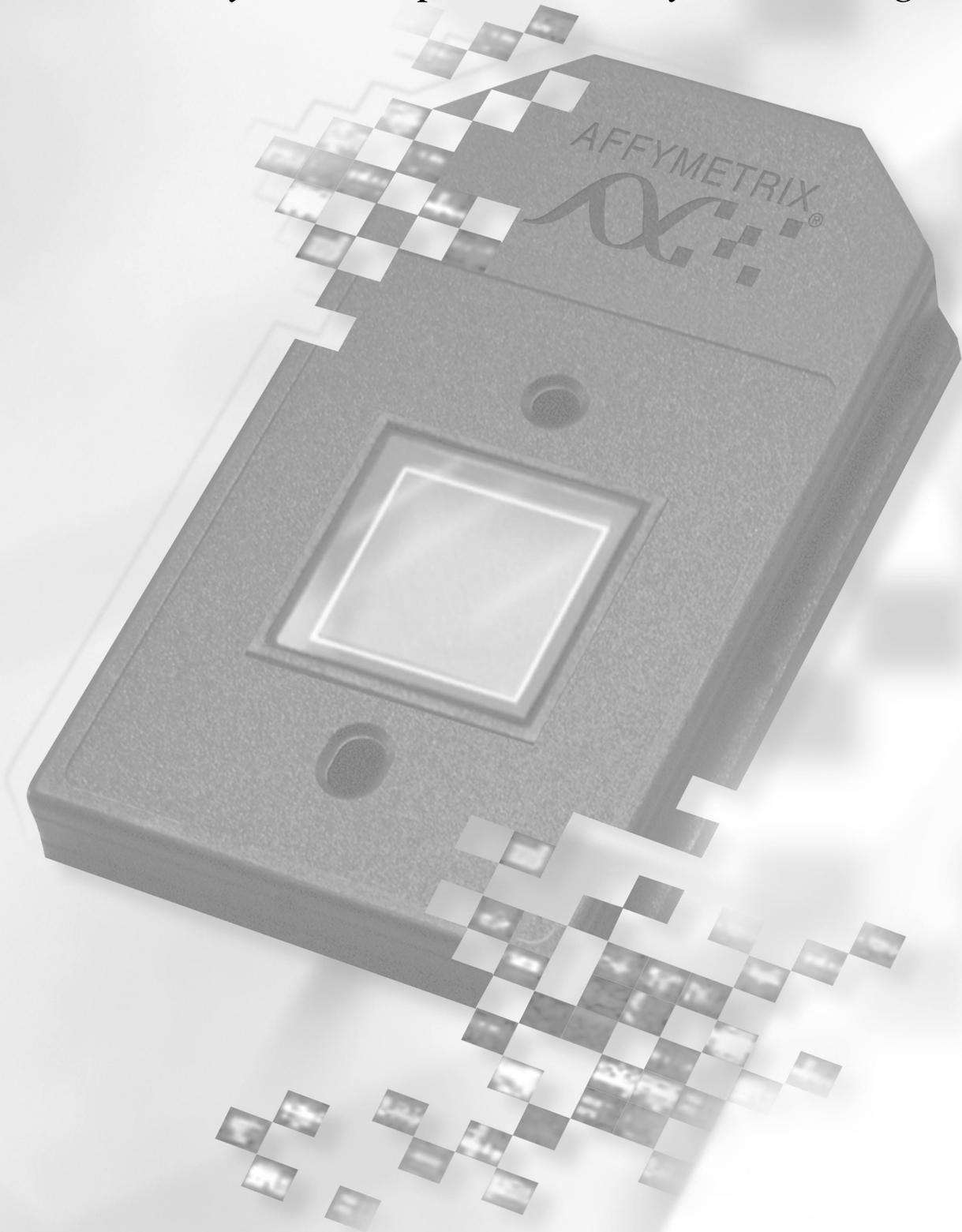
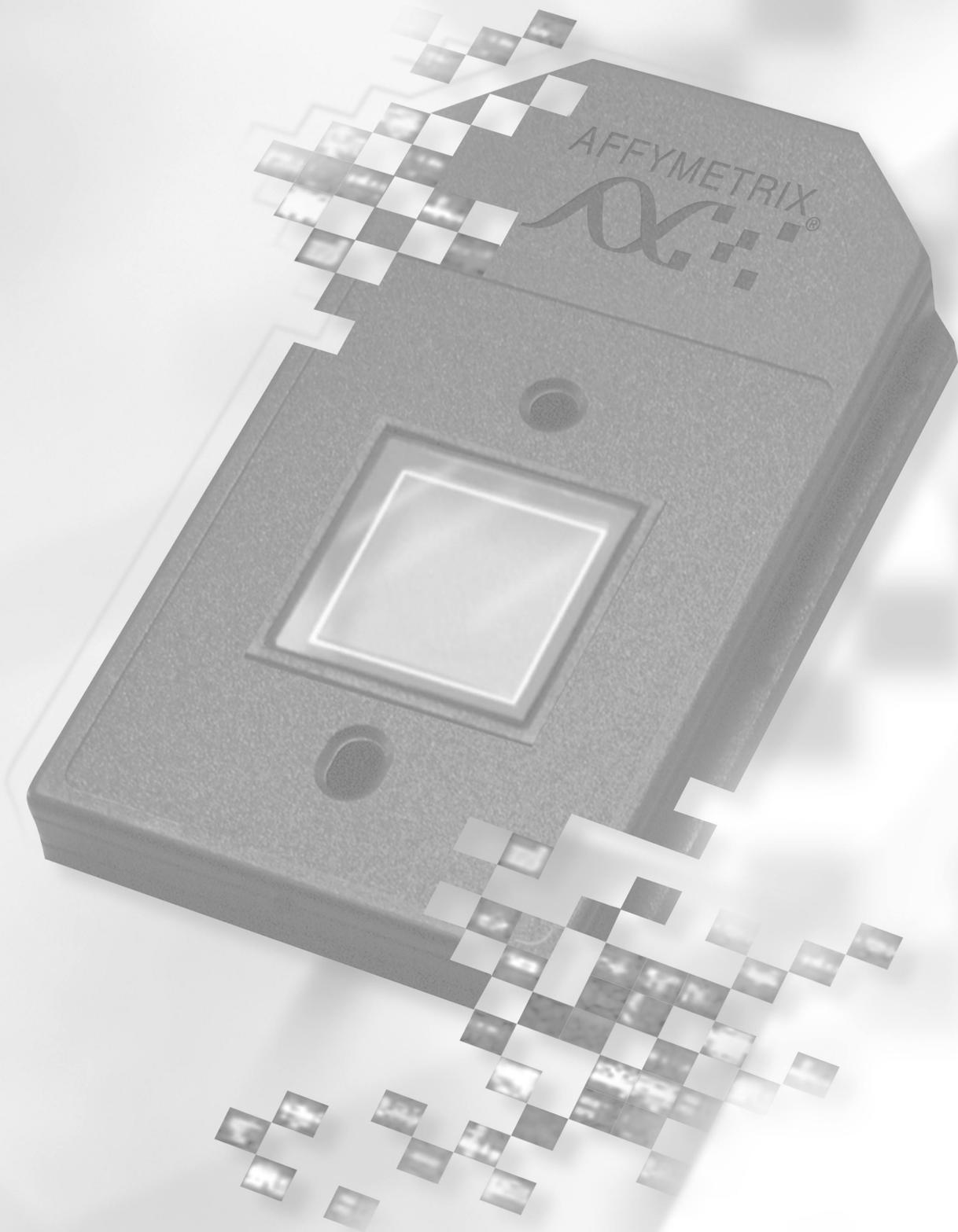


Section 2:

