$ L	10 mM
10 mM dNTPs	3 $\mu$ L	0.5 mM
SUPERase <sup>•</sup> In (20 U/ $\mu$ L)	1.5 $\mu$ L	0.5 U/ $\mu$ L
SuperScript II (200 U/ $\mu$ L)	7.5 $\mu$ L	25 U/ $\mu$ L
Total Volume	60 $\mu$ L	

4. Incubate the reaction at the following temperatures:

- 25°C for 10 minutes
- 37°C for 60 minutes
- 42°C for 60 minutes
- Inactivate SuperScript II at 70°C for 10 minutes
- Chill to 4°C

### STEP 3: REMOVAL OF RNA

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

#### **STEP 4: PURIFICATION AND QUANTITATION OF cDNA**

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

**NOTE** 

---

**Typical yields of cDNA are 3 to 7  $\mu\text{g}$ . A minimum of 1.5  $\mu\text{g}$  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 4.4**  
Fragmentation Reaction

Component	Volume	Dilution
10X DNase I Buffer	2 $\mu$ L	1X
cDNA	10 $\mu$ L	–
DNase I (see note below)	X $\mu$ L	0.6 U/ $\mu$ g of cDNA
Nuclease-free H <sub>2</sub> O	Up to 20 $\mu$ L	–
Total Volume	20 $\mu$ L	

**NOTE** 

Use all remaining cDNA purified from the previous step in this reaction. Do not proceed if the yield is lower than 1.5  $\mu$ g. Dilute DNase I to 0.6 U/ $\mu$ L 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  $\mu$ g of cDNA) to be used in the fragmentation reaction. 0.6U for each  $\mu$ g of cDNA can be used as a starting point for the titration.

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

**NOTE** 

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

## Terminal Labeling

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

1. Prepare the following reaction mix:

**Table 4.5**  
Terminal Label Reaction

Component	Volume
5X Reaction Buffer	10 µL
GeneChip DNA Labeling Reagent, 7.5 mM	2 µL
Terminal Deoxynucleotidyl Transferase	2 µL
Fragmentation cDNA Product	Up to 20 µL
H <sub>2</sub> O	16 µL
Total Volume	50 µL

2. Incubate the reaction at 37°C for 60 minutes.
3. Stop the reaction by adding 2 µL of 0.5M EDTA.
4. The target is ready to be hybridized onto probe arrays, as described in *Prokaryotic Target Hybridization* on page 83. Alternatively, it may be stored at –20°C for later use.

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

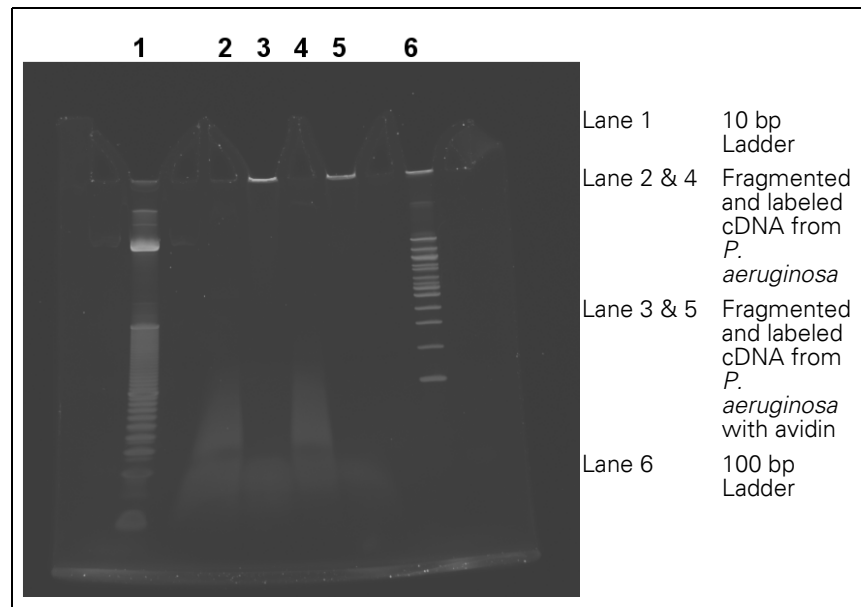
### Gel-Shift Assay

The efficiency of the labeling procedure can be assessed using the following procedure. This quality control protocol prevents hybridizing poorly labeled target onto the probe array. The addition of biotin residues is monitored in a gel-shift assay, where the fragments are incubated with avidin prior to electrophoresis. The nucleic acids are then detected by staining, as shown in the gel photograph [Figure 4.3](#). 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.





**Figure 4.3**  
Gel-shift for monitoring *P. aeruginosa* target labeling efficiency. Notice that the majority of the avidin-conjugated product, in both lanes 3 and 5, is highlighted as a bright band towards the top of the gel.

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

10. While the gel is running, prepare at least 100 mL of a 1X solution of SYBR Gold for staining.

**NOTE** 

---

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

---

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

Chapter 5



**Prokaryotic Target Hybridization**

Chapter 5



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### **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* arrays. The hybridized probe array is then ready for washing, staining, and scanning as detailed in [Chapter 6](#).

## Reagents and Materials Required

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

- GeneChip Hybridization, Wash and Stain Kit: Affymetrix P/N 900720 (30 reactions)
  - Hybridization Module, Box 1
    - 2X Hybridization Mix
    - DMSO
    - Nuclease-free Water
- Control Oligo B2, 3 nM: Affymetrix, P/N 900301

### **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

## Prokaryotic Target Hybridization

After determining that the fragmented cDNA is labeled with biotin, prepare the hybridization cocktail. 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 cocktail.

**NOTE** 

**DMSO will solidify when stored at 4°C. Please ensure that the reagent is completely thawed prior to use. After the first use, it is recommended to store DMSO at room temperature.**

**Table 5.1**  
Hybridization Cocktail for Single Probe Array\*

Component	49 Format (Standard)	100 Format (Midi)	169 Format (Mini)	Final Dilution or Amount
Fragmented and Labeled cDNA	Up to 50 µL	Up to 50 µL	25 µL	0.5 – 7.0 µg
Control Oligonucleotide B2 (3 nM)	3.3 µL	2.2 µL	1.3 µL	50 pM
2X Hybridization Mix	100 µL	65 µL	40 µL	1X
DMSO	–	10.2 µL	6.2 µL	7.8% (or 0%)
Nuclease-free Water	to a final volume of 200 µL	to a final volume of 130 µL	7.5 µL	
Total Volume	200 µL	130 µL	80 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 equilibrate 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. Based on the format of the array type used, refer to [Table 5.2](#) to add the appropriate volume of hybridization cocktail.

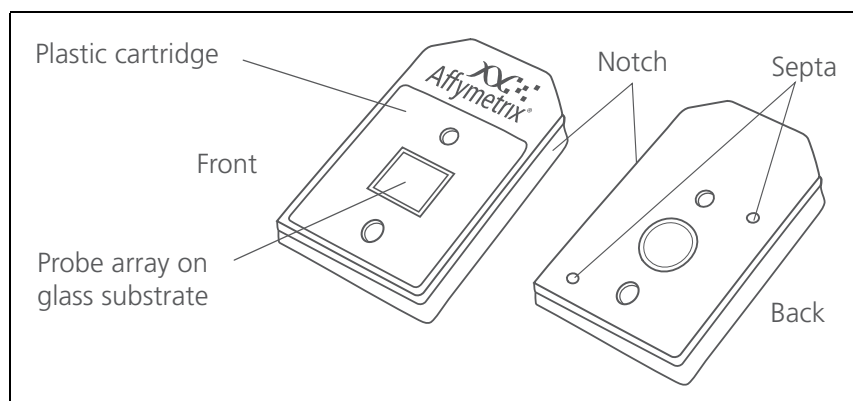
**Table 5.2**

Probe Array Cartridge Volumes for Hybridization Cocktail

Array	Volume
49 Format (Standard)	200 $\mu\text{L}$
100 Format (Midi)	130 $\mu\text{L}$
169 Format (Mini)	80 $\mu\text{L}$

**NOTE**

Each array has two septa (see [Figure 5.1](#) for location of the probe array septa). In order to fill the array, first vent the array chamber by inserting a clean, unused pipette tip into one of the septa; then insert the pipette tip of a micropipettor into the remaining septum to fill.



**Figure 5.1**  
GeneChip<sup>®</sup> Probe Array

4. Place probe array in the hybridization oven set at the temperatures indicated below.
  - *P. aeruginosa* 50°C
  - *E. coli* 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. To 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 [Chapter 6](#) to prepare reagents for the washing and staining steps required immediately after completion of hybridization.



Chapter 6

**Prokaryotic Arrays:  
Washing, Staining and Scanning**

Chapter 6



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### 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* 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 have been tested and evaluated by Affymetrix scientists. Information and part numbers listed are based on U.S. catalog information. For supplier information, please refer to the Supplier Reference List in [Appendix G](#) of this manual.

