




# GeneChip<sup>®</sup> Mapping 10K 2.0 Assay Manual

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701722 Rev. 3



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# Introduction

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*This manual serves as a guide for technical personnel conducting GeneChip® Mapping 10K 2.0 experiments in the laboratory. It contains protocols for sample preparation, array hybridization, information for washing, staining, and scanning arrays, and generating genotype calls. It also includes extensive troubleshooting information. This technical manual is divided into chapters which describe in detail specific aspects of the GeneChip Mapping 10K Assay. A description of each chapter follows.*

**Chapter 1: Scientific Overview.** A description of the overall concept behind the GeneChip Mapping assay, including the biochemical process, data generation, potential applications, and a list of references.

**Chapter 2: Laboratory Setup.** An explanation of the appropriate laboratory configuration for running GeneChip Mapping experiments including how to set up a workflow to minimize the possibility of carryover contamination.

**Chapter 3: Genomic DNA Preparation.** The requirements for genomic DNA, with recommended sources and methods for purification and quantitation.

**Chapter 4: Experimental Protocols.** Detailed, step-by-step protocols for preparation of sample from genomic DNA, including restriction digestion, adaptor ligation, amplification, purification, quantitation, fragmentation, labeling and array hybridization. A description of quality control checkpoints included at various stages of the protocol which enable array performance to be monitored.

**Chapter 5: Washing, Staining, and Scanning.** Basic protocols for fluidics station and scanner operation.

**Chapter 6: Fluidics Station Maintenance Procedures.** Describes a weekly fluidics station bleach protocol and a monthly fluidics station decontamination protocol.

**Chapter 7: Data Analysis.** Contents describe the generation from scanned arrays of multiple file types to aid technical personnel to assess experimental results and perform simple analyses. The user is referred to a separate software manual, *Affymetrix GeneChip DNA Analysis Software User's Guide*, for a more in-depth description of software capabilities.

**Chapter 8: Troubleshooting.** Additional guidelines for obtaining optimal assay results including troubleshooting.

**Chapter 9: Affymetrix Technical Support.** Contact information.

**Chapter 10: Reagents, Instruments, and Supplies.** A complete list of the reagents required for the GeneChip Mapping Assay including those provided in the assay kit and recommended suppliers of supplementary items.

This manual is intended to be used in conjunction with the following:

- Affymetrix® GeneChip® Operating Software User's Guide (P/N 701439)
- Affymetrix® GeneChip® DNA Analysis Software User's Guide (P/N 701454)

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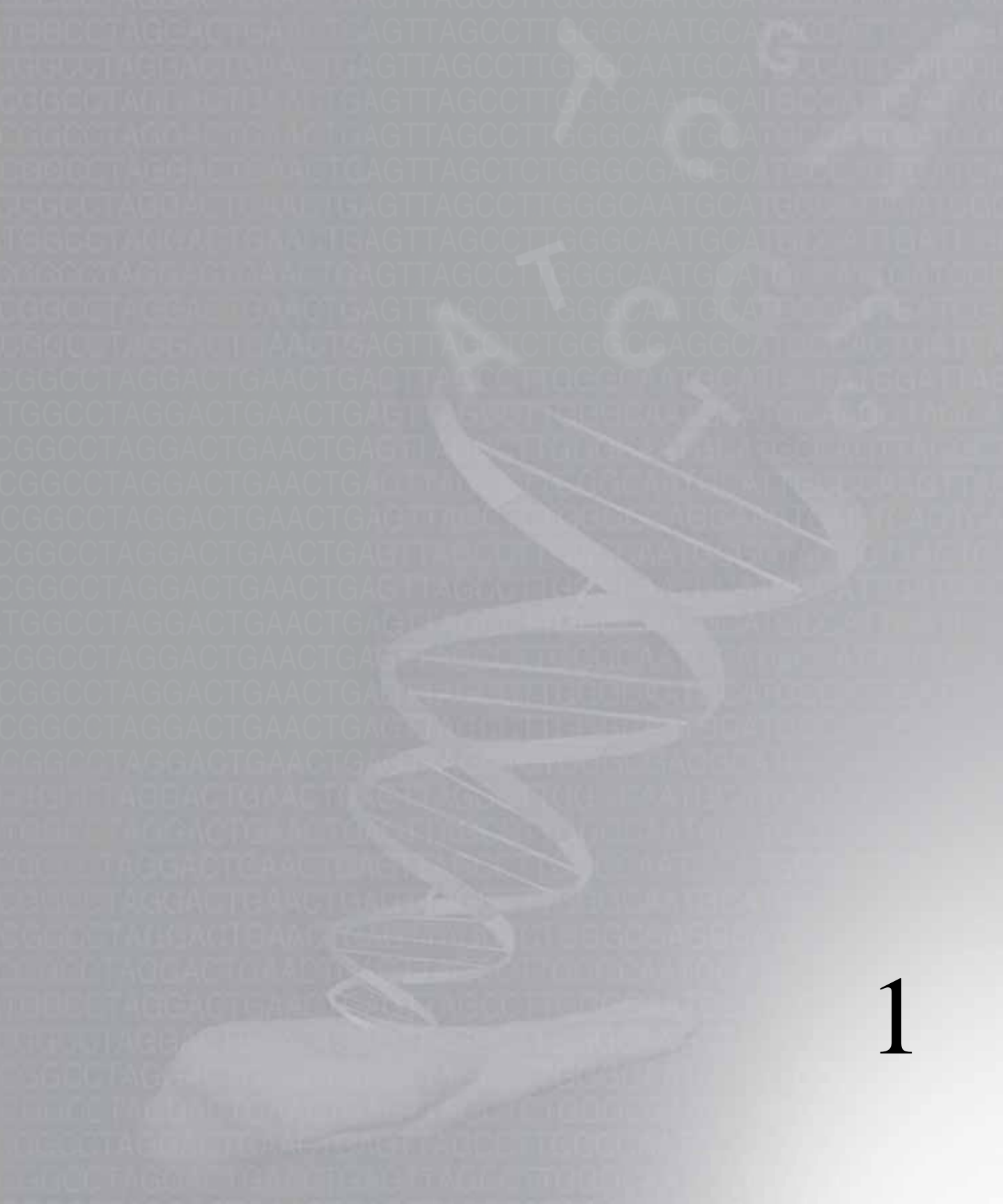
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# Chapter 1