Eukaryotic Sample and Array Processing



Section 2

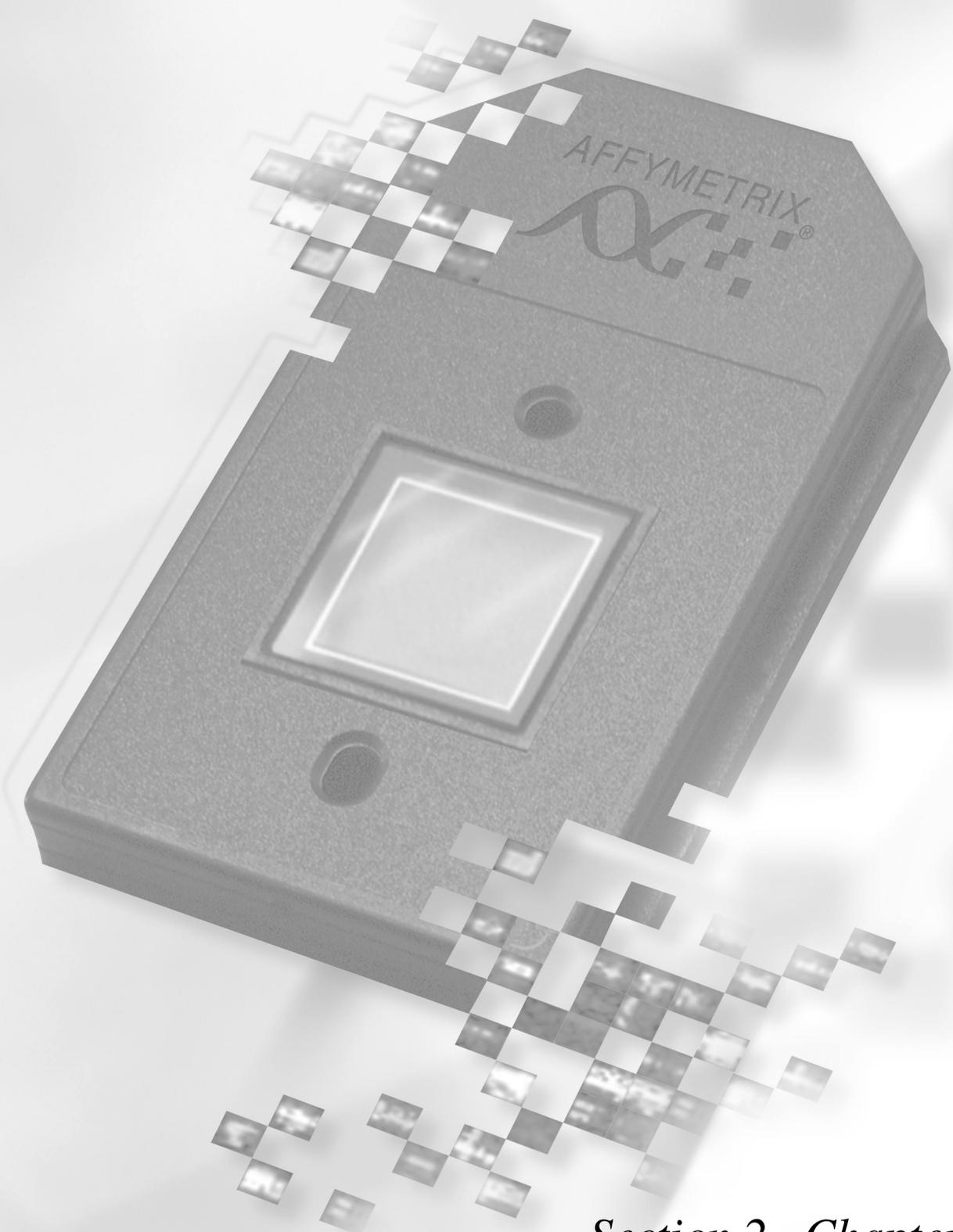




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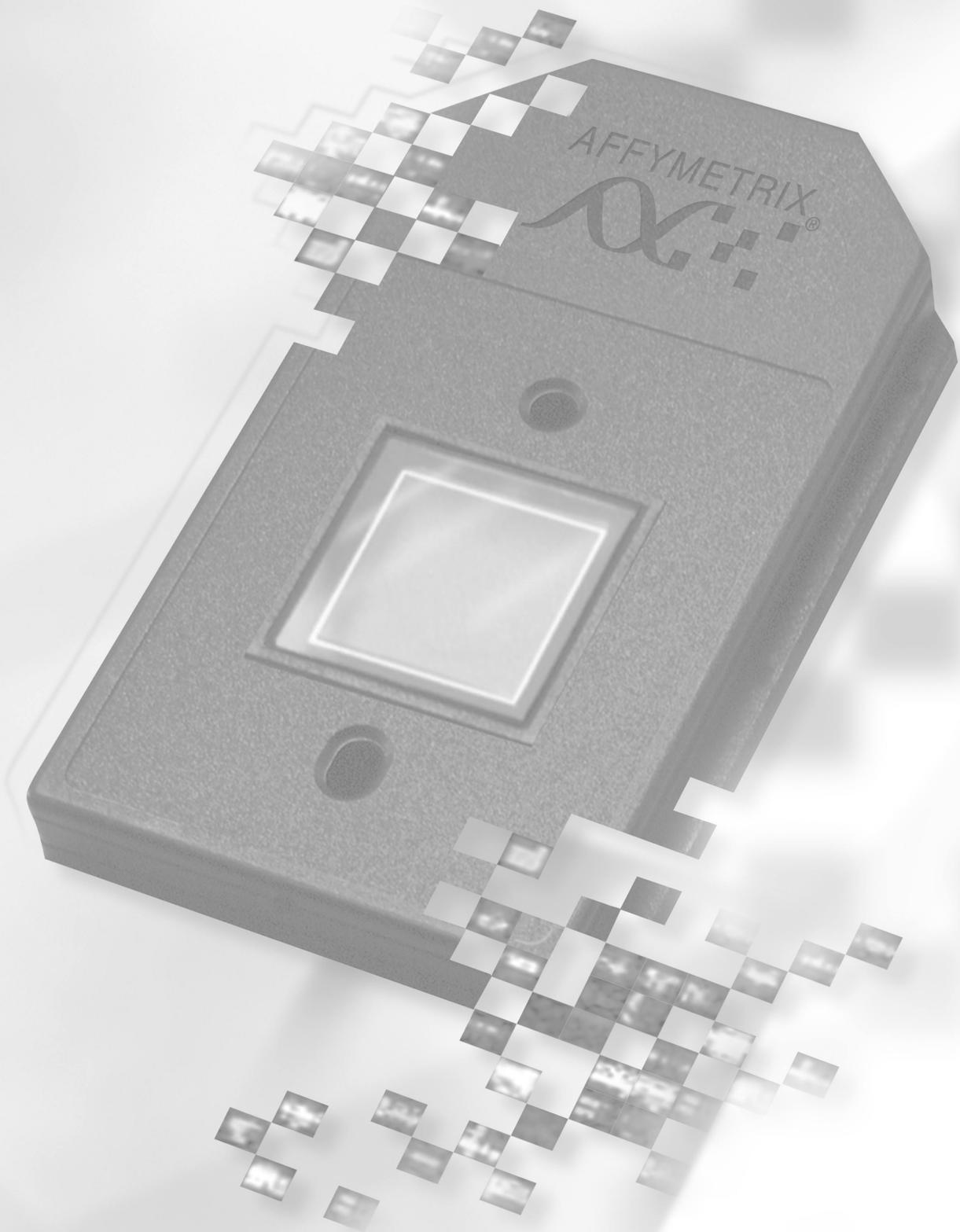
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Eukaryotic Target Preparation

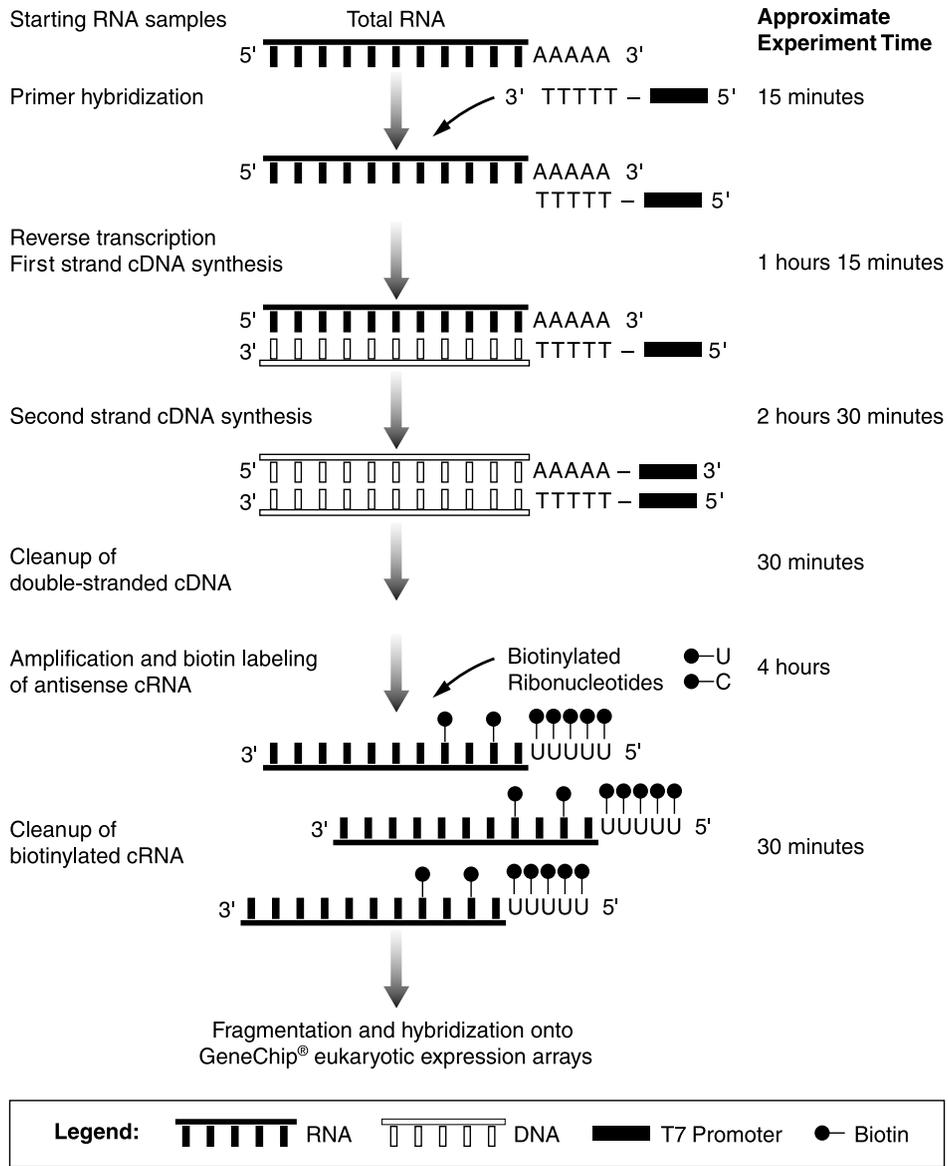
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This Chapter Contains:

- General Guidelines for extracting RNA from eukaryotic cells or tissues using commercially available reagents and kits
- Detailed steps for making double-stranded cDNA from extracted RNA.
- Guidelines for producing biotin-labeled antisense cRNA (target) using *in vitro* transcription reaction (IVT) and the ENZO® BioArray™ HighYield™ RNA Transcript Labeling Kit
- Instructions for fragmenting the labeled cRNA target

After completing the procedures described in this chapter, the labeled and fragmented cRNA target is hybridized to the probe array as described in Section 2, Chapter 3.

Eukaryotic Target Labeling GeneChip® Probe Arrays



Reagents and Materials Required

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

IMPORTANT

Do not store enzymes in a frost-free freezer.

Total RNA Isolation

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

Poly-A mRNA Isolation

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

cDNA Synthesis

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

✓ Note

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

- *E. coli* DNA Ligase, Invitrogen Life Technologies, P/N 18052-019
- *E. coli* DNA Polymerase I, Invitrogen Life Technologies, P/N 18010-025
- *E. coli* RNaseH, Invitrogen Life Technologies, P/N 18021-071
- T4 DNA Polymerase, Invitrogen Life Technologies, P/N 18005-025
- 5X Second strand buffer, Invitrogen Life Technologies, P/N 10812-014
- 10 mM dNTP, Invitrogen Life Technologies, P/N 18427-013
- GeneChip Sample Cleanup Module, Affymetrix, P/N 900371

Synthesis of Biotin-Labeled cRNA

 RNA Transcript Labeling Kit, Affymetrix, P/N 900182

IVT cRNA Cleanup and Quantification

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

cRNA Fragmentation

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

Alternative Protocol for Cleanup of Double-Stranded cDNA

- Phase Lock Gel, Brinkmann Instruments, P/N 955 15 415
- Phenol/chloroform/isoamyl alcohol, Ambion, P/N 9732
- 7.5 M Ammonium Acetate (NH₄OAc), Sigma-Aldrich, P/N A2706
- Water, Molecular Biology Grade, BioWhittaker Molecular Applications / Cambrex, P/N 51200

Miscellaneous Reagents

- Absolute ethanol (stored at -20°C for RNA precipitation; store ethanol at room temperature for use with the GeneChip Sample Cleanup Module)
- 80% ethanol (stored at -20°C for RNA precipitation; store ethanol at room temperature for use with the GeneChip Sample Cleanup Module)
- SYBR Green II, Cambrex, P/N 50523; or Molecular Probes, P/N S7586 (optional)
- Pellet Paint, Novagen, P/N 69049-3 (optional)
- Glycogen, Ambion, P/N 9510 (optional)
- 3 M Sodium Acetate (NaOAc), Sigma-Aldrich, P/N S7899
- Ethidium Bromide, Sigma-Aldrich, P/N E8751
- 1 N NaOH
- 1 N HCl
- 50 mM MgCl₂
- 0.5 M EDTA

Miscellaneous Supplies

- Sterile, RNase-free, microcentrifuge tubes, 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
- Vacuum filter units (1 liter capacity, 0.22 µm or 0.45 µm), VWR Scientific Products, P/N 28199-730
- UV spectrophotometer
- Cooling waterbath

Isolation of RNA

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

Please review precautions and interfering conditions in Section 1.

IMPORTANT

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

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

Isolation of RNA from Yeast

Total RNA

We have successfully isolated good quality total RNA 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 yields of yeast mRNA and is not generally recommended.

Isolation of RNA from Arabidopsis

Total RNA

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

Poly-A mRNA

We have successfully isolated Arabidopsis poly-A RNA using QIAGEN Oligotex products. However, other standard isolation products are likely to be adequate.

Isolation of RNA from Mammalian Cells or Tissues

Total RNA

We have successfully isolated high-quality total RNA 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, we recommend isolating total RNA with a commercial reagent such as TRIzol.

IMPORTANT

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

Poly-A mRNA

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

Precipitation of RNA

Total RNA

It is not necessary to precipitate total RNA following isolation or cleanup with RNeasy Mini Kit. Please adjust elution volumes from the RNeasy column to prepare for cDNA synthesis based upon expected RNA yields from your experiment. Ethanol precipitation is required following TRIzol isolation and hot phenol extraction methods; see methods on page 2.1.9.