- GeneChip® Hybridization, Wash and Stain Kit: Affymetrix, P/N 900720 (30 reactions)
  - Stain Module, Box 1
    - Stain Cocktail 1
    - Stain Cocktail 2
    - Array Holding Buffer
  - Wash Buffers A and B, Box 2
    - Wash Buffer A (P/N 900721)
    - Wash Buffer B (P/N 900722)
- RNase-free water

### Miscellaneous Supplies

- Fluidics Station 450: Affymetrix, P/N 00-0079
- GeneChip® Scanner 3000: Affymetrix, P/N 00-00212
- Sterile, RNase-free, microcentrifuge vials, 1.5 mL: USA Scientific, P/N 1415-2600 (or equivalent)
- Sterile, RNase-free, microcentrifuge vials, 1.5 mL, amber: USA Scientific, P/N 1615-5507 (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
- Media Bottle, SQ, 1,000 mL: Affymetrix, P/N 400119
- Media Bottle, SQ, 500 mL (set of 3): Affymetrix, P/N 400118
- Bottle Cap, Pre-Drilled (set of 4): Affymetrix, P/N 400137

## Experiment and Fluidics Station Setup

### STEP 1: DEFINING FILE LOCATIONS

Before working with Affymetrix® Microarray Suite it is important to define where the program will store and look 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 *GeneChip® Operating Software User’s Guide* or *Microarray Suite User’s Guide*.

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

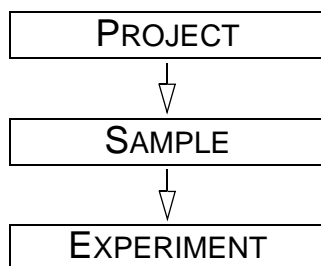
- Experiment Name
- Probe Array Type

In GCOS, three additional fields are required:

- Sample Name
- Sample Type
- Project

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

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



## STEP 3: PREPARING THE FLUIDICS STATION

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

### Setting Up the Fluidics Station

1. Turn on the Fluidics Station using the switch on the lower left side of the machine.
2. Select **Run** → **Fluidics** from the menu bar.

The Fluidics Station dialog box appears with a drop-down list for selecting the experiment name for each of the fluidics station modules. A second list is accessed for choosing the Protocol for each of the four fluidics station modules.

#### NOTE

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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. Ensure that the designated Fluidics Station Wash A and Wash B media bottles are clean. Transfer **Wash Buffer A** and **Wash Buffer B** from the kit to the clean, empty Fluidics Station bottles.
  4. If using Microarray Suite, click **Run** for each module to begin priming. If using GCOS, select the **All Modules** check box, then click **Run**.



## Probe Array Wash and Stain

**NOTE** 

The wash and stain procedure described below is different from previous recommended protocols. Closely adhere to the following recommendations for best results.

After 16 hours of hybridization remove the array from the hybridization oven. Vent the array by inserting a clean pipette tip into one of the septa, and extract the hybridization cocktail with a pipettor through the remaining septum. Refill the probe array completely with the appropriate volume of **Wash Buffer A**, as given in [Table 6.1](#).

**Table 6.1**  
Probe Array Cartridge Volumes for Wash Buffer A and Array Holding Buffer

Array	Total Fill Volume
49 Format (Standard)	250 $\mu$ L
100 Format (Midi)	160 $\mu$ L
169 Format (Mini)	100 $\mu$ L

**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 procedure takes approximately 75 to 90 minutes to complete.

## PREPARING THE STAIN REAGENTS

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

1. Remove **Stain Cocktail 1**, **Stain Cocktail 2**, and **Array Holding Buffer** from the Stain Module, Box 1.
2. Gently tap the bottles to mix well.
3. Aliquot the following reagents:
  - A. 600  $\mu$ L of **Stain Cocktail 1** into a 1.5 mL amber microcentrifuge vial.
  - B. 600  $\mu$ L of **Stain Cocktail 2** into a 1.5 mL (clear) microcentrifuge vial.
  - C. 800  $\mu$ L of **Array Holding Buffer** into a 1.5 mL (clear) microcentrifuge vial.
4. Spin down all vials to remove the presence of any air bubbles.

**NOTE** 

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**Stain Cocktail 1 is light-sensitive. Please be sure to use amber microcentrifuge vials when aliquoting.**

---

**IMPORTANT** 

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**The 600  $\mu$ L of Stain Cocktail 1 will be used for the first and third stain. If using the Fluidics Station 400, after the first staining step is completed, save the vial with the Stain Cocktail 1 solution and reuse for the third staining step.**

---

For instructions on how to use the solutions and determining which Fluidics Script(s) fits your application, please follow the instructions for using the Fluidics Station 450/250, which can be found [on page 98](#), or the instructions for using the Fluidics Station 400, which can be found [on page 102](#).

## 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.

- 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 6.2](#) and [Table 6.3](#).

**Table 6.2**  
Fluidics Protocols for Fluidics Station 450/250

	FS450_0006	Flex FS450_0002	Modified Flex FS450_0002 for <i>P. aeruginosa</i> Array*	FS450_0005
<b>Array Format</b>	169	100	100	49
<b>Post Hyb Wash #1</b>	10 cycles of 2 mixes/ cycle with Wash Buffer A at 30°C	10 cycles of 2 mixes/ cycle with Wash Buffer A at <b>30°C</b>	10 cycles of 2 mixes/ cycle with Wash Buffer A at <b>25°C</b>	10 cycles of 2 mixes/ cycle with Wash Buffer A at 25°C
<b>Post Hyb Wash #2</b>	4 cycles of 15 mixes/ cycle with Wash Buffer B at 50°C	<b>6</b> cycles of 15 mixes/ cycle with Wash Buffer B at 50°C	<b>4</b> 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
<b>1<sup>st</sup> Stain</b>	Stain the probe array for 300 seconds with Stain Cocktail 1 at 35°C	Stain the probe array for <b>300 seconds</b> with Stain Cocktail 1 at <b>35°C</b>	Stain the probe array for <b>600 seconds</b> with Stain Cocktail 1 at <b>25°C</b>	Stain the probe array for 600 seconds with Stain Cocktail 1 at 25°C.
<b>Post Stain Wash</b>	10 cycles of 4 mixes/ cycle with Wash Buffer A at 30°C	10 cycles of 4 mixes/ cycle with Wash Buffer A at 30°C	10 cycles of 4 mixes/ cycle with Wash Buffer A at 30°C	10 cycles of 4 mixes/ cycle with Wash Buffer A at 30°C
<b>2<sup>nd</sup> Stain</b>	Stain the probe array for 300 seconds with Stain Cocktail 2 at 35°C	Stain the probe array for <b>300 seconds</b> with Stain Cocktail 2 at <b>35°C</b>	Stain the probe array for <b>600 seconds</b> with Stain Cocktail 2 at <b>25°C</b>	Stain the probe array for 600 seconds with Stain Cocktail 2 at 25°C
<b>3<sup>rd</sup> Stain</b>	Stain the probe array for 300 seconds with Stain Cocktail 1 at 35°C	Stain the probe array for <b>300 seconds</b> with Stain Cocktail 1 at <b>35°C</b>	Stain the probe array for <b>600 seconds</b> with Stain Cocktail 1 at <b>25°C</b>	Stain the probe array for 600 seconds with Stain Cocktail 1 at 25°C.
<b>Final Wash</b>	15 cycles of 4 mixes/ cycle with Wash Buffer A at 35°C	15 cycles of 4 mixes/ cycle with Wash Buffer A at <b>35°C</b>	15 cycles of 4 mixes/ cycle with Wash Buffer A at <b>30°C</b>	15 cycles of 4 mixes/ cycle with Wash Buffer A at 30°
<b>Array Holding Buffer</b>	Fill the probe array with Array Holding Buffer	Fill the probe array with Array Holding Buffer	Fill the probe array with Array Holding Buffer	Fill the probe array with Array Holding Buffer

\*Refer to [Table 6.3](#) for instructions on how to modify Flex FS450\_0002.

**Table 6.3**  
Modification of Flex FS450\_0002 for GeneChip® *P. aeruginosa* Array

GeneChip *P. aeruginosa* Genome Array requires a modification to the Flex FS450\_0002 protocol. See below for details.

The Flex FS450\_0002 fluidics protocol must be modified. Please follow the instructions carefully to make the modifications. Additionally, it is highly recommended that you save your new *P. aeruginosa* fluidics protocol under a different name to avoid confusion.

1. Modify and save the fluidics protocol for the assay:
  - a. Modify the fluidics protocol by using **Tools** → **Edit Protocol** drop-down list and selecting **Flex FS450\_0002** within the Protocol Name window.
  - b. Change the following parameters: (Enter the new parameters by highlighting the default values and typing in the new values.)
    - i) **Wash A1 Temperature** from 30°C to 25°C;
    - ii) **Number of Wash B Cycles** from 6 to 4;
    - iii) **Stain Temperature (C)** from 35°C to 25°C;
    - iv) **First Stain Time (seconds)** from 300 to 600 seconds;
    - v) **Second Stain Time (seconds)** from 300 to 600 seconds;
    - vi) **Third Stain Time (seconds)** from 300 to 600 seconds; and
    - vii) **Wash A3 Temperature** from 35°C to 30°C.
  - c. Save the modified fluidics protocol by highlighting **Flex FS450\_0002** 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 450/250.