# Scientific Overview

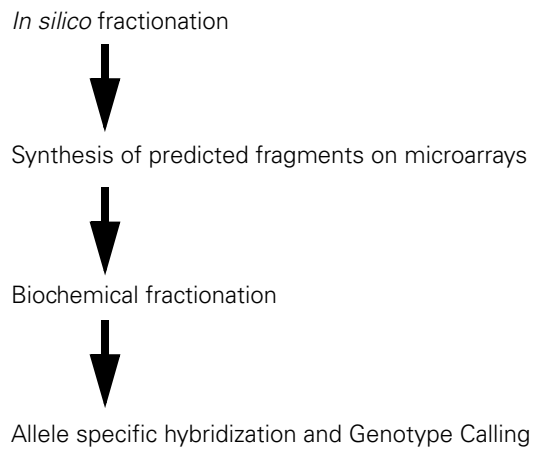
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The GeneChip® Mapping Assay genotypes greater than 10,000 human single nucleotide polymorphisms (SNPs) on a single array, using a single polymerase chain reaction (PCR) primer. As its name implies, it is a mapping tool designed to identify regions of the genome that are linked to, or associated with, a particular trait or phenotype. In addition, it is also useful for determination of allele frequencies in various populations and for mapping regions with chromosomal copy number changes during cancer progression. While linkage mapping of simple and complex traits has been successful using microsatellite markers, recent work has shown that linkage studies can be conducted more quickly with higher accuracy and better results with 10,000 SNPs (Kaindl, *et al.* 2004, Middleton, *et al.* 2004, Puffenberger, *et al.* 2004, Shrimpton, *et al.* 2004).

Genetic mapping studies aimed toward understanding the molecular basis of complex human phenotypes require the genotyping of many thousands of SNPs across large numbers of individuals (reviewed in Ardlie, *et al.* 2002). Public efforts have identified over two million common human SNPs (Sachidanandam, *et al.* 2001), however the scoring of these SNPs by currently available methods has been labor intensive, requiring a substantial amount of automation. The GeneChip Mapping Assay is a highly parallel genotyping platform that does not require the use of robots or automation. It takes advantage of GeneChip brand microarrays by coupling a highly reproducible generic sample preparation method with allele-specific hybridization (Kennedy, *et al.* 2003, Matsuzaki, *et al.* 2004).

We set out to devise a genotyping approach that would satisfy three criteria. First, the genomic fractionation strategy should leverage the large numbers of SNPs deposited in public databases. Second, due to the large numbers of SNPs being interrogated, sample preparation must avoid the use of individual SNP-specific primers. Finally, the reduction and amplification must be highly reproducible, capturing a majority of the same SNPs across many samples.

The GeneChip Mapping Assay strategy is outlined in Figure 1.1. After *in silico* prediction of SNPs residing in desired genomic fractions, oligonucleotides corresponding to these SNP-containing fragments were used to design high-density microarrays. Following biochemical fractionation that mirrors the *in silico* fractionation, the target is hybridized to arrays and SNPs are genotyped by allele-specific hybridization.