✓ Note

Affymetrix recommends starting the cDNA synthesis protocol with a minimum of 0.2 µg poly-A mRNA at a minimum concentration of 0.02 µg/µL, or 5 µg of total RNA at a minimum concentration of 0.5 µg/µL, in order to obtain sufficient quantity of labeled cRNA for target assessment and hybridization to GeneChip expression probe arrays. There are two major advantages to starting with at least the recommended amount of material:

- 1. Enough material to check sample yield and quality at the various steps of this protocol.*
- 2. Production of enough cRNA for hybridization of the target to multiple probe arrays.*

For smaller amounts of starting material, please refer to the alternative research protocol for target labeling described in *GeneChip Eukaryotic Small Sample Target Labeling Technical Note*, available at www.affymetrix.com.

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

*Addition of Carrier to Ethanol Precipitations

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

■ Pellet Paint

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

■ Glycogen

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

Quantification of RNA

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

- 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).

Synthesis of Double-Stranded cDNA 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 Kit¹ 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 be checked by gel electrophoresis.

T7-oligo(dT) primer

5' - GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-(dT)₂₄ - 3'

Step 1: First Strand cDNA Synthesis

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



Note

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

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

Before starting cDNA synthesis, the correct volumes of DEPC-treated H₂O and Reverse Transcriptase (RT) must be determined. These volumes will depend on both the concentration and total volume of RNA that is being added to the reaction.



IMPORTANT

Use Table 2.1.1 and Table 2.1.2 for variable component calculations. Determine the volumes of RNA and SuperScript II RT required in Table 2.1.1, then calculate the amount of DEPC-treated H₂O needed in Step 1 Table 2.1.2 to bring the final volume in Step 3 Table 2.1.2 to 20 µL.

1. 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. 5,569,584, 5,716,785, 5,891,636, 6,291,170 and 5,545,522 or to purchase another licensed kit.

Table 2.1.1
Reverse Transcriptase Volumes for First Strand cDNA Synthesis Reaction

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



Note

The combined volume of RNA DEPC-treated H_2O and SuperScript II RT should not exceed 11 μL as indicated in Table 2.1.2.

Table 2.1.2
First Strand cDNA Synthesis Components

	Reagents in reaction	Volume	Final concentration or amount in reaction
1: Primer Hybridization Incubate at 70°C for 10 minutes Quick spin and put on ice	DEPC-treated H_2O (variable) T7-oligo(dT) primer, 50 μM RNA (variable)	for final reaction volume of 20 μL 2 μL 5.0 to 20 μg	100 pmol 5.0 to 20 μg
2: Temperature Adjustment Add to the above tube and mix well Incubate at 42°C for 2 minutes	5X First strand cDNA buffer 0.1 M DTT 10 mM dNTP mix	4 μL 2 μL 1 μL	1X 10 mM DTT 500 μM each
3: First Strand Synthesis Add to the above tube and mix well Incubate at 42°C for 1 hour	SuperScript II RT (variable) (200 U/ μL)	See Table 2.1.1	200 U to 1000 U
Total Volume		20 μL	



Note

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

Step 2: Second Strand cDNA Synthesis

1. Place First Strand reactions on ice. Centrifuge briefly to bring down condensation on sides of tube.
2. Add to the First Strand synthesis tube the reagents listed in the following Second Strand Final Reaction Composition Table (Table 2.1.3).

Table 2.1.3
Second Strand Final Reaction Composition

Component	Volume	Final Concentration or Amount in Reaction
DEPC-treated water	91 μ L	
5X Second Strand Reaction Buffer	30 μ L	1X
10 mM dNTP mix	3 μ L	200 μ M each
10 U/ μ L <i>E. coli</i> DNA Ligase	1 μ L	10 U
10 U/ μ L <i>E. coli</i> DNA Polymerase I	4 μ L	40 U
2 U/ μ L <i>E. coli</i> RNase H	1 μ L	2 U
Final Volume	150 μ 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 μ L [10 U] T4 DNA Polymerase.
5. Return to 16°C for 5 minutes.
6. Add 10 μ L 0.5 M EDTA.
7. Proceed to cleanup procedure for cDNA, *Cleanup of Double-Stranded cDNA* on page 2.1.15, or store at -20°C for later use.

Synthesis of Double-Stranded cDNA 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 Kit² 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 be checked by gel electrophoresis.

T7-oligo(dT) primer

5' - GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-(dT)₂₄ - 3'

Step 1: First Strand cDNA Synthesis

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

✓ Note

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

Before starting cDNA synthesis, the correct volumes of DEPC-treated H₂O and Reverse Transcriptase (RT) must be determined. These volumes will depend on both the concentration and total volume of mRNA that is being added to the reaction. For every µg of mRNA, you will need to add 1 µL of SuperScript II RT (200 U/µL). For mRNA quantity ≤ 1 µg, use 1 µL of SuperScript II RT. Synthesis reactions should be done in a polypropylene tube (RNase-free).

➔ IMPORTANT

*Use Table 2.1.4 for variable component calculations. Determine volumes of mRNA and SuperScript II RT required, and then calculate the amount of DEPC-treated H₂O needed in the **Primer Hybridization Mix** step to bring the final First Strand Synthesis reaction volume to 20 µL.*

2. 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. 5,569,584, 5,716,785, 5,891,636, 6,291,170 and 5,545,522 or to purchase another licensed kit.

Table 2.1.4

First Strand cDNA Synthesis Components

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

Step 2: Second Strand cDNA Synthesis

- Place First Strand reactions on ice. Centrifuge briefly to bring down condensation on sides of tube.
- Add to the First Strand synthesis tube the reagents listed in the following Second Strand Final Reaction Composition Table (Table 2.1.5).

Table 2.1.5

Second Strand Final Reaction Composition

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

- 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.
- Add 2 μL [10 U] T4 DNA Polymerase.
- Return to 16°C for 5 minutes.
- Add 10 μL 0.5 M EDTA.
- Proceed to cleanup procedure for cDNA, *Cleanup of Double-Stranded cDNA* on page 2.1.15, or store at -20°C for later use.

Cleanup of 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 μ L cDNA Binding Buffer to the 162 μ L final double-stranded cDNA synthesis preparation (page 2.1.10 or 2.1.13). Mix by vortexing for 3 seconds.
2. Check that the color of the mixture is yellow (similar to cDNA Binding Buffer without the cDNA synthesis reaction).

✓ Note

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

3. Apply 500 μ L of the sample to the cDNA Cleanup Spin Column sitting in a 2 mL Collection Tube, 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 (262 μ L) and centrifuge as above. Discard flow-through and Collection Tube.
5. Transfer spin column into a new 2 mL Collection Tube (supplied). Pipet 750 μ L 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 above 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.
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 counter-clockwise 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 μ 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 μ L from 14 μ L 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.

8. An aliquot of the cDNA prepared from isolated poly-A RNA can be analyzed for size distribution and yield on a 1% agarose gel. One microliter of double-stranded cDNA should be sufficient to detect on an agarose gel stained with ethidium bromide. A representative gel is shown in **Figure 2.1.1** on page 2.1.22. We do not recommend gel analysis for cDNA prepared from total RNA.

✓ Note

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

9. After cleanup, please proceed to *Synthesis of Biotin-Labeled cRNA* on page 2.1.17.

Synthesis of Biotin-Labeled cRNA

✓ Note

The purity and quality of template cDNA is important for high yields of biotin-labeled RNA.

Use only RNase-free water, buffers, and pipette tips.

▶ IMPORTANT

Store all reagents at -20°C, in a freezer that is not self-defrosting.

Prior to use, centrifuge all reagents briefly to ensure that the components remain at the bottom of the tube.

The product should be used only until the expiration date stated on the label.

1. Enzo BioArray HighYield RNA Transcript Labeling Kit³ (Affymetrix, P/N 900182) is used for generating labeled cRNA target. Use the following tables to determine the amount of cDNA used for each IVT reaction. Done properly, each reaction should produce sufficient biotin-labeled target to hybridize to at least three standard format GeneChip expression probe arrays in parallel.

Table 2.1.6
cDNA in IVT (Total RNA)

Total RNA (μg)	Volume of cDNA to use in IVT*
5.0 to 8.0	10 μL
8.1 to 16.0	5 μL
16.1 to 20.0	3.3 μL

* assuming 12 μL was eluted from the column, as previously described.

Table 2.1.7
cDNA in IVT (Poly-A RNA)

Poly-A RNA (μg)	Volume cDNA*
0.2 - 0.5	10 μL
0.6 - 1.0	8 μL
1 - 2	5 μL

* assuming 12 μL was eluted from the column, as previously described.

3. For Research Use Only. This product is manufactured by ENZO LIFE SCIENCES, INC. for distribution by Affymetrix, Inc. for research purposes only by the end-user and is not intended for diagnostic or therapeutic use. Purchase does not include a license or the right to utilize this product except for research purposes. Purchase does not include the right to distribute or sell this product commercially. As distributed by Affymetrix, Inc., this product may be used only in conjunction with and is permitted for use only with Affymetrix® GeneChip® probe arrays.

Enzo is a registered trademark of Enzo Biochem, Inc. and BioArray is a trademark of Enzo Biochem, Inc.

This product or the use of this product is covered by one or more claims of Enzo patents including, but not limited to, the following: U.S. Patent Nos. 5,328,824; 5,449,767; 5,476,928; 4,711,955 and 4,994,373; EP 0 063 879 B1; EP 0 329 198 B1; DK 171 822 B; Canadian Patent Nos. 1,219,824 and 1,309,672; Japanese Patent Nos. 2,131,266; 1,416,584 and other patents pending.

IMPORTANT

Each GeneChip® Sample Cleanup Module contains 30 cDNA cleanup columns and 30 IVT cRNA cleanup columns. If more than one IVT is carried out from a single cDNA sample and is purified on separate IVT cRNA cleanup columns, there will not be sufficient IVT cRNA columns in each kit for 30 samples.

2. Add to RNase-free microfuge tubes template cDNA and additions of other reaction components in the order indicated in the following table. Keep reactions at room temperature while additions are made to avoid precipitation of DTT.

Table 2.1.8
IVT cRNA Labeling

Reagent	Volume
Template cDNA	Variable. Refer to Table 2.1.6 and Table 2.1.7.
Distilled or deionized water	Variable (to give a final reaction volume of 40 μ L).
10X HY Reaction Buffer (Vial 1)	4 μ L
10X Biotin-Labeled Ribonucleotides (Vial 2)	4 μ L
10X DTT (Vial 3)	4 μ L
10X RNase Inhibitor Mix (Vial 4)	4 μ L
20X T7 RNA Polymerase (Vial 5)	2 μ L
Total Volume	40 μL

3. Carefully mix the reagents and collect the mixture in the bottom of the tube by brief (5 second) microcentrifugation.
4. Immediately place the tube in a 37°C water bath. Incubate for 4 to 5 hours, gently mixing the contents of the tube every 30-45 minutes during the incubation.

✓ Note

Overnight incubation may produce shorter products which is less desirable.

5. Store labeled cRNA at -70°C, or -20°C if not purifying immediately.

Cleanup and Quantification of 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 *in vitro* transcription 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 µL 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 counter-clockwise direction). This avoids damage of the caps. Centrifugation with open caps allows complete drying of the membrane.
8. Transfer spin column into a new 1.5 mL Collection Tube (supplied), and pipet 11 μL of RNase-free Water directly onto the spin column membrane. Ensure that the water is dispensed directly onto the membrane. Centrifuge 1 minute at maximum speed ($\leq 25,000 \times g$) to elute.
9. Pipet 10 μL of RNase-free Water directly onto the spin column membrane. Ensure that the water is dispensed directly onto the membrane. Centrifuge 1 minute at maximum speed ($\leq 25,000 \times g$) to elute.
For subsequent photometric quantification of the purified cRNA, we recommend dilution of the eluate between 1:100 fold and 1:200 fold.