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

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

4. Insert the appropriate probe array into the designated module of the fluidics station while the cartridge lever is down, or in the eject position. When finished, verify that the cartridge lever is returned to the up, or engaged, position.
5. Remove any microcentrifuge vial(s) remaining in the sample holder of the fluidics station module(s) being used.
6. Follow the instructions on the LCD window of the fluidics station by placing 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 600  $\mu$ L **Stain Cocktail 1** in sample holder 1.
  - B. Place one vial containing 600  $\mu$ L **Stain Cocktail 2** in sample holder 2.
  - C. Place one vial containing 800  $\mu$ L of **Array Holding Buffer** in sample holder 3.
  - D. Press down on the needle lever to snap needles into position and to start the run.

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

7. When the protocol is complete, the LCD window displays the message **EJECT & INSPECT CARTRIDGE**.
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 the probe array has no large bubbles, it is ready to scan on the GeneArray<sup>®</sup> Scanner, or the GeneChip<sup>®</sup> Scanner 3000. Pull up on the cartridge lever to close the washblock and proceed to [Probe Array Scan on page 107](#).
  - If bubbles are present, do the following:

Return the probe array to the probe array holder. Follow instructions on the LCD window. 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 **Array Holding Buffer**. When it is finished, the LCD window will display **EJECT AND INSPECT CARTRIDGE**. Again, remove the probe array and

inspect it for bubbles. If no bubbles are present, it is ready to scan. Pull up on the cartridge lever to close the washblock and proceed to [Probe Array Scan on page 107](#).

If attempt to fill the probe array without bubbles is unsuccessful, the array should be filled manually with **Array Holding Buffer** using a micropipette. Excessive washing will result in a loss of signal intensity.

10. Keep the probe arrays at 4°C and in the dark until ready for scanning.
11. If there are no more samples to hybridize, shut down the fluidics station following the procedure in [Shutting Down the Fluidics Station on page 109](#).

**NOTE** 

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For proper cleaning and maintenance of the fluidics station, including the bleach protocol, refer to the appropriate *GeneChip® Fluidics Station User's Guide* for instructions.

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## 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 6.4](#) and [Table 6.5](#).

**Table 6.4**  
Fluidics Protocols for Fluidics Station 400

	Mini_prok2v1	FlexMidi_euk2v3	Modified FlexMidi_euk2v3 for <i>P. aeruginosa</i> Array*	ProkGE_WS2
<b>Array Format</b>	169	100	100	49
<b>Post Hyb Wash #1</b>	10 cycles of 2 mixes/ cycle with Wash Buffer A at 30°C	10 cycles of 2 mixes/ cycle with Wash Buffer A at <b>30°C</b>	10 cycles of 2 mixes/ cycle with Wash Buffer A at <b>25°C</b>	10 cycles of 2 mixes/ cycle with Wash Buffer A at 25°C
<b>Post Hyb Wash #2</b>	4 cycles of 15 mixes/ cycle with Wash Buffer B at 50°C	<b>6</b> cycles of 15 mixes/ cycle with Wash Buffer B at 50°C	<b>4</b> 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
<b>1<sup>st</sup> Stain</b>	Stain the probe array for 300 seconds with Stain Cocktail 1 at 35°C	Stain the probe array for <b>300 seconds</b> with Stain Cocktail 1 at <b>35°C</b>	Stain the probe array for <b>600 seconds</b> with Stain Cocktail 1 at <b>25°C</b>	Stain the probe array for 600 seconds with Stain Cocktail 1 at 25°C.
<b>Post Stain Wash</b>	10 cycles of 4 mixes/ cycle with Wash Buffer A at 30°C	10 cycles of 4 mixes/ cycle with Wash Buffer A at 30°C	10 cycles of 4 mixes/ cycle with Wash Buffer A at 30°C	10 cycles of 4 mixes/ cycle with Wash Buffer A at 30°C
<b>2<sup>nd</sup> Stain</b>	Stain the probe array for 300 seconds with Stain Cocktail 2 at 35°C	Stain the probe array for <b>300 seconds</b> with Stain Cocktail 2 at <b>35°C</b>	Stain the probe array for <b>600 seconds</b> with Stain Cocktail 2 at <b>25°C</b>	Stain the probe array for 600 seconds with Stain Cocktail 2 at 25°C
<b>3<sup>rd</sup> Stain</b>	Stain the probe array for 300 seconds with Stain Cocktail 3 at 35°C	Stain the probe array for <b>300 seconds</b> with Stain Cocktail 3 at <b>35°C</b>	Stain the probe array for <b>600 seconds</b> with Stain Cocktail 3 at <b>25°C</b>	Stain the probe array for 600 seconds with Stain Cocktail 3 at 25°C
<b>Final Wash</b>	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 <b>35°C</b> . The holding temperature is 25°C.	15 cycles of 4 mixes/ cycle with Wash Buffer A at <b>30°C</b> . The holding temperature is 25°C.	15 cycles of 4 mixes/ cycle with Wash Buffer A at 30°. The holding temperature is 25°C.
<b>Array Holding Buffer</b>	N/A - manual process	N/A - manual process	N/A - manual process	N/A - manual process

\* Refer to Table 6.5 for instructions on how to modify FlexMidi\_euk2v3.

**Table 6.5**Modification of FlexMidi\_euk2v3 for GeneChip® *P. aeruginosa* Array

GeneChip *P. aeruginosa* Genome Array requires a modification to the **FlexMidi\_euk2v3** protocol. See below for details.

The **FlexMidi\_euk2v3** fluidics protocol must be modified. Please follow the instructions carefully to make the modifications. Additionally, it is highly recommended that you save your new *P. aeruginosa* fluidics protocol under a different name to avoid confusion.

1. Modify and save the fluidics protocol for the assay:
  - a. Modify the fluidics protocol by using **Tools** → **Edit Protocol** drop-down list and selecting **FlexMidi\_euk2v3** within the Protocol Name window.
  - b. Change the following parameters: (Enter the new parameters by highlighting the default values and typing in the new values.)
    - i) **Wash A1 Temperature** from 30°C to 25°C;
    - ii) **Number of Wash B Cycles** from 6 to 4;
    - iii) **Stain Temperature (C)** from 35°C to 25°C;
    - iv) **First Stain Time (seconds)** from 300 to 600 seconds;
    - v) **Second Stain Time (seconds)** from 300 to 600 seconds;
    - vi) **Third Stain Time (seconds)** from 300 to 600 seconds; and
    - vii) **Wash A3 Temperature** from 35°C to 30°C.
  - c. Save the modified fluidics protocol by highlighting **FlexMidi\_euk2v3** 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.



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

If you are unfamiliar with inserting and removing probe arrays from the fluidics station modules, please refer to the *Fluidics Station 400 User's Guide*, *Fluidics Station 400 Video In-Service CD* (P/N 900374), or *Quick Reference Card* (P/N 08-0072).

4. Insert the appropriate probe array into the designated module of the fluidics station while the cartridge lever is in the EJECT position. When finished, verify that the cartridge lever is returned to the ENGAGE position.
5. Remove any microcentrifuge tube remaining in the sample holder of the fluidics station module(s) being used.
6. When the LCD window indicates **LOAD 1<sup>ST</sup> STAIN**, place a microcentrifuge tube containing 600  $\mu$ L of **Stain Cocktail 1** into the sample holder, making sure that the metal sampling needle is in the tube with its tip near the bottom.

The Fluidics Station dialog box and the LCD window display the status of the washing and staining as they progress.

7. When the LCD window indicates **LOAD 2<sup>ND</sup> STAIN**, replace the microcentrifuge vial containing the **Stain Cocktail 1** with a microcentrifuge vial containing **Stain Cocktail 2** into the sample holder, making sure that the metal sampling needle is in the vial with its tip near the bottom.

**IMPORTANT !**

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**Do not dispose the vial containing Stain Cocktail 1. Be sure to save the Stain Cocktail 1 vial for use in [Step 8](#).**

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8. When the LCD window indicates **LOAD 3<sup>RD</sup> STAIN**, replace the microcentrifuge vial containing **Stain Cocktail 2** with the saved microcentrifuge vial containing 600  $\mu$ L of **Stain Cocktail 1** (retained from the previous step) 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**.

9. Remove microcentrifuge vial containing stain and replace with an empty microcentrifuge tube.
10. Remove the probe arrays from the fluidics station modules by first moving the cartridge lever to the **EJECT** position.
11. Fill the probe array manually with **Array Holding Buffer** as follows:
  - A. Vent one of the septa with a clean pipette tip. Drain the **Wash Buffer A** from the remaining septum of the probe array using a micropipettor. Refill the array completely with **Array Holding Buffer**. Refer to [Table 6.1](#) for the fill volume specific to the array type used.
  - B. Inspect the array and ensure that no bubbles are present. The probe array is ready to be scanned on the GeneChip Scanner 3000 or the GeneArray Scanner.
  - C. Pull up on the cartridge lever to engage wash board and proceed to [Probe Array Scan on page 107](#).

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 109](#).