IMPORTANT

The minimum concentration for purified cRNA is 0.6 $\mu\text{g}/\mu\text{L}$ before starting the following fragmentation reaction in "Fragmenting the cRNA for Target Preparation" on page 2.1.21.

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:

$$\text{adjusted cRNA yield} = \text{RNA}_m - (\text{total RNA}_i)(y)$$

RNA_m = amount of cRNA measured after IVT (μg)

total RNA_i = starting amount of total RNA (μg)

y = fraction of cDNA reaction used in IVT

Example: Starting with 10 μg total RNA, 50% of the cDNA reaction is added to the IVT, giving a yield of 50 μg cRNA. Therefore, adjusted cRNA yield = 50 μg cRNA - (10 μg total RNA) (0.5 cDNA reaction) = 45.0 μg .

Use adjusted yield in *Eukaryotic Target Hybridization* on page 2.3.3.

Note

Please refer to Table 2.3.1 on page 2.3.7 for the amount of cRNA required for one array hybridization experiment. The amount varies depending on the array format. Please refer to a specific probe array package insert for information on the array format.

Step 3: Checking Unfragmented Samples by Gel Electrophoresis

Gel electrophoresis of the IVT product is done to estimate the yield and size distribution of labeled transcripts. Parallel gel runs of unpurified and purified IVT product can help determine the extent of a loss of sample during the cleanup process.

- Run 1% of each sample on a 1% agarose gel.
- Mix RNA (samples or markers) with loading dye and heat to 65°C for 5 minutes before loading on the gel.
- Ethidium bromide can be used to visualize the RNA in the gel. Alternatively, gels can be stained with SYBR Green II at a 1:10,000 dilution in 1X TBE buffer. SYBR Green II stains single-stranded RNA with greater sensitivity than ethidium bromide, but it requires a special photographic filter available from Molecular Probes to photograph stained bands.
- As an option, run a denaturing gel to obtain a more accurate estimation of the RNA size distribution. Please refer to Figure 2.1.1 for the typical size distribution of unfragmented cRNA.

Fragmenting the cRNA for Target Preparation

5X Fragmentation Buffer is supplied with the GeneChip Sample Cleanup Module.

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. The cRNA must be at a minimum concentration of 0.6 µg/µL. Fragment an appropriate amount of cRNA for hybridization cocktail and gel analysis (see Section 2, Chapter 3, Table 2.3.1).

1. Add 2 µL of 5X Fragmentation Buffer for every 8 µL of RNA plus H₂O. The fragmentation buffer has been optimized to break down full-length cRNA to 35 to 200 base fragments by metal-induced hydrolysis.

The final concentration of RNA in the fragmentation mix can range from 0.5 µg/µL to 2 µg/µL. The following table shows an example of a fragmentation mix for a 20 µg cRNA sample at a final concentration of 0.5 µg/µL.

For fragmentation, use **adjusted** cRNA concentration, as described in *Step 2: Quantification of the cRNA* on page 2.1.20.

Example for 0.5 µg/µL final concentration:

Table 2.1.9
Example of Fragmentation Reaction

Component	Volume
20 µg cRNA	1 to 21 µL
5X Fragmentation Buffer	8 µL
RNase-free water	to 40 µL

2. Incubate at 94°C for 35 minutes. Put on ice following the incubation.

3. Save an aliquot for gel analysis.

At least 1 μg fragmented cRNA is needed if using ethidium bromide for staining the gel. Less RNA can be used with SYBR Green II staining. See *Step 3: Checking Unfragmented Samples by Gel Electrophoresis* on page 2.1.21, for information regarding gel electrophoresis. The standard fragmentation procedure should produce a distribution of RNA fragment sizes from approximately 35 to 200 bases. An example of a gel with cRNA samples before and after fragmentation is shown below.

4. Store undiluted, fragmented sample RNA at -20°C until ready to perform the hybridization, as described in Section 2, Chapter 3.

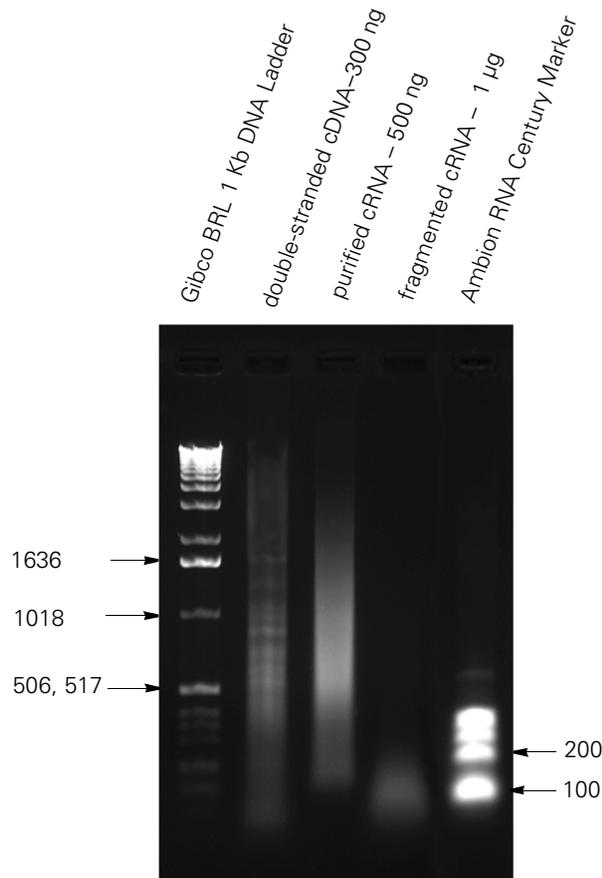


Figure 2.1.1
Monitoring of target preparation by agarose gel electrophoresis

Alternative Protocol for Cleanup of Double-Stranded cDNA

✓ Note

The following procedure may be followed as an alternative to using the cDNA cleanup columns in the GeneChip Sample Cleanup Module. Global concordance on array results obtained from using the two protocols has been established. However, Affymetrix encourages customers to evaluate their unique samples carefully before comparing results from the two cleanup protocols directly.

Step 1: Phase Lock Gels (PLG)-Phenol/Chloroform Extraction

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

✓ Note

A standard phenol/chloroform extraction can be performed as an alternative to the PLG procedure.

1. Pellet the Phase Lock Gel (1.5 mL tube with PLG I - heavy) in a microcentrifuge at 12,000 x g for 20 to 30 seconds.
2. Add 162 μ L (equal volume) of (25:24:1) Phenol:chloroform:isoamyl alcohol (saturated with 10 mM Tris-HCl pH 8.0, 1 mM EDTA) to the final cDNA synthesis preparation (162 μ L) to a final volume of 324 μ L. Vortex briefly.
(See Reagents and Materials Required on page 2.1.5 for ordering information for phenol:chloroform:isoamyl alcohol.)

✓ Note

Store phenol:chloroform:isoamyl alcohol at 4°C. Dispose of solution when it turns pink.

3. Transfer the entire cDNA-phenol/chloroform mixture to the PLG tube.
4. **DO NOT VORTEX.** PLG will now become part of the suspension. Microcentrifuge at full speed (12,000 x g) for 2 minutes.
5. Transfer the aqueous upper phase to a fresh 1.5 mL tube.

Step 2: Ethanol Precipitation

Please refer to Precipitation of RNA on page 2.1.9 for information on the use of carriers during ethanol precipitation.

1. Add 0.5 volumes of 7.5 M NH₄OAc and 2.5 volumes of absolute ethanol (stored at -20°C) to the sample and vortex.
2. Immediately centrifuge at 12,000 x g in a microcentrifuge at room temperature for 20 minutes.
3. Remove supernatant. Wash pellet with 0.5 mL of 80% ethanol (stored at -20°C).
4. Centrifuge at 12,000 x g at room temperature for 5 minutes.
5. Remove the 80% ethanol very carefully; the pellet may be loose. Repeat the 80% ethanol wash one additional time.

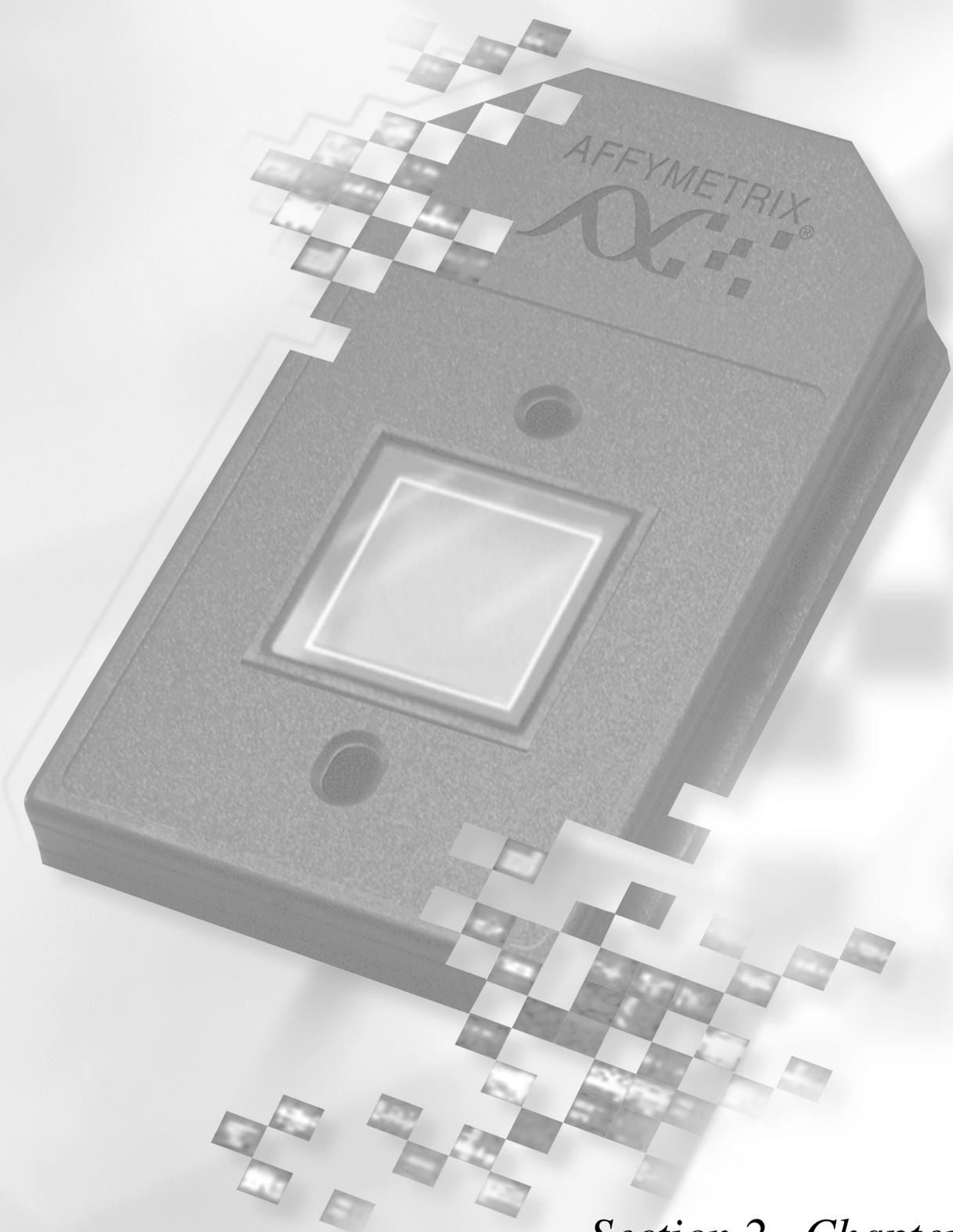
6. Air dry the pellet. Check pellet for dryness before proceeding.
7. Resuspend dried pellet in a small volume of RNase-free water. We recommend resuspending in 12 μ L.

Alternative Protocol for Preparing 5X Fragmentation Buffer

5X RNA Fragmentation Buffer (200 mM Tris-acetate, pH 8.2, 500 mM KOAc, 150 mM MgOAc)

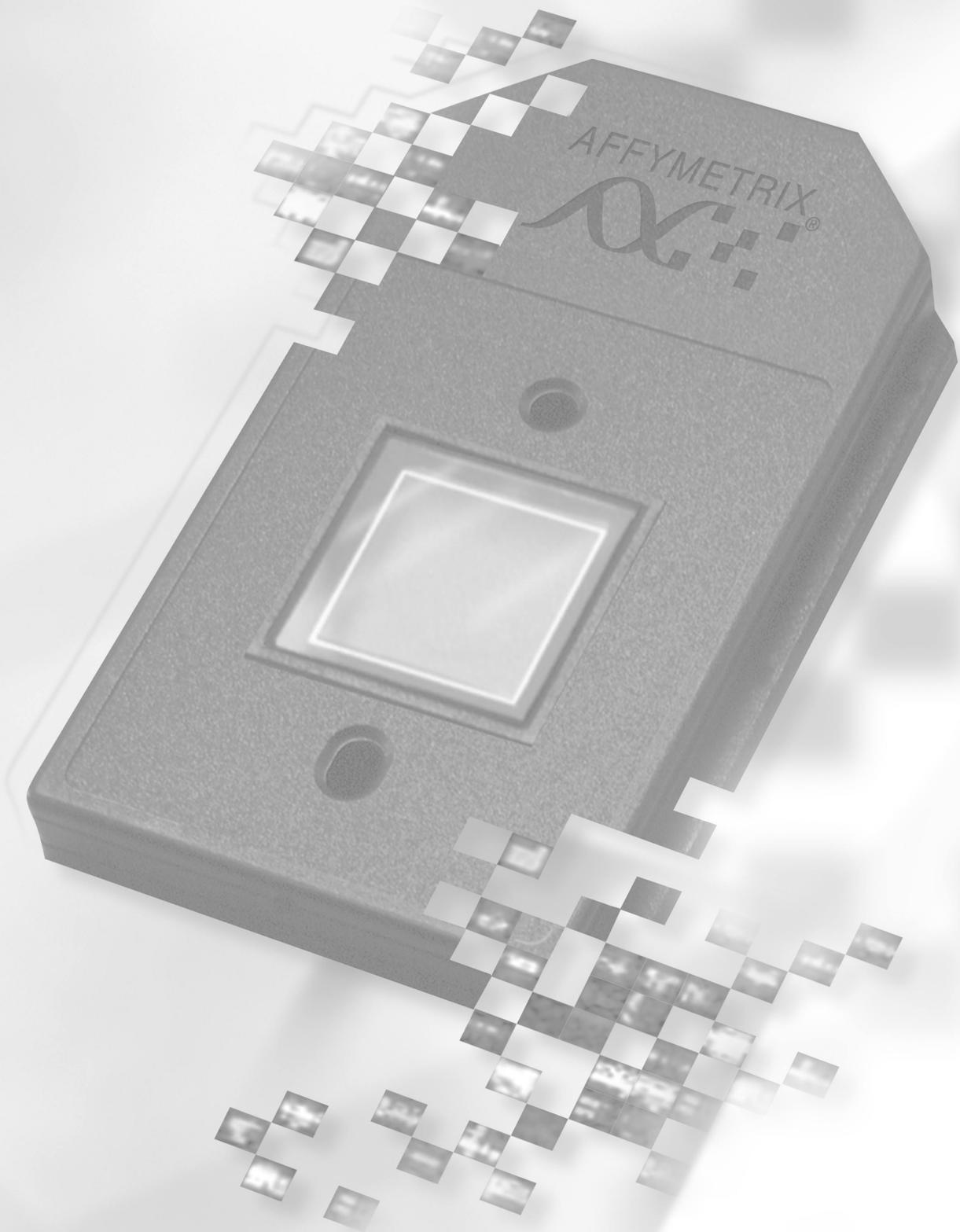
The fragmentation buffer is made with RNase-free reagents. Tris-containing solutions should not be treated with DEPC; however, once H₂O has been DEPC-treated and autoclaved it can be used for making the Tris solution.

1. Combine the following components to a total volume of 20 mL.
 - 4.0 mL 1 M Tris acetate pH 8.1 (Trizma Base, pH adjusted with glacial acetic acid)
 - 0.64 g MgOAc
 - 0.98 g KOAc
 - DEPC-treated H₂O to 20 mLFinal pH without adjustment should be 8.2.
2. Mix thoroughly and filter through a 0.2 μ m vacuum filter unit. This reagent should be aliquotted and stored at room temperature.



Section 2, Chapter 2

Section 2, Chapter 2





Controls for Eukaryotic Arrays

Reagents and Materials Required	2.2.5
Hybridization Control Kit	2.2.7
Poly-A Spike Controls	2.2.7

This Chapter Contains:

- General guidelines for producing controls for eukaryotic arrays.

After completing the procedures described in this chapter, the control transcripts are combined in variable concentrations before adding to the target hybridization mix as explained in Section 2, Chapter 3.

Reagents and Materials Required

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

GeneChip Eukaryotic Hybridization Control Kit (Complete Kit)

- Affymetrix, P/N 900299 (30 reactions) or P/N 900362 (150 reactions)

Poly-A Spike Controls

- pGIBS-lys ATCC 87482
- pGIBS-phe ATCC 87483
- pGIBS-thr ATCC 87484
- pGIBS-trp ATCC 87485
- pGIBS-dap ATCC 87486

Hybridization Control Kit

Each commercially available eukaryotic probe array contains probe sets for several prokaryotic genes as controls. These probe sets are readily identified by the AFFX prefix in the probe set name. The .chp data for these control probe sets can be examined in the Summary Report File (.rpt).

Control Oligo B2

Control Oligo B2 hybridizes to features along the outer edge of all expression arrays and to the checkerboard pattern in each corner. These predefined patterns provide signals for the Microarray Suite software to perform automatic grid alignment during image analysis. They can also be used to align the grid manually. The fluorescence intensities for Control Oligo B2 are not used for analyzing data.

A 60X stock of the B2 oligo is provided as part of the GeneChip Eukaryotic Hybridization Control Kit (P/N 900299 or 900362, for 30 or 150 reactions, respectively), or can be purchased alone (P/N 900301). Please refer to the instructions in Section 2, Chapter 3 for detailed information on including the B2 oligo in preparing the hybridization cocktail.

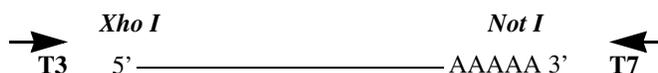
Biotinylated Hybridization Controls: *bioB*, *bioC*, *bioD*, and *cre*

BioB, *bioC*, and *bioD* are genes of the biotin synthesis pathway from the bacteria *E. coli*, and *cre* is the recombinase gene from P1 bacteriophage. A ready-prepared mixture of these biotinylated controls at staggered concentrations can be added with labeled eukaryotic cRNA samples to hybridize onto GeneChip probe arrays. Signal intensities obtained on these genes provide information on how well the hybridization, washing and staining procedures have performed.

Affymetrix provides a kit that contains a 20X pre-mixed control reagents (P/N 900299 or 900362) and the final concentrations in the hybridization cocktail are 1.5 pM, 5 pM, 25 pM and 100 pM for the four transcripts *bioB*, *bioC*, *bioD*, and *cre*, respectively.

Poly-A Spike Controls

Five poly-A-tailed control clones encoding *B. subtilis* genes (*dap*, *thr*, *trp*, *phe*, *lys*) are cloned into pBluescript as an *Xho I* to *Not I* insert, 5' to 3', respectively.

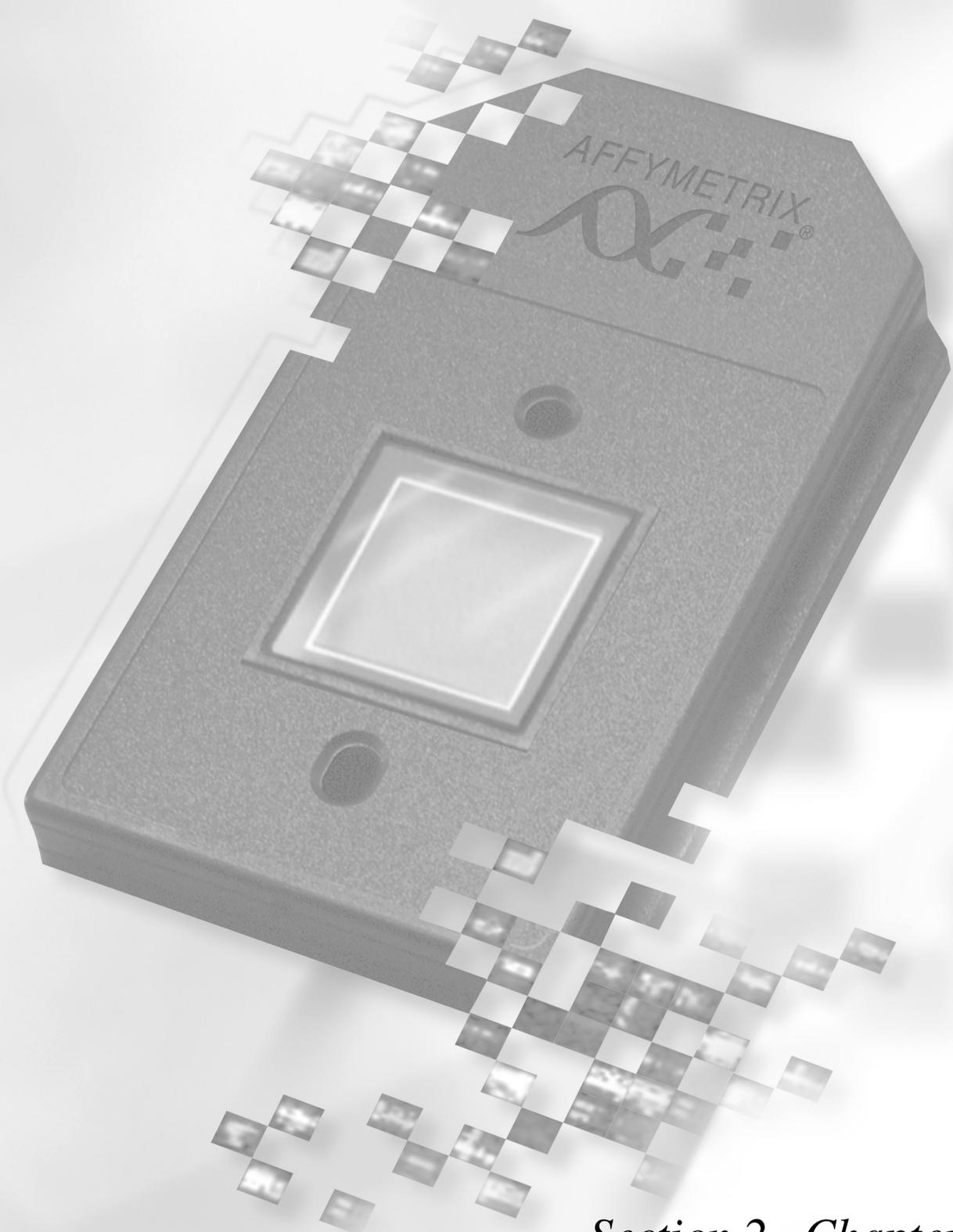


Poly-A-tailed constructs (*dap*, *thr*, *trp*, *phe*, *lys*)

These clones can be cut with different restriction enzymes to produce template DNA for either sense strand RNA synthesis or antisense RNA synthesis. The antisense control RNA for each *B. subtilis* gene is synthesized from linearized plasmid using T7 RNA polymerase with biotinylated nucleotides. The sense RNA for each *B. subtilis* gene is synthesized from linearized plasmid using T3 RNA polymerase with unlabeled nucleotides. For detailed preparation of sense RNA controls, please refer to Section 3, Chapter 2.