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**NOTE** 

**For proper cleaning and maintenance of the fluidics station, including the bleach protocol, refer to the appropriate *GeneChip® Fluidics Station User's Guide* for instructions.**

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## 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<sup>®</sup> Scanner, or 10 minutes if you are using the GeneChip<sup>®</sup> Scanner 3000. If the probe array was stored at 4°C, warm to room temperature before scanning. Refer to the Microarray Suite or GCOS online help and the appropriate scanner user's manual for more information on scanning.

**WARNING** 

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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.

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**NOTE** 

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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), *GeneChip Operating Software User's Guide* (P/N 701439), or to the *GeneChip<sup>®</sup> Scanner 3000 Quick Reference Card* (P/N 08-0075).

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## HANDLING THE GENECHIP<sup>®</sup> PROBE ARRAY

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

Before scanning the probe array cartridge, follow this procedure to apply Tough-Spots<sup>™</sup> to the probe array cartridge to prevent the leaking of fluids from the cartridge during scanning.

**IMPORTANT** 

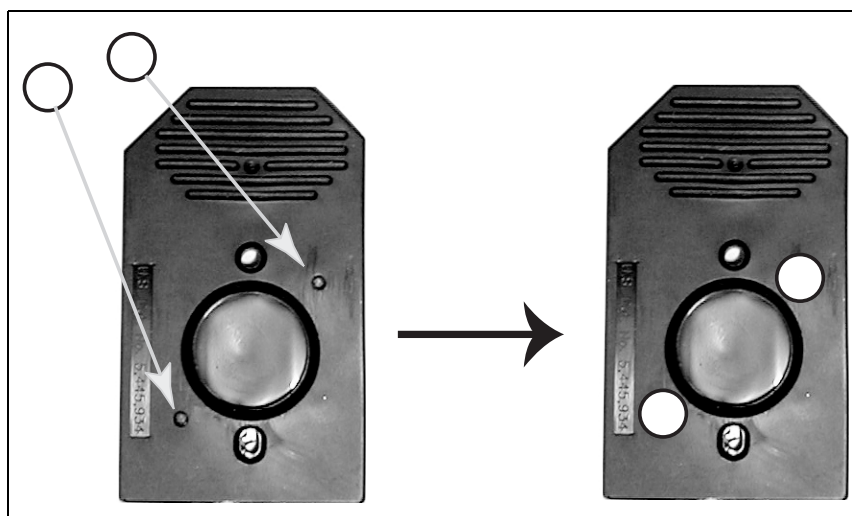
---

**Apply the spots just before scanning.**

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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 6.1](#).



**Figure 6.1**  
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 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  $\mu\text{m}$
  - Wavelength = 570 nmIf you are using the GeneChip Scanner 3000, pixel resolution and wavelength are preset and cannot be changed.
6. Open the sample door on the scanner and insert the probe array into the holder. Do not force the probe array into the holder. Close the sample door of the scanner. If you are using the GeneChip Scanner 3000, do not attempt to close the door by hand. The door closes automatically through the User Interface when start scan is selected or the scanner goes into stand-by mode.
7. Click **OK** in the Start Scanner dialog box.

The scanner begins scanning the probe array and acquiring data. When Scan in Progress is Selected from the **View** menu, the probe array image appears on the screen as the scan progresses.

## Shutting Down the Fluidics Station

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

If you are using the FS-450, gently lift up the cartridge lever to engage, or close, the washblock.

The fluidics station automatically performs a Cleanout procedure. The LCD window indicates the progress of the Cleanout procedure.
3. When the fluidics station LCD window indicates **REMOVE VIALS**, the Cleanout procedure is complete.
4. Remove the sample microcentrifuge vial(s) from the sample holder(s).

5. If no other hybridizations are to be performed, place wash lines into a bottle filled with deionized water.
6. Select **Shutdown** or **Shutdown\_450** for all modules from the drop-down **Protocol** list in the Fluidics Station dialog box. Click the **Run** button for all modules.

The Shutdown protocol is critical to instrument reliability. Refer to the appropriate *Fluidics Station User's Guide* for more information.

7. After Shutdown protocol is complete, flip the ON/OFF switch of the fluidics station to the OFF position.

**IMPORTANT** 

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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 the appropriate *GeneChip® Fluidics Station User's Guide* for further detail.

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## Customizing the Protocol

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

1. Select **Tools** → **Edit Protocol** from the menu bar.
2. In the Edit Protocol dialog box under Protocol Name, click the arrow to open a list of protocols. Click the protocol to be changed. The name of the protocol is displayed in the **Protocol Name** text box. The conditions for that protocol are displayed on the right side of the Edit Protocol dialog box.
3. Select the items to be changed and input the new parameters as needed, keeping parameters within the ranges shown below in [Table 6.6](#).

**Table 6.6**  
Valid Ranges for Wash/Stain Parameters

Parameter	Valid Range
Wash Temperature for A1, B, A2, or A3 (°C)	15 to 50
Number of Wash Cycles for A1, B, A2, or A3	0 to 99
Mixes / Wash Cycle for A1, B, A2, or A3	1 to 99
Stain Time (seconds)	0 to 86,399
Stain Temperature (°C)	15 to 50
Holding Temperature (°C)	15 to 50
<ul style="list-style-type: none"> <li>• Wash A1 corresponds to Post Hyb Wash #1 in <a href="#">Table 6.2</a> and <a href="#">Table 6.4</a>.</li> <li>• Wash B corresponds to Post Hyb Wash #2 in <a href="#">Table 6.2</a> and <a href="#">Table 6.4</a>.</li> <li>• Wash A2 corresponds to Post Stain Wash in <a href="#">Table 6.2</a> and <a href="#">Table 6.4</a>.</li> <li>• Wash A3 corresponds to Final Wash in <a href="#">Table 6.2</a> and <a href="#">Table 6.4</a>.</li> </ul>	

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

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.



Appendix **A**

**Alternative Protocols for One-Cycle  
cDNA Synthesis**

Appendix **A**



## Appendix Contents

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## Reagents and Materials Required

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

- GeneChip® T7-Oligo(dT) Promoter Primer Kit,  
5' - GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-(dT)<sub>24</sub> - 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* RNase H: 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

## 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<sup>1</sup> 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)<sub>24</sub> - 3'

## STEP 1: FIRST-STRAND cDNA SYNTHESIS

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

### NOTE

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

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

Before starting cDNA synthesis, the correct volumes of DEPC-treated H<sub>2</sub>O 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.

<sup>1</sup> 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.

**IMPORTANT** 

Use [Table A.1](#) and [Table A.2](#) for variable component calculations. Determine the volumes of RNA and SuperScript II RT required in [Table A.1](#), then calculate the amount of DEPC-treated H<sub>2</sub>O needed in Step 1 [Table A.2](#) to bring the final First-Strand Synthesis volume to 20 µL.

**Table A.1**  
Reverse Transcriptase Volumes for First-Strand cDNA Synthesis Reaction

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

**NOTE** 

The combined volume of RNA, DEPC-treated H<sub>2</sub>O and SuperScript II RT should not exceed 11 µL as indicated in [Table A.2](#).

**Table A.2**  
First-Strand cDNA Synthesis Components

	Reagents in Reaction	Volume	Final Dilution or Amount in Reaction
1: Primer Hybridization Incubate at 70°C for 10 minutes. Quick spin and put on ice	DEPC-treated H <sub>2</sub> O (variable)	for final reaction volume of 20 µL	
	T7-Oligo(dT) Primer, 50 µM RNA (variable)	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 <b>42°C</b> for 2 minutes	5X First-Strand cDNA buffer	4 µL	1X
	0.1M DTT	2 µL	10 mM DTT
	10 mM dNTP mix	1 µL	500 µM each
3: First-Strand Synthesis Add to the above tube and mix well Incubate at <b>42°C</b> for 1 hr	SuperScript II RT (variable) (200 U/µL)	See <a href="#">Table A.1</a>	200U to 600U
Total Volume		20 µL	

**NOTE** 

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

## STEP 2: SECOND-STRAND cDNA SYNTHESIS

1. Place First-Strand reactions on ice. Centrifuge briefly to bring down condensation on sides of tube.
2. Add to the First-Strand synthesis tube the reagents listed in the following Second-Strand Final Reaction Composition Table (Table A.3).

**Table A.3**  
Second-Strand Final Reaction Composition

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

3. Gently tap tube to mix. Then, briefly spin in a microcentrifuge to remove condensation and incubate at 16°C for 2 hours in a cooling waterbath.
4. Add 2  $\mu$ L [10U] T4 DNA Polymerase.
5. Return to 16°C for 5 minutes.
6. Add 10  $\mu$ L 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 48, 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<sup>1</sup> 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)<sub>24</sub> - 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<sub>2</sub>O 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).

<sup>1</sup> 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.

**IMPORTANT !**

Use [Table A.4](#) for variable component calculations. Determine volumes of mRNA and SuperScript II RT required, and then calculate the amount of DEPC-treated H<sub>2</sub>O needed in the Primer Hybridization Mix step to bring the final First-Strand Synthesis reaction volume to 20  $\mu$ L.

**Table A.4**  
First-Strand cDNA Synthesis Components

	Reagents in Reaction	Volume	Final Dilution or Amount in Reaction
1: Primer Hybridization Incubate at 70°C for 10 minutes Quick spin and put on ice	DEPC-treated H <sub>2</sub> O (variable)	for final reaction volume of 20 $\mu$ L	
	T7-Oligo(dT) Primer, 50 $\mu$ M mRNA (variable)	2 $\mu$ L 0.2 to 2 $\mu$ g	100 pmol 0.2 to 2 $\mu$ g
2: Temperature Adjustment Add to the above tube and mix well Incubate at 37°C for 2 minutes	5X First-Strand cDNA buffer	4 $\mu$ L	1X
	0.1M DTT	2 $\mu$ L	10 mM
	10 mM dNTP mix	1 $\mu$ L	500 $\mu$ M 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/ $\mu$ L)	1 $\mu$ L per $\mu$ g mRNA	200U to 400U
Total Volume		20 $\mu$ L	



## 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 A.5).

**Table A.5**  
Second-Strand Final Reaction Composition

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

3. Gently tap tube to mix. Then, briefly spin in a microcentrifuge to remove condensation and incubate at 16°C for 2 hours in a cooling waterbath.
4. Add 2  $\mu$ L [10 U] T4 DNA Polymerase.
5. Return to 16°C for 5 minutes.
6. Add 10  $\mu$ L 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 48, or store at -20°C for later use.



Appendix **B**

**Alternative Protocol for  
Eukaryotic Target Hybridization**

Appendix **B**



## Appendix Contents

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Eukaryotic Target Hybridization . . . . .	129

### **This Appendix Contains:**

- Introduction and objectives for information provided in this Appendix.
- 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 the *GeneChip® Expression Wash, Stain and Scan User Manual*, P/N 702731.

## Introduction and Objectives

Affymetrix offers a complete set of GeneChip reagents developed specifically for use with GeneChip® 3' eukaryotic arrays in cartridge format and GeneChip® prokaryotic arrays in cartridge format. GeneChip® reagent kits include the One- and Two-Cycle cDNA Synthesis Kits, IVT Labeling Kits, Sample Cleanup Modules, and Hybridization, Wash, and Stain Kits. For best results, it is highly recommended that all GeneChip reagents are used for target preparation, hybridization, wash, and staining when using GeneChip arrays.

The information and protocols described in this Appendix have been provided by Affymetrix in previous revisions of the *GeneChip® Expression Analysis Technical Manual* describing hybridization, wash, and staining procedures for GeneChip 3' eukaryotic arrays in cartridge format. These protocols should be closely followed when the GeneChip® Hybridization, Wash, and Stain Kit is not used.

## Reagents and Materials Required

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

- Water, Molecular Biology Grade: 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)
- 5 M 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, 0.5 M 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

(1.22 M MES, 0.89M [Na<sup>+</sup>])

For 1,000 mL:

64.61 g of MES hydrate

193.3 g 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, 1 M [Na<sup>+</sup>], 20 mM EDTA, 0.01% Tween-20)

For 50 mL:

8.3 mL of 12X MES Stock Buffer

17.7 mL of 5 M NaCl

4.0 mL of 0.5 M 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 [Table B.1](#) 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 to 20  $\mu\text{L}$ ) 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 B.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 Dilution
Fragmented cRNA <sup>†</sup>	15 $\mu\text{g}$	10 $\mu\text{g}$	5 $\mu\text{g}$	0.05 $\mu\text{g}/\mu\text{L}$
Control Oligonucleotide B2 (3 nM)	5 $\mu\text{L}$	3.3 $\mu\text{L}$	1.7 $\mu\text{L}$	50 pM
20X Eukaryotic Hybridization Controls ( <i>bioB</i> , <i>bioC</i> , <i>bioD</i> , <i>cre</i> )	15 $\mu\text{L}$	10 $\mu\text{L}$	5 $\mu\text{L}$	1.5, 5, 25, and 100 pM respectively
2X Hybridization Buffer	150 $\mu\text{L}$	100 $\mu\text{L}$	50 $\mu\text{L}$	1X
Herring Sperm DNA (10 mg/mL)	3 $\mu\text{L}$	2 $\mu\text{L}$	1 $\mu\text{L}$	0.1 mg/mL
BSA (50 mg/mL)	3 $\mu\text{L}$	2 $\mu\text{L}$	1 $\mu\text{L}$	0.5 mg/mL
<b>DMSO<sup>‡</sup></b>	30 $\mu\text{L}$	20 $\mu\text{L}$	10 $\mu\text{L}$	10%
H <sub>2</sub> O	to final volume of 300 $\mu\text{L}$	to final volume of 200 $\mu\text{L}$	to final volume of 100 $\mu\text{L}$	
Total Volume	300 $\mu\text{L}$	200 $\mu\text{L}$	100 $\mu\text{L}$	

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

<sup>†</sup>Please see [Chapter 2](#), for amount of adjusted fragmented cRNA to use when starting from total RNA.

<sup>‡</sup>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 B.1](#) for location of the probe array septa) with appropriate volume of 1X Hybridization Buffer using a micropipettor and appropriate tips [Table B.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.

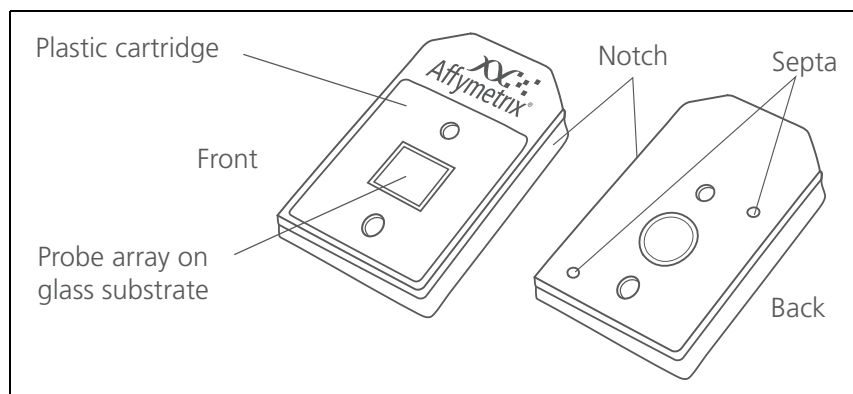
5. Incubate the probe array filled with 1X Hybridization Buffer at 45°C for 10 minutes with rotation.

**Table B.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 B.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 the *GeneChip® Expression Wash, Stain and Scan User Manual*, P/N 702731, to prepare reagents required immediately after completion of hybridization.



**Figure B.1**  
GeneChip® Probe Array



Appendix **C**

**Alternative Protocol for  
Prokaryotic Target Hybridization**

Appendix C



## Appendix Contents

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### **This Appendix Contains:**

- Introduction and objectives for information provided in this Appendix.
- Steps for preparing the hybridization mix, and instructions for hybridizing the target mix to the GeneChip® *P. aeruginosa* Genome Array and GeneChip® *E. coli* arrays. The hybridized probe array is then ready for washing, staining, and scanning as detailed in [Appendix D](#).

## Introduction and Objectives

Affymetrix offers a complete set of GeneChip reagents developed specifically for use with GeneChip® 3' eukaryotic arrays in cartridge format and GeneChip® prokaryotic arrays in cartridge format. GeneChip® reagent kits include the One- and Two-Cycle cDNA Synthesis Kits, IVT Labeling Kits, Sample Cleanup Modules, and Hybridization, Wash, and Stain Kits. For best results, it is highly recommended that all GeneChip reagents are used for target preparation, hybridization, wash, and staining when using GeneChip arrays.

The information and protocols described in this Appendix have been provided by Affymetrix in previous revisions of the *GeneChip® Expression Analysis Technical Manual* describing hybridization, wash, and staining procedures for GeneChip prokaryotic arrays in cartridge format. These protocols should be closely followed when the GeneChip® Hybridization, Wash, and Stain Kit is not used.

## Reagents and Materials Required

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

- Water, Molecular Biology Grade: Cambrex, P/N 51200
- Bovine Serum Albumin (BSA) solution, 50 mg/mL: Invitrogen Life Technologies, P/N 15561-020
- Herring Sperm DNA: Promega Corporation, P/N D1811
- Control Oligo B2, 3 nM: Affymetrix, P/N 900301 (can be ordered separately)
- NaCl, 5M, RNase-free, DNase-free: Ambion, P/N 9760G
- MES hydrate SigmaUltra: Sigma-Aldrich, P/N M5287
- MES Sodium Salt: Sigma-Aldrich, P/N M5057
- EDTA Disodium Salt, 0.5M solution (100 mL): Sigma-Aldrich, P/N E7889

### Miscellaneous Reagents

- 100% DMSO: Sigma-Aldrich, P/N D2650
- Surfact-Amps 20 (Tween-20), 10%: Pierce Chemical, P/N 28320

### Miscellaneous Supplies

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



## Reagent Preparation

### 12X MES Stock Buffer

(1.22M MES, 0.89 M [Na<sup>+</sup>])

**For 1,000 mL:**

64.61 g of MES hydrate

193.3 g of MES Sodium Salt

800 mL of Molecular Biology Grade water

Mix and adjust volume to 1,000 mL.