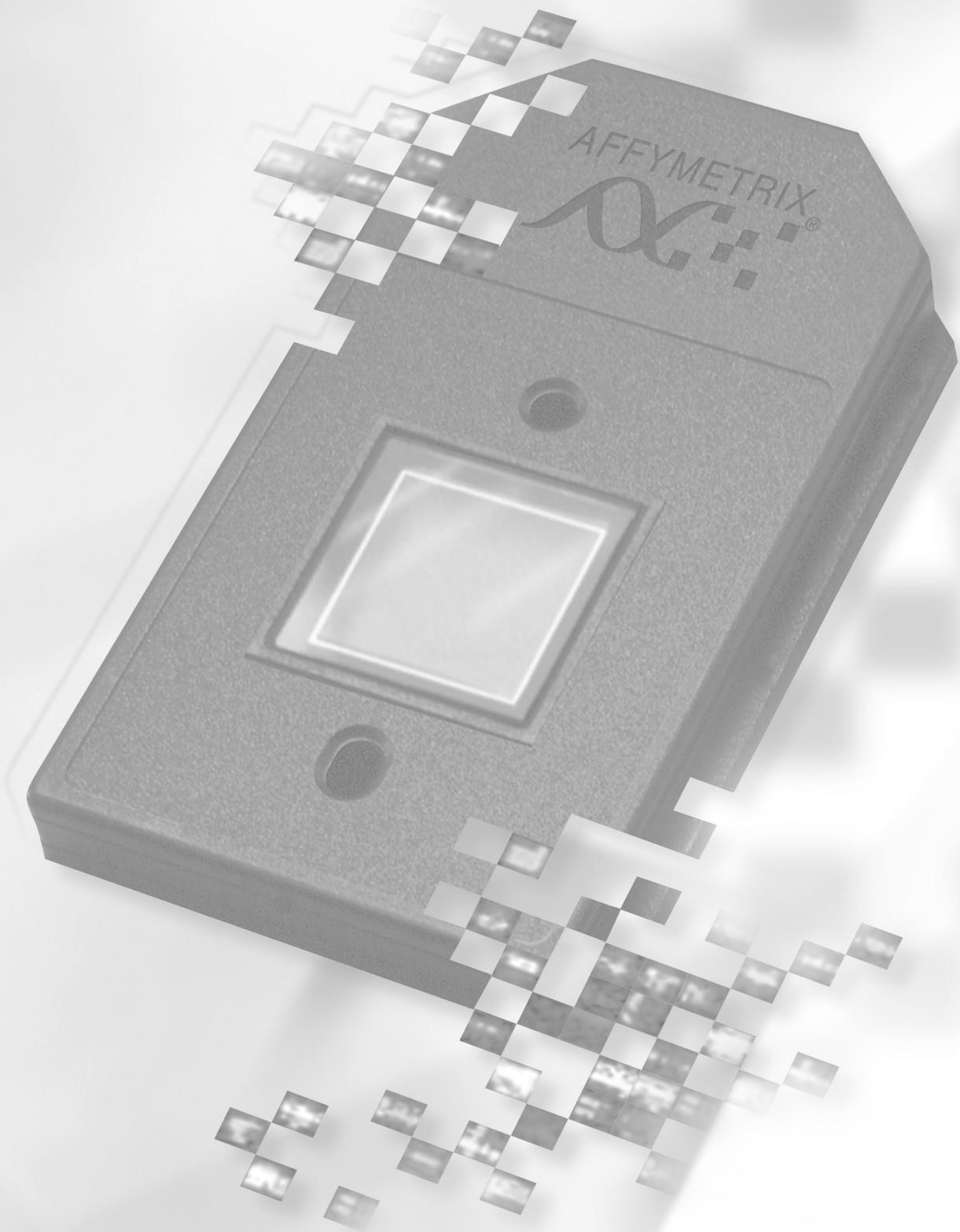
The antisense strand *B. subtilis* RNA controls are used as described above for *bioB*, *bioC*, and *bioD* genes. The sense strand RNA controls can be spiked into samples during mRNA preparation to monitor the efficiency of target preparation, hybridization, wash, and stain.

Bacteria containing these recombinant plasmids can be obtained from the American Type Culture Collection (ATCC). See *Reagents and Materials Required* on page 2.2.5 for details.



Section 2, Chapter 3

Section 2, Chapter 3





Eukaryotic Target Hybridization

Reagents and Materials Required	2.3.5
Reagent Preparation	2.3.6
Eukaryotic Target Hybridization	2.3.7

This Chapter Contains:

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

After completing the procedures described in this chapter, the hybridized probe array is ready for washing, staining, and scanning as detailed in Section 2, Chapter 4.

Reagents and Materials Required

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

- Water, Molecular Biology Grade, BioWhittaker Molecular Applications / Cambrex, P/N 51200
- Acetylated Bovine Serum Albumin (BSA) solution (50 mg/mL), Invitrogen Life Technologies, P/N 15561-020
- Herring Sperm DNA, Promega Corporation, P/N D1811
- Micropure Separator, Millipore, P/N 42512 (optional)
- GeneChip Eukaryotic Hybridization Control Kit, Affymetrix, P/N 900299 (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 Free Acid Monohydrate 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

Miscellaneous Reagents

- Tough Spots, Label Dots, USA Scientific, P/N 9185 (optional)
- Surfact-Amps 20 (Tween-20), 10%, Pierce Chemical, P/N 28320

Miscellaneous Supplies

- Hybridization Oven 640, Affymetrix, P/N 800139
- Sterile, RNase-free, microcentrifuge tubes, 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

(1.22 M MES, 0.89 M [Na⁺])

For 1000 mL:

70.4 g MES-free acid monohydrate

193.3 g MES Sodium Salt

800 mL of Molecular Biology Grade water

Mix and adjust volume to 1000 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⁺], 20 mM EDTA, 0.01% Tween 20)

For 50 mL:

8.3 mL of 12X MES Stock

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

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

Table 2.3.1
Hybridization Cocktail for Single Probe Array*

Component	Micro/Mini Array	Midi Array	Standard Array	Final Concentration
Fragmented cRNA **	5 μg	10 μg	15 μg	0.05 $\mu\text{g}/\mu\text{L}$
Control Oligonucleotide B2 (3 nM)	1.7 μL	3.3 μL	5 μL	50 pM
20X Eukaryotic Hybridization Controls (<i>bioB</i> , <i>bioC</i> , <i>bioD</i> , <i>cre</i>)	5 μL	10 μL	15 μL	1.5, 5, 25 and 100 pM respectively
Herring Sperm DNA (10 mg/mL)	1 μL	2 μL	3 μL	0.1 mg/mL
Acetylated BSA (50 mg/mL)	1 μL	2 μL	3 μL	0.5 mg/mL
2X Hybridization Buffer	50 μL	100 μL	150 μL	1X
H ₂ O	to final volume of 100 μL	to final volume of 200 μL	to final volume of 300 μL	
Final volume	100 μL	200 μL	300 μL	

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

**Please see Section 2, Chapter 1, page 2.1.20 for amount of adjusted fragmented cRNA to use when starting from total RNA.

IMPORTANT

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

- 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.

- Heat the hybridization cocktail to 99°C for 5 minutes in a heat block.
- Meanwhile, wet the array by filling it through one of the septa (see **Figure 2.3.1** for location of the probe array septa) with appropriate volume 1X Hybridization Buffer using a micropipettor and appropriate tips (**Table 2.3.2**).

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. After the addition of hybridization cocktails to the array, the septa may be covered with Tough Spots to prevent evaporation.

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

Table 2.3.2
Probe Array Cartridge Volumes

Array	Hybridization Volume	Total Fill Volume
Standard	200 μ L	250 μ L
Midi	130 μ L	160 μ L
Mini	80 μ L	100 μ L
Micro	80 μ L	100 μ L

- Transfer the hybridization cocktail that has been heated at 99°C, in step 3, to a 45°C heat block for 5 minutes.
- Spin hybridization cocktail(s) at maximum speed in a microcentrifuge for 5 minutes to remove any insoluble material from the hybridization mixture.
- Remove the buffer solution from the probe array cartridge and fill with appropriate volume (Table 2.3.2 on page 2.3.8) of the clarified hybridization cocktail avoiding any insoluble matter in the volume at the bottom of the tube.
- Place probe array in rotisserie box in 45°C oven.
Avoid stress to rotisserie motor; load probe arrays in a balanced configuration around rotisserie axis. Rotate at 60 rpm.
- Hybridize for 16 hours.
During the latter part of the 16-hour hybridization, proceed to Section 2, Chapter 4 to prepare reagents required immediately after completion of hybridization.