The pH should be between 6.5 and 6.7. Filter through a 0.2 µm filter.

#### **IMPORTANT**

---

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

---

### 2X Hybridization Buffer (50 mL)

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

**For 50 mL:**

8.3 mL of 12X MES Stock Buffer

17.7 mL of 5 M NaCl

4.0 mL of 0.5 M 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 C.1**  
Hybridization Cocktail for Single Probe Array\*

Component	49 Format (Standard)	100 Format (Midi)	169 Format (Mini)	Final Dilution or Amount
Fragmented and Labeled cDNA	Up to 50 µL	Up to 50 µL	25 µL	0.5 – 7.0 µg
Control Oligonucleotide B2 (3 nM)	3.3 µL	2.2 µL	1.3 µL	50 pM
2X Hybridization Buffer	100 µL	65 µL	40 µL	1X
100% DMSO	–	10.2 µL	6.2 µL	7.8% (or 0%)
10 mg/mL Herring Sperm DNA	2.0 µL	1.3 µL	0.8 µL	0.1 mg/mL
50 mg/mL BSA	2.0 µL	1.3 µL	0.8 µL	0.5 mg/mL
Molecular Biology Grade Water	42.7 µL	–	5.9 µL	
Total Volume	200 µL	130 µL	80 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* Array 50°C
  - *E. coli* Antisense arrays 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 [Appendix D, Alternative Protocol for Prokaryotic Arrays: Washing, Staining and Scanning](#) to prepare reagents required immediately after completion of hybridization.



Appendix **D**

**Alternative Protocol for  
Prokaryotic Arrays:  
Washing, Staining and Scanning**

Appendix **D**



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### This Appendix Contains:

- Introduction and objectives for information provided in this Appendix.
- Instructions for using the Fluidics Station 400 or 450/250 to automate the washing and staining of GeneChip *P. aeruginosa* and GeneChip *E. coli* 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).

## Introduction and Objectives

Affymetrix offers a complete set of GeneChip reagents developed specifically for use with GeneChip® 3' eukaryotic arrays in cartridge format and GeneChip® prokaryotic arrays in cartridge format. GeneChip® reagent kits include the One- and Two-Cycle cDNA Synthesis Kits, IVT Labeling Kits, Sample Cleanup Modules, and Hybridization, Wash, and Stain Kits. For best results, it is highly recommended that all GeneChip reagents are used for target preparation, hybridization, wash, and staining when using GeneChip arrays.

The information and protocols described in this Appendix have been provided by Affymetrix in previous revisions of the *GeneChip® Expression Analysis Technical Manual* describing hybridization, wash, and staining procedures for GeneChip prokaryotic arrays in cartridge format. These protocols should be closely followed when the GeneChip® Hybridization, Wash, and Stain Kit is not used.



## Reagents and Materials Required

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

- Water, Molecular Biology Grade: Cambrex, P/N 51200
- Nuclease-free Water: Ambion, P/N 9930
- Bovine Serum Albumin (BSA) solution, 50 mg/mL: Invitrogen Life Technologies, P/N 15561-020
- R-Phycoerythrin Streptavidin: Molecular Probes, P/N S-866
- NaCl, 5M, RNase-free, DNase-free: Ambion, P/N 9760G
- PBS, pH 7.2: Invitrogen Life Technologies, P/N 20012-027
- 20X SSPE (3 M NaCl, 0.2M NaH<sub>2</sub>PO<sub>4</sub>, 0.02 M EDTA): Cambrex, P/N 51214
- Goat IgG, Reagent Grade: Sigma-Aldrich, P/N I 5256
- Anti-streptavidin antibody (goat), biotinylated: Vector Laboratories, P/N BA-0500
- Surfact-Amps 20 (Tween-20), 10%: Pierce Chemical, P/N 28320
- Bleach (5.25% Sodium Hypochlorite): VWR Scientific, P/N 37001-060 (or equivalent)
- ImmunoPure Streptavidin: Pierce Chemical, P/N 21125

### Miscellaneous Supplies

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

## Reagent Preparation

### Wash Buffer A: Non-Stringent Wash Buffer

(6X SSPE, 0.01% Tween-20)

For 1,000 mL:

300 mL of 20X SSPE

1.0 mL of 10% Tween-20

699 mL of water

Filter through a 0.2 µm filter.

Store at room temperature.

### Wash Buffer B: Stringent Wash Buffer

(100 mM MES, 0.1 M [Na<sup>+</sup>], 0.01% Tween-20)

For 1,000 mL:

83.3 mL of 12 X MES Stock Buffer (see [Appendix C](#) 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, 1 M [Na<sup>+</sup>], 0.05% Tween-20)

For 250 mL:

41.7 mL of 12X MES Stock Buffer (see [Appendix C](#))

92.5 mL of 5M NaCl

2.5 mL of 10% Tween-20

113.3 mL of water

Filter through a 0.2 µm filter.

Store at 2°C to 8°C and shield from light.

### 10 mg/mL Goat IgG Stock

Resuspend 50 mg in 5 mL 150 mM NaCl.

Store at 4°C.

**NOTE** 

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

### 1 mg/mL Streptavidin Stock

Resuspend 5 mg in 5 mL of PBS.

Store at 4°C.

## Experiment and Fluidics Station Setup

### STEP 1: DEFINING FILE LOCATIONS

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

**NOTE** 

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

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

## STEP 2: ENTERING EXPERIMENT INFORMATION

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

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

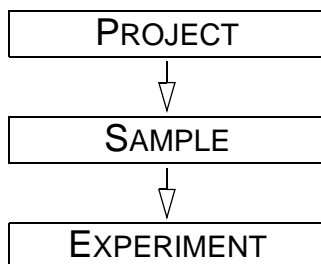
- Experiment Name
- Probe Array Type

In GCOS, three additional fields are required:

- Sample Name
- Sample Type
- Project

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

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



## STEP 3: PREPARING THE FLUIDICS STATION

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

### Setting Up the Fluidics Station

1. Turn on the Fluidics Station using the switch on the lower left side of the machine.
2. Select **Run** → **Fluidics** from the menu bar.

The Fluidics Station dialog box appears with a drop-down list for selecting the experiment name for each of the fluidics station modules. A second list is accessed for choosing the Protocol for each of the four fluidics station modules.

#### NOTE

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

### Priming the Fluidics Station

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

Priming should be done:

- When the fluidics station is first started
  - When wash solutions are changed
  - Before washing if a shutdown has been performed
  - If the LCD window instructs the user to prime
1. Select **Protocol** in the Fluidics Station dialog box.
  2. Choose **Prime** or **Prime\_450** for the respective modules in the **Protocol** drop-down list.
  3. Change the intake buffer reservoir A to **Non-stringent Wash Buffer** and intake buffer reservoir B to **Stringent Wash Buffer**.
  4. For MAS, click **Run** for each module to begin priming. In GCOS, select the **All Modules** check box, then click **Run**.

## Probe Array Wash and Stain

Following hybridization, the wash and stain procedures are carried out by the Fluidics Station. A modified FlexMidi\_euk2v3 fluidics script (FlexMidi\_euk2v3\_450, if you are using the FS-450) is used for the GeneChip *P. aeruginosa* Genome Array, and the ProkGE-WS2 fluidics script (ProkGE-WS2\_450, if you are using the FS-450) is used for the GeneChip *E. coli* arrays. The procedures take approximately 75 and 90 minutes, respectively, to complete. The use of streptavidin in the first part of the stain procedure enhances the overall signal.

After 16 hours of hybridization, remove the hybridization cocktail from the probe array and fill the probe array completely with the appropriate volume of Non-Stringent Wash Buffer (Wash Buffer A), as given in [Table D.1](#).

### NOTE

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

**Table D.1**  
Probe Array Cartridge Volumes

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

## Preparing the Staining Reagents

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

Streptavidin Phycoerythrin (SAPE) should be stored in the dark at 4°C, either foil wrapped or kept in an amber tube. Remove SAPE from the refrigerator and tap the tube to mix well before preparing stain solution.

Do not freeze SAPE. Always prepare the SAPE stain solution fresh, on the day of use.

**Table D.2**  
Streptavidin Solution Mix - Vial 1

Component	Volume	Final Dilution
2X Stain Buffer	300.0 $\mu$ L	1X
50 mg/mL BSA	24.0 $\mu$ L	2 mg/mL
1 mg/mL Streptavidin	6.0 $\mu$ L	10 $\mu$ g/mL
Nuclease-free H <sub>2</sub> O	270.0 $\mu$ L	—
Total Volume	600 $\mu$ L	

**Table D.3**  
Antibody Solution Mix - Vial 2

Component	Volume	Final Concentration
2X MES Stain Buffer	300.0 $\mu$ L	1X
50 mg/mL BSA	24.0 $\mu$ L	2 mg/mL
10 mg/mL Normal Goat IgG	6.0 $\mu$ L	0.1 mg/mL
0.5 mg/mL Anti-streptavidin Antibody, biotinylated	6.0 $\mu$ L	5 $\mu$ g/mL
Nuclease-free H <sub>2</sub> O	264.0 $\mu$ L	—
Total Volume	600 $\mu$ L	

**Table D.4**  
SAPE Solution Mix - Vial 3

Component	Volume	Final Concentration
2X MES Stain Buffer	300.0 $\mu$ L	1X
50 mg/mL BSA	24.0 $\mu$ L	2 mg/mL
1 mg/mL Streptavidin Phycoerythrin	6.0 $\mu$ L	10 $\mu$ g/mL
Nuclease-free H <sub>2</sub> O	270.0 $\mu$ L	—
Total Volume	600 $\mu$ L	

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.