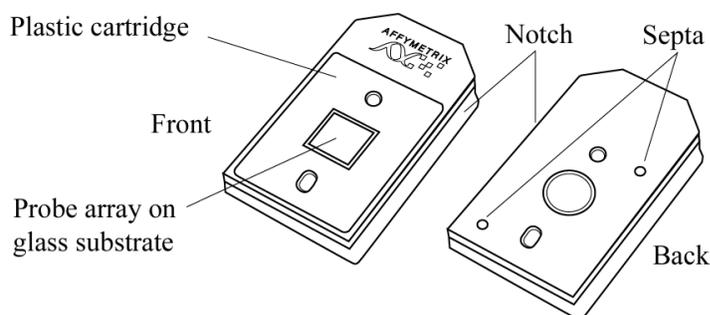
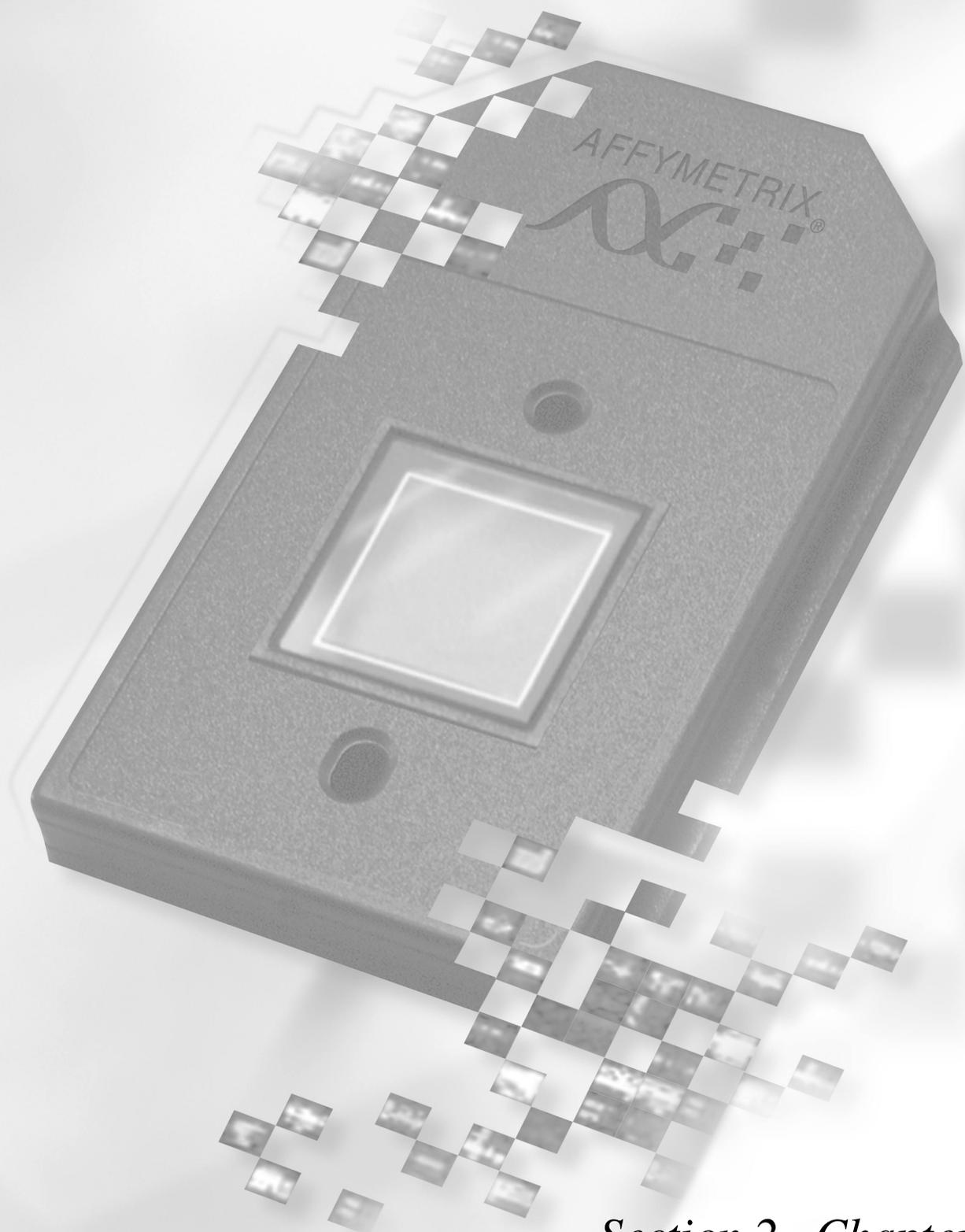
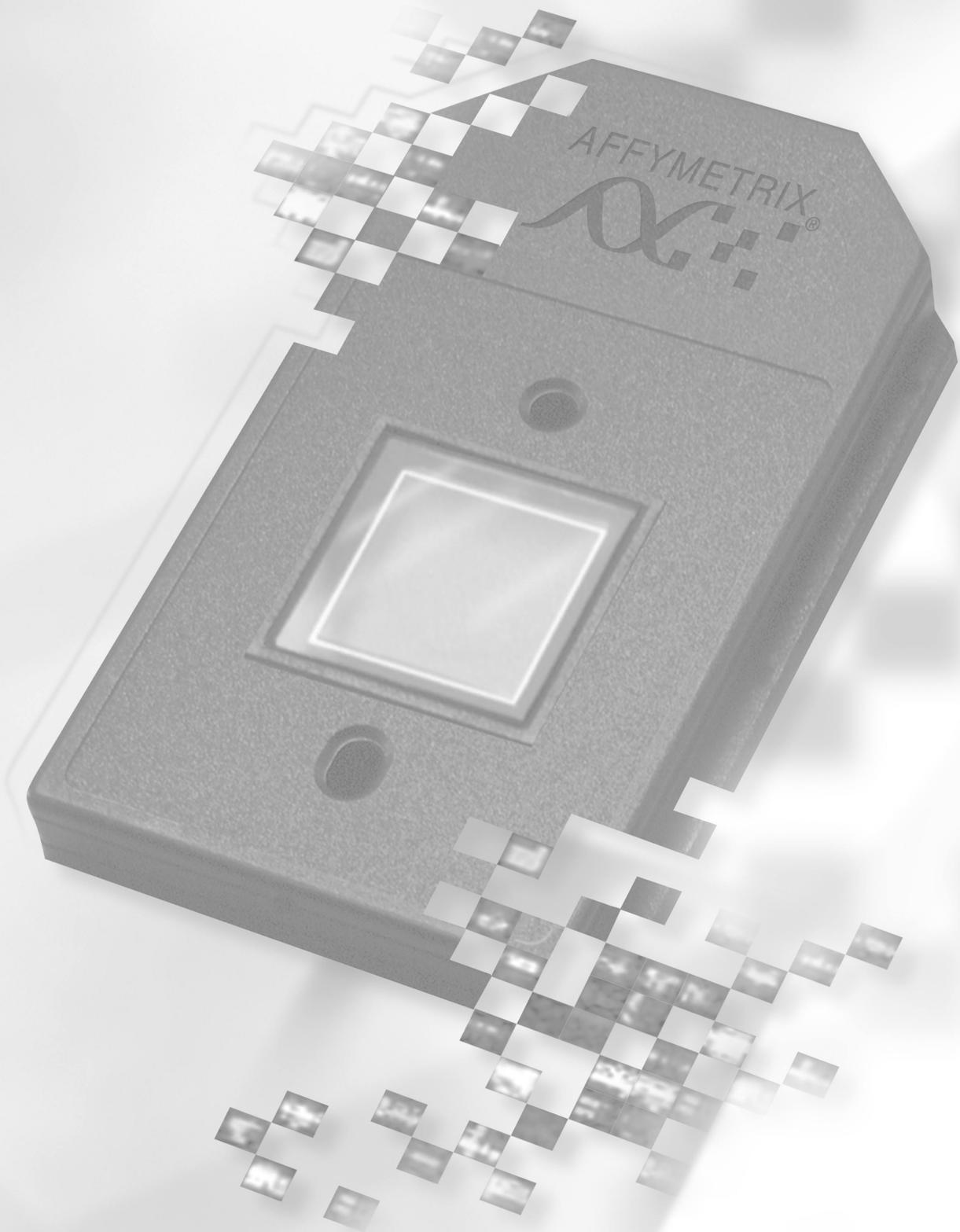


Figure 2.3.1
GeneChip® Probe Array



Section 2, Chapter 4

Section 2, Chapter 4





Eukaryotic Arrays: Washing, Staining, and Scanning

Reagents and Materials Required	2.4.5
Reagent Preparation	2.4.6
Experiment and Fluidics Station Setup	2.4.7
Step 1: Defining File Locations	2.4.7
Step 2: Entering Experiment Information.	2.4.7
Step 3: Preparing the Fluidics Station.	2.4.8
Probe Array Wash and Stain	2.4.9
Washing and Staining Procedure 1: Single Stain for Eukaryotic Targets	2.4.9
Washing and Staining Procedure 2: Antibody Amplification for Eukaryotic Targets	2.4.12
Probe Array Scan	2.4.15
Shutting Down the Fluidics Station	2.4.16
Customizing the Protocol	2.4.17

This Chapter Contains:

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

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

Reagents and Materials Required

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

- Water, Molecular Biology Grade, BioWhittaker Molecular Applications / Cambrex, P/N 51200
- Distilled water, Invitrogen Life Technologies, P/N 15230147
- Acetylated Bovine Serum Albumin (BSA) solution (50 mg/mL), Invitrogen Life Technologies, P/N 15561-020
- R-Phycoerythrin Streptavidin, Molecular Probes, P/N S-866
- 5 M NaCl, RNase-free, DNase-free, Ambion, P/N 9760G
- PBS, pH 7.2, Invitrogen Life Technologies, P/N 20012-027
- 20X SSPE (3 M NaCl, 0.2 M NaH₂PO₄, 0.02 M EDTA), BioWhittaker Molecular Applications / Cambrex, P/N 51214
- Goat IgG, Reagent Grade, Sigma-Aldrich, P/N I 5256
- Anti-streptavidin antibody (goat), biotinylated, Vector Laboratories, P/N BA-0500
- 10% surfact-Amps20 (Tween-20), Pierce Chemical, P/N 28320
- Bleach (5.25% Sodium Hypochlorite), VWR Scientific, P/N 21899-504 (or equivalent)

Miscellaneous Supplies

- Sterile, RNase-free, microcentrifuge tubes, 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-Palmer, P/N H-06418-04
- Water, Molecular Biology Grade, BioWhittaker Molecular Applications / Cambrex, P/N 51200

Reagent Preparation

Wash A: Non-Stringent Wash Buffer

(6X SSPE, 0.01% Tween 20)

For 1000 mL:

300 mL of 20X SSPE

1.0 mL of 10% Tween-20

699 mL of water

Filter through a 0.2 μ m filter

Wash B: Stringent Wash Buffer

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

For 1000 mL:

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

5.2 mL of 5 M NaCl

1.0 mL of 10% Tween 20

910.5 mL of water

Filter through a 0.2 μ m filter

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

2X Stain Buffer

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

For 250 mL:

41.7 mL 12X MES Stock Buffer (see Section 2, Chapter 3 for reagent preparation)

92.5 mL 5 M NaCl

2.5 mL 10% Tween 20

113.3 mL water

Filter through a 0.2 μ m 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.

Experiment and Fluidics Station Setup

Step 1: Defining File Locations

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

1. Launch Microarray Suite from the workstation and select **Tools** → **Defaults** → **File Locations** from the menu bar.
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 defined in Microarray Suite.

1. Select **Run** → **Experiment Info** from the menu bar. Alternatively, click the New Experiment icon on the tool bar.
 - ⇒ The Experiment Information dialog box appears allowing the experiment name to be defined along with several other parameters, such as probe array type, sample description, and comments.
2. Type in the **Experiment Name**.
3. In the **Probe Array Type** box, click the arrow and select the probe array type from the drop-down list.

Experiment name and probe array type are required. Complete as much of the other information as desired. The protocol information at the bottom of the dialog box is exported to the experiment information dialog box after the hybridization and scan are completed.
4. Save the experiment by selecting **Save**.

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

Step 3: Preparing the Fluidics Station

The Fluidics Station 400 is used to wash and stain the probe arrays. It is operated using Microarray Suite.

Setting Up the Fluidics Station

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

**Note**

Refer to the Fluidics Station 400 User's Guide for instructions on connecting and addressing multiple fluidics stations.

Priming the Fluidics Station

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

Priming should be done:

- when the fluidics station is first started.
 - when wash solutions are changed.
 - before washing, if a shutdown has been performed.
 - if the LCD window instructs the user to prime.
1. To prime the fluidics station, select **Protocol** in the Fluidics Station dialog box.
 2. Choose **Prime** for the respective modules in the Protocol drop-down list.
 3. Change the intake buffer reservoir A to **Non-Stringent Wash Buffer** and intake buffer reservoir B to **Stringent Wash Buffer**.
 4. Click **Run** for each module to begin priming.