#### Arrays & Fluidics Protocols

- Array: GeneChip *E. coli* Genome 2.0 Array  
Protocol: Mini\_prok2v1(if using FS-450, Mini\_prok2v1\_450)
- Array: GeneChip *E. coli* Antisense Genome Array  
Protocol: ProkGE-WS2 (if using FS-450, ProkGE-WS2\_450)
- Array: GeneChip *P. aeruginosa* Genome Array  
Protocol: Modified FlexMidi\_euk2v3\*  
(\*See [Table D.5](#). If using FS-450, FlexMidi\_euk2v3\_450)

#### IMPORTANT !

---

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

---

3. Choose **Run** in the Fluidics Station dialog box to begin the washing and staining. Follow the instructions in the LCD window on the fluidics station when using the Fluidics Station 400.

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



**Table D.5**  
Modification of FlexMidi\_euk2v3 for GeneChip® *P. aeruginosa* Array

GeneChip *P. aeruginosa* Genome Array requires a modification to the **FlexMidi\_euk2v3** (or the **FlexMidi\_euk2v3\_450**) protocol. See below for details.

The **FlexMidi\_euk2v3** (or the **FlexMidi\_euk2v3\_450**) fluidics protocol must be modified. Please follow the instructions carefully to make the modifications. Additionally, it is highly recommended that you save your new *P. aeruginosa* fluidics protocol under a different name to avoid confusion.

1. Modify and save the fluidics protocol for the assay:
  - a. Modify the fluidics protocol by using **Tools** → **Edit Protocol** drop-down list and selecting **FlexMidi\_euk2v3** (or the **FlexMidi\_euk2v3\_450**) within the Protocol Name window.
  - b. Change the following parameters: (Enter the new parameters by highlighting the default values and typing in the new values.)
    - i) **Wash A1 Temperature** from 30°C to 25°C;
    - ii) **Number of Wash B Cycles** from 6 to 4;
    - iii) **Stain Temperature (C)** from 35°C to 25°C;
    - iv) **First Stain Time (seconds)** from 300 to 600 seconds;
    - v) **Second Stain Time (seconds)** from 300 to 600 seconds;
    - vi) **Third Stain Time (seconds)** from 300 to 600 seconds; and
    - vii) **Wash A3 Temperature** from 35°C to 30°C.
  - c. Save the modified fluidics protocol by highlighting **FlexMidi\_euk2v3** (or the **FlexMidi\_euk2v3\_450**) within the Protocol Name window and typing over with an assigned protocol name (e.g., Pae\_cDNA). Click **Save**.  
The new fluidics protocol should be present in the Protocol drop-down list and is used in the subsequent steps.
2. Select the name of the newly modified protocol (e.g., Pae\_cDNA) from the **Protocol** drop-down list in the Fluidics Station dialog box. Select **Run** in the Fluidics Station dialog box to begin the washing and staining. Follow the instructions in the LCD window on the fluidics station.

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

**Table D.6**  
Fluidics Scripts Prokaryotic Arrays

Format	Fluidics Scripts *
169	Mini_prok2v1
100	modified FlexMidi_Euk2v3
49	ProkGE_WS2

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

**Table D.7**  
Fluidics Protocols

	Mini_prok2v1*	FlexMidi_euk2v3†	Modified FlexMidi_euk2v3 for <i>P. aeruginosa</i> Array	ProkGE_WS2‡
<b>Post Hyb Wash #1</b>	10 cycles of 2 mixes/ cycle with Wash Buffer A at 30°C	10 cycles of 2 mixes/ cycle with Wash Buffer A at 30°C	10 cycles of 2 mixes/cycle with Wash Buffer A at 25°C	10 cycles of 2 mixes/ cycle with Wash Buffer A at 25°C
<b>Post Hyb Wash #2</b>	4 cycles of 15 mixes/ cycle with Wash Buffer B at 50°C	6 cycles of 15 mixes/ cycle with Wash Buffer B at 50°C	4 cycles of 15 mixes/cycle with Wash Buffer B at 50°C	4 cycles of 15 mixes/ cycle with Wash Buffer B at 45°C
<b>1<sup>st</sup> Stain</b>	Stain the probe array for 300 seconds in Streptavidin Solution Mix at 35°C	Stain the probe array for <b>300 seconds</b> in SAPE Solution Mix at <b>35°C</b>	Stain the probe array for <b>600 seconds</b> in Streptavidin Solution Mix at <b>25°C</b>	Stain the probe array for 600 seconds in Streptavidin Solution Mix at 25°C.
<b>Post Stain Wash</b>	10 cycles of 4 mixes/ cycle with Wash Buffer A at 30°C	10 cycles of 4 mixes/ cycle with Wash Buffer A at 30°C	10 cycles of 4 mixes/cycle with Wash Buffer A at 30°C	10 cycles of 4 mixes/ cycle with Wash Buffer A at 30°C
<b>2<sup>nd</sup> Stain</b>	Stain the probe array for 300 seconds in Antibody Solution Mix at 35°C	Stain the probe array for <b>300 seconds</b> in Antibody Solution Mix at <b>35°C</b>	Stain the probe array for <b>600 seconds</b> in Antibody Solution Mix at <b>25°C</b>	Stain the probe array for 600 seconds in Antibody Solution Mix at 25°C.
<b>3<sup>rd</sup> Stain</b>	Stain the probe array for 300 seconds in SAPE Solution Mix at 35°C	Stain the probe array for <b>300 seconds</b> in SAPE Solution Mix at <b>35°C</b>	Stain the probe array for <b>600 seconds</b> in SAPE Solution Mix at <b>25°C</b>	Stain the probe array for 600 seconds in SAPE Solution Mix at 25°C.
<b>Final Wash</b>	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 <b>35°C</b> . The holding temperature is 25°C.	15 cycles of 4 mixes/cycle with Wash Buffer A at <b>30°C</b> . 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.

\*Mini\_prok2v1\_450 for the FS-450/250

†FlexMidi\_Euk2v3\_450 for the FS-450/250

‡ProkGE\_WS2\_450 for the FS-450/250

## 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 D.6](#).
3. Choose **Run** in the Fluidics Station dialog box to begin the washing and staining. Follow the instructions in the LCD window on the fluidics station.

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

4. Insert the appropriate probe array into the designated module of the fluidics station while the cartridge lever is down, or in the eject position. When finished, verify that the cartridge lever is returned to the up, or engaged, position.
5. Remove any microcentrifuge vial(s) remaining in the sample holder of the fluidics station module(s) being used.
6. If prompted to "Load Vials 1-2-3," place the three experiment sample vials (the microcentrifuge vials) into the sample holders 1, 2, and 3 on the fluidics station.
  - A. Place one vial containing streptavidin solution in sample holder 1.
  - B. Place one vial containing the anti-streptavidin biotinylated antibody solution in sample holder 2.
  - C. Place one vial containing the streptavidin phycoerythrin (SAPE) solution in sample holder 3.
  - D. Press down on the needle lever to snap needles into position and to start the run.

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

7. When the protocol is complete, the LCD window displays the message **EJECT CARTRIDGE**.
8. Remove the probe arrays from the fluidics station modules by first pressing down the cartridge lever to the eject position.
9. Lift up on the needle lever to disengage the needles from the microcentrifuge vials. Remove the three microcentrifuge vials from the needle holders.
10. Check the probe array window for large bubbles or air pockets.
  - If bubbles are present, refer to [Table D.8](#).
  - If the probe array has no large bubbles, it is ready to scan on the GeneArray® Scanner, or the GeneChip® Scanner 3000. Pull up on the cartridge lever to close the washblock and proceed to [Probe Array Scan on page 159](#).
11. If there are no more samples to hybridize, shut down the fluidics station following the procedure in [Shutting Down the Fluidics Station on page 161](#).
12. Keep the probe arrays at 4°C and in the dark until ready for scanning.
13. Lift up on the cartridge lever to close the washblock.

**NOTE** 

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For proper cleaning and maintenance of the fluidics station, including the bleach protocol, refer to the appropriate *GeneChip® Fluidics Station User's Guide* for instruction.

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**Table D.8**

If Bubbles Are Present

<p>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 <b>EJECT CARTRIDGE</b>. Again, remove the probe array and inspect it for bubbles. If no bubbles are present, it is ready to scan. Proceed to <a href="#">Probe Array Scan on page 159</a>.</p> <p>If several attempts to fill the probe array without bubbles are unsuccessful, the array should be filled with <b>Wash Buffer A (non-stringent buffer)</b> manually, using a micropipette. Excessive washing will result in a loss of signal intensity.</p>
--

## 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 D.6](#).
3. Choose **Run** in the Fluidics Station dialog box to begin the washing and staining. Follow the instructions on the LCD window on the fluidics station.

If you are unfamiliar with inserting and removing probe arrays from the fluidics station modules, please refer to the *Fluidics Station 400 User's Guide*, *Fluidics Station 400 Video In-Service CD* (P/N 900374), or *Quick Reference Card* (P/N 08-0072).

4. Insert the appropriate probe array into the designated module of the fluidics station while the cartridge lever is in the **EJECT** position. When finished, verify that the cartridge lever is returned to the **ENGAGE** position.
5. Remove any microcentrifuge tube remaining in the sample holder of the fluidics station module(s) being used.
6. Place a microcentrifuge tube containing the streptavidin solution into the sample holder, making sure that the metal sampling needle is in the tube with its tip near the bottom  
The Fluidics Station dialog box and the LCD window display the status of the washing and staining as they progress.
7. When the LCD window indicates, replace the microcentrifuge vial containing the streptavidin stain with a microcentrifuge vial containing antibody stain solution into the sample holder, making sure that the metal sampling needle is in the vial with its tip near the bottom.
8. When the LCD window indicates, replace the microcentrifuge vial containing antibody solution with the microcentrifuge vial containing the streptavidin phycoerythrin (SAPE) solution.
9. When the protocol is complete, the LCD window displays the message **EJECT CARTRIDGE**.

10. Remove microcentrifuge vial containing stain and replace with an empty microcentrifuge tube.
11. Remove the probe arrays from the fluidics station modules by first moving the cartridge lever to the **EJECT** position.
12. Check the probe array window for large bubbles or air pockets.
  - If bubbles are present, refer to [Table D.9](#).
  - If the probe array has no large bubbles, it is ready to scan on the GeneChip Scanner 3000 or the GeneArray® Scanner. ENGAGE washblock and proceed to [Probe Array Scan on page 159](#).

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 161](#).

**NOTE** 

---

**For proper cleaning and maintenance of the fluidics station, including the bleach protocol, refer to the appropriate GeneChip® Fluidics Station User's Guide for instruction.**

---

**Table D.9**  
If Bubbles Are Present

<p>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 <b>ENGAGE</b> 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 <b>EJECT CARTRIDGE</b> again, remove the probe array and inspect it again for bubbles. If no bubbles are present, it is ready to scan. Proceed to <a href="#">Probe Array Scan on page 159</a>.</p> <p>If several attempts to fill the probe array without bubbles are unsuccessful, the array should be filled with <b>Wash Buffer A (non-stringent buffer)</b> manually, using a micropipette. Excessive washing will result in a loss of signal intensity.</p>
---

## Probe Array Scan

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

**WARNING** 

---

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

---

**NOTE** 

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

---

## HANDLING THE GENECHIP® PROBE ARRAY

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

Before scanning the probe array cartridge, follow this procedure to apply Tough-Spots™ to the probe array cartridge to prevent the leaking of fluids from the cartridge during scanning.

**IMPORTANT** 

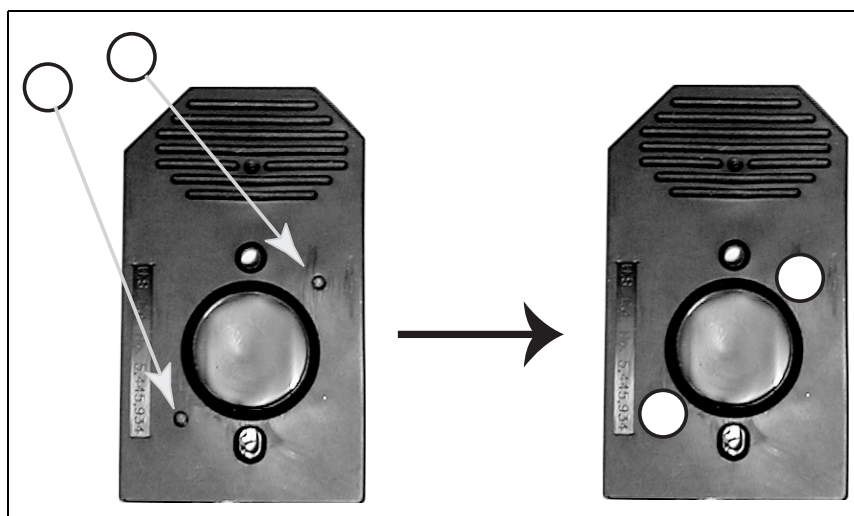
---

**Apply the spots just before scanning. Do not use them in the hybridization 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 D.1](#).



**Figure D.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  $\mu\text{m}$
  - Wavelength = 570 nmIf you are using the GeneChip Scanner 3000, pixel resolution and wavelength are preset and cannot be changed.
6. Open the sample door on the scanner and insert the probe array into the holder. Do not force the probe array into the holder. Close the sample door of the scanner. If you are using the GeneChip Scanner 3000, do not attempt to close the door by hand. The door closes automatically through the User Interface when start scan is selected or the scanner goes into stand-by mode.
7. Click OK in the Start Scanner dialog box.

The scanner begins scanning the probe array and acquiring data. When Scan in Progress is Selected from the View menu, the probe array image appears on the screen as the scan progresses.

## Shutting Down the Fluidics Station

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

If you are using the FS-450, gently lift up the cartridge lever to engage, or close, the washblock.

The fluidics station automatically performs a Cleanout procedure. The LCD window indicates the progress of the Cleanout procedure.
3. When the fluidics station LCD window indicates **REMOVE VIALS**, the Cleanout procedure is complete.
4. Remove the sample microcentrifuge vial(s) from the sample holder(s).

5. If no other hybridizations are to be performed, place wash lines into a bottle filled with deionized water.
6. Select Shutdown or Shutdown\_450 for all modules from the drop-down **Protocol** list in the Fluidics Station dialog box. Click the **Run** button for all modules.

The Shutdown protocol is critical to instrument reliability. Refer to the appropriate *Fluidics Station User's Guide* for more information.

7. After Shutdown protocol is complete, flip the ON/OFF switch of the fluidics station to the OFF position.

**IMPORTANT !**

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**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 the appropriate *GeneChip® Fluidics Station User's Guide* for further detail.**

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## Customizing the Protocol

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

1. Select **Tools** → **Edit Protocol** from the menu bar.
2. In the Edit Protocol dialog box under Protocol Name, click the arrow to open a list of protocols. Click the protocol to be changed.  
The name of the protocol is displayed in the **Protocol Name** text box. The conditions for that protocol are displayed on the right side of the Edit Protocol dialog box.
3. Select the items to be changed and input the new parameters as needed, keeping parameters within the ranges shown in [Table D.10](#).

**Table D.10**  
Valid Ranges for Wash/Stain Parameters

Parameter	Valid Range
Wash Temperature for A1, B, A2, or A3 (°C)	15 to 50
Number of Wash Cycles for A1, B, A2, or A3	0 to 99
Mixes / Wash Cycle for A1, B, A2, or A3	1 to 99
Stain Time (seconds)	0 to 86,399
Stain Temperature (°C)	15 to 50
Holding Temperature (°C)	15 to 50
<ul style="list-style-type: none"> <li>• Wash A1 corresponds to Post Hyb Wash #1 in <a href="#">Table D.7</a>.</li> <li>• Wash B corresponds to Post Hyb Wash #2 in <a href="#">Table D.7</a>.</li> <li>• Wash A2 corresponds to Post Stain Wash in <a href="#">Table D.7</a>.</li> <li>• Wash A3 corresponds to Final Wash in <a href="#">Table D.7</a>.</li> </ul>	

- To return to the default values for the protocol selected, click the Defaults button.
- 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.**

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



Appendix **E**

**List of Controls on GeneChip® Probe Arrays**

Appendix **E**



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### Control Genes on GeneChip® Eukaryotic Probe Arrays

**Table E.1**  
Control Genes on GeneChip® Eukaryotic Probe Arrays

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

## Control Genes on GeneChip® Prokaryotic Probe Arrays

**Table E.2**  
Control Genes on GeneChip® Prokaryotic Probe Arrays

Origin of Organism	Control Gene Name	Utility for GeneChip® Experiments	Associated Affymetrix Products
synthetic	B2 Oligo	Grid alignment.	Control Oligo B2, P/N 900301, also as part of the Hybridization Control Kit P/N 900454 or 900457
<i>B. subtilis</i>	<i>lys</i> <i>phe</i> <i>thr</i> <i>dap</i>	Sense RNA can be spiked into purified sample RNA as control for the labeling and hybridization process. The spikes can also be used to estimate assay sensitivity.	GeneChip Eukaryotic Poly-A RNA Control Kit, P/N 900433



Appendix **G**

**Contact Information**

Appendix **G**



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### Affymetrix Technical Support

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

**Affymetrix Inc.**  
3420 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

UK and Others Tel: +44 (0) 1628 552550  
France Tel: 0800919505  
Germany Tel: 01803001334  
E-mail: supporteurope@affymetrix.com

**Affymetrix Japan, K.K.**  
Mita NN Bldg., 16 F  
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](http://www.affymetrix.com)

## Supplier Contact Information

**Table 1.G.1**

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