Probe Array Wash and Stain

Affymetrix offers two staining protocols: 1) the single stain protocol for eukaryotic targets (page 4.9), and 2) a signal amplification protocol for eukaryotic targets (page 4.12). Please use the *Antibody Amplification Washing and Staining Protocol* for all arrays with probe cells of 24 μm or smaller.

1. After 16 hours of hybridization, remove the hybridization cocktail from the probe array and set it aside in a microcentrifuge tube. Store on ice during the procedure or at -80°C for long-term storage.
2. Fill the probe array completely with the appropriate volume of Non-Stringent Wash Buffer, as given in Table 2.3.2 on page 2.3.8.

✓ Note

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

Washing and Staining Procedure 1: Single Stain for Eukaryotic Targets

✓ Note

Volumes needed will be the same for all fluidics protocols. This procedure takes approximately 75 minutes to complete.

Preparing the SAPE Stain Solution

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

For each probe array to be stained, combine the following components in a microcentrifuge tube:

Table 2.4.1
SAPE Solution Mix

Components	Volume	Final Concentration
2X MES Stain Buffer	300.0 μL	1X
50 mg/mL acetylated BSA	24.0 μL	2 mg/mL
1 mg/mL Streptavidin Phycoerythrin (SAPE)	6.0 μL	10 $\mu\text{g/mL}$
DI H_2O	270.0 μL	—
Total	600 μL	

Table 2.4.2
Fluidics Protocols - Single Stain for Eukaryotic Targets

	Standard Format EukGE-WS1	Mini Format Mini_euk1
Post Hyb Wash #1	10 cycles of 2 mixes/cycle with Wash Buffer A at 25°C	10 cycles of 2 mixes/cycle with Wash Buffer A at 25°C
Post Hyb Wash #2	4 cycles of 15 mixes/cycle with Wash Buffer B at 50°C	8 cycles of 15 mixes/cycle with Wash Buffer B at 50°C
Stain	Stain the probe array for 30 minutes in SAPE solution at 25°C	Stain the probe array for 10 minutes in SAPE solution at 25°C
Final Wash	10 cycles of 4 mixes/cycle with Wash Buffer A at 25°C. The holding temperature is 25°C	10 cycles of 4 mixes/cycle with Wash Buffer A at 30°C. The holding temperature is 25°C

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

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 specific single stain protocol to control the washing and staining of the probe array format being used: **Table 2.4.2**.
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 probe array lever is in the **EJECT** position. When finished, verify that the probe array 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 the microcentrifuge tube containing the SAPE stain solution into the sample holder, verifying 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. When the wash is complete, the LCD window displays the message **EJECT CARTRIDGE**.
7. Remove microcentrifuge tube containing stain and replace with an empty microcentrifuge tube.
8. Remove the probe arrays from the fluidics station modules by first moving the probe array holder lever to the **EJECT** position.
9. Check the probe array window for large bubbles or air pockets.

- If bubbles are present, proceed to **Table 2.4.3**.
- If the probe array has no large bubbles, it is ready to scan on the GeneArray Scanner. **ENGAGE** wash block and proceed to *Probe Array Scan* on page 2.4.15.

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

If there are no more samples to hybridize, shut down the fluidics station following the procedure outlined in the section, *Shutting Down the Fluidics Station* on page 2.4.16.

✓ Note

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

Table 2.4.3

If Bubbles are Present

Return the probe array to the probe array holder. Latch the probe array holder by gently pushing it up until a light click is heard. Engage the wash block by firmly pushing up on the probe array 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, if the LCD window displays **EJECT CARTRIDGE** again, remove the probe array and inspect it again for bubbles. If no bubbles are present, it is ready to scan. Proceed to *Probe Array Scan* on page 2.4.15. If several attempts to fill the probe array without bubbles are unsuccessful, the array should be filled with **Wash A (non-stringent buffer)** manually, using a micropipette. Excessive washing will result in a loss of signal intensity.

Washing and Staining Procedure 2: Antibody Amplification for Eukaryotic Targets

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

Preparing the Staining Reagents

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

SAPE Stain Solution

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

Table 2.4.4
SAPE Solution Mix

Components	Volume	Final Concentration
2X MES Stain Buffer	600.0 μL	1X
50 mg/mL acetylated BSA	48.0 μL	2 mg/mL
1 mg/mL Streptavidin Phycoerythrin (SAPE)	12.0 μL	10 $\mu\text{g}/\text{mL}$
DI H ₂ O	540.0 μL	—
Total	1200 μL	

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

Antibody Solution

Table 2.4.5
Antibody Solution Mix

Components	Volume	Final Concentration
2X MES Stain Buffer	300.0 μL	1X
50 mg/mL acetylated BSA	24.0 μL	2 mg/mL
10 mg/mL Normal Goat IgG	6.0 μL	0.1 mg/mL
0.5 mg/mL biotinylated antibody	3.6 μL	3 $\mu\text{g}/\text{mL}$
DI H ₂ O	266.4 μL	—
Total	600 μL	

Table 2.4.6

Fluidics Protocols - Antibody Amplification for Eukaryotic Targets

	Standard Format EukGE-WS2	Midi Format Midi_euk2	Micro / Mini Format Micro_1v1 / Mini_euk2
Post Hyb Wash #1	10 cycles of 2 mixes/cycle with Wash Buffer A at 25°C	10 cycles of 2 mixes/cycle with Wash Buffer A at 30°C	10 cycles of 2 mixes/cycle with Wash Buffer A at 25°C
Post Hyb Wash #2	4 cycles of 15 mixes/cycle with Wash Buffer B at 50°C	6 cycles of 15 mixes/cycle with Wash Buffer B at 50°C	8 cycles of 15 mixes/cycle with Wash Buffer B at 50°C
Stain	Stain the probe array for 10 minutes in SAPE solution at 25°C	Stain the probe array for 5 minutes in SAPE solution at 35°C	Stain the probe array for 10 minutes in SAPE solution at 25°C
Post Stain Wash	10 cycles of 4 mixes/cycle with Wash Buffer A at 25°C	10 cycles of 4 mixes/cycle with Wash Buffer A at 30°C	10 cycles of 4 mixes/cycle with Wash Buffer A at 30°C
2nd Stain	Stain the probe array for 10 minutes in antibody solution at 25°C	Stain the probe array for 5 minutes in antibody solution at 35°C	Stain the probe array for 10 minutes in antibody solution at 25°C
3rd Stain	Stain the probe array for 10 minutes in SAPE solution at 25°C	Stain the probe array for 5 minutes in SAPE solution at 35°C	Stain the probe array for 10 minutes in SAPE solution at 25°C
Final Wash	15 cycles of 4 mixes/cycle with Wash Buffer A at 30°C The holding temperature is 25°C	15 cycles of 4 mixes/cycle with Wash Buffer A at 35°C The holding temperature is 25°C	15 cycles of 4 mixes/cycle with Wash Buffer A at 35°C The holding temperature is 25°C

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

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.

✓ Note

Three-stain protocols require the user to replace stain solutions as directed by the LCD window during staining steps.

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 *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 probe array lever is in the **EJECT** position. When finished, verify that the probe array 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 the microcentrifuge tube containing the SAPE stain solution into the sample holder, verifying 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. When the wash is complete, the LCD window displays the message **EJECT CARTRIDGE**.
7. Remove microcentrifuge tube containing stain and replace with an empty microcentrifuge tube.
8. Remove the probe arrays from the fluidics station modules by first moving the probe array holder lever to the **EJECT** position.
9. Check the probe array window for large bubbles or air pockets.
 - If bubbles are present, proceed to **Table 2.4.3**.
 - If the probe array has no large bubbles, it is ready to scan on the GeneArray Scanner. **ENGAGE** wash block and proceed to *Probe Array Scan* on page 2.4.15.

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

If there are no more samples to hybridize, shut down the fluidics station following the procedure outlined in the section, *Shutting Down the Fluidics Station* on page 2.4.16.

**Note**

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

Table 2.4.7

If Bubbles are Present

Return the probe array to the probe array holder. Latch the probe array holder by gently pushing it up until a light click is heard. Engage the washblock by firmly pushing up on the probe array 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, if the LCD window displays **EJECT CARTRIDGE** again, remove the probe array and inspect it again for bubbles. If no bubbles are present, it is ready to scan. Proceed to *Probe Array Scan* on page 2.4.15.

If several attempts to fill the probe array without bubbles are unsuccessful, the array should be filled with **Wash A (non-stringent buffer)** manually, using a micropipette. Excessive washing will result in a loss of signal intensity.

Probe Array Scan

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

If necessary, clean the glass surface of probe array with a non-abrasive towel or tissue before scanning. **Do not use alcohol to clean glass.**

✓ Note

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

1. Click **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, after selecting the experiment the number [2] is displayed in the **Number of Scans** box to perform the recommended 2X image scan.
4. Once the experiment has been selected, click the **Start** button.
 - ⇒ A dialog box prompts you to load a sample into the scanner.
5. Click the **Options** button to check for the correct pixel value and wavelength of the laser beam.

For Probe Arrays with Probe Cells 24 µm or Less

 - Pixel value = 3 µm
 - Wavelength = 570 nm

For a 50 µm Probe Array with a Phycoerythrin Stain

 - Pixel value = 6 µm
 - Wavelength = 570 nm
6. Open the sample door on the scanner and insert the probe array into the holder. Do not force the probe array into the holder. Close the sample door of the scanner.
7. Click **OK** in the Start Scanner dialog box.
 - ⇒ The scanner begins scanning the probe array and acquiring data. When **Scan in Progress** is selected from the **View** menu, the probe array image appears on the screen as the scan progresses.

Shutting Down the Fluidics Station

1. After removing a probe array from the probe array holder, the LCD window displays the message **ENGAGE WASHBLOCK**.
2. Engage the washblock by firmly pushing up on the probe array lever to the **ENGAGE** position.
⇒ 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 VIAL**, the Cleanout procedure is complete.
4. Remove the sample microcentrifuge tube from the sample holder.
5. If no other hybridizations are to be performed, place wash lines into a bottle filled with deionized water.
6. Choose **Shutdown** 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 *Fluidics Station 400 User's Guide* for more information.
7. After Shutdown protocol is complete, flip the ON/OFF switch of the fluidics station to the OFF position.

▶ IMPORTANT

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

Customizing the Protocol

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

1. Select **Tools** → **Edit Protocol** from the menu bar.
⇒ The Edit Protocol dialog box appears.
2. Select the protocol to be changed from the **Protocol Name** drop-down list.
⇒ The name of the protocol is displayed in the Protocol Name box. The conditions for that protocol are displayed on the right side of the Edit Protocol dialog box.
3. Select the item to be changed and input the new parameters as needed, keeping the parameters within the ranges shown below in **Table 2.4.8**.

Table 2.4.8
Valid Ranges for Wash/Stain Parameters

Parameter	Valid Range
Wash Temperature for A1, B, A2, or A3 (°C)	15 to 50
Number of Wash Cycles for A1, B, A2, or A3	0 to 99
Mixes / Wash cycle for A1, B, A2, or A3	15 to 50
Stain Time (seconds)	0 to 86399
Stain Temperature (°C)	15 to 50
Holding Temperature (°C)	15 to 50
<ul style="list-style-type: none"> • Wash A1 corresponds to Post Hyb wash #1 in Tables 2.4.2 and 2.4.6. • Wash B corresponds to Post Hyb wash #2 in Tables 2.4.2 and 2.4.6. • Wash A2 corresponds to Post Stain Wash in Tables 2.4.2 and 2.4.6. • Wash A3 corresponds to Final Wash in Tables 2.4.2 and 2.4.6. 	

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

! CAUTION

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

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