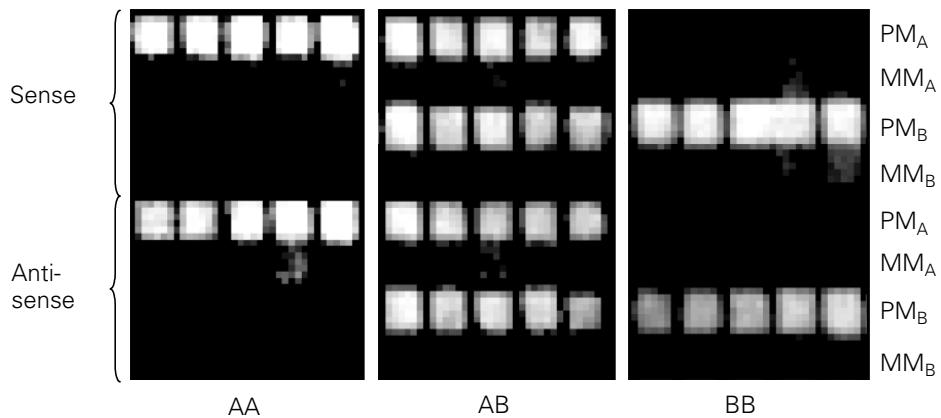


**Figure 1.1**  
GeneChip Mapping Assay Strategy

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## Principles of Allele-Specific Hybridization on GeneChip® Probe Arrays

Allele-specific hybridization (ASH) is a method of allele discrimination in genotyping (Chee, *et al.* 1996; Wang, *et al.* 1998; Lindblad-Toh, *et al.* 2000). By synthesizing probes on the array corresponding to both of the two possible alleles at each SNP and hybridizing the target to the array, we can determine whether a SNP is AA, AB, or BB by analyzing the resulting signal from the allele-specific probes. We synthesize 25-mer probes corresponding to a perfect match of the A allele sequence (PMA) and to the perfect match of the B allele sequence (PMB). To determine specificity in binding, we include a single basepair mismatch at the center position of each 25-mer, for each allele (MMA and MMB). In fact, for each SNP we tile 40 different 25 bp oligonucleotides, each with a slight variation in perfect matches, mismatches, and flanking sequence around the SNP. An example of hybridized array images is shown in Figure 1.2.



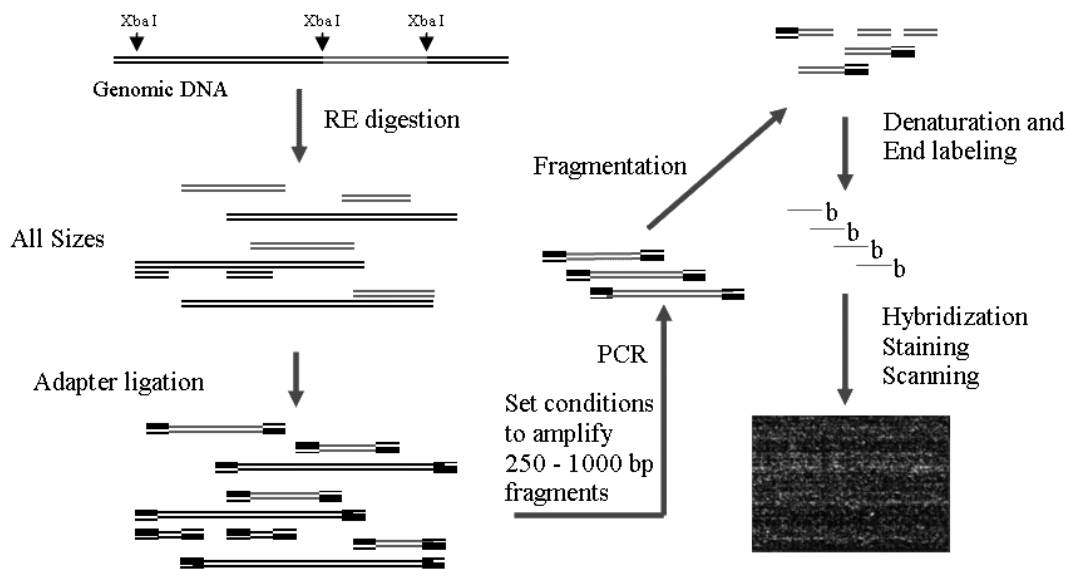
**Figure 1.2**

Example of Allele-Specific Hybridization with 40 probes / SNP

While the ASH signal can be visualized on the scanned images of the microarrays in Figure 1.2, the genotyping algorithm uses hybridization

signal intensity gathered across many individuals to assign center points (medoids) to each genotype cluster for a particular SNP. This is necessary, as the signal intensities for the A and B allele are seldom equivalent and must be determined empirically on training data. The training data consisted of an ethnically diverse set of samples to maximize utility of SNPs across major populations. Principles of the GeneChip Mapping Algorithm have recently been published and are described in greater detail in the GDAS 3.0 software manual. (Liu, *et al.* 2003).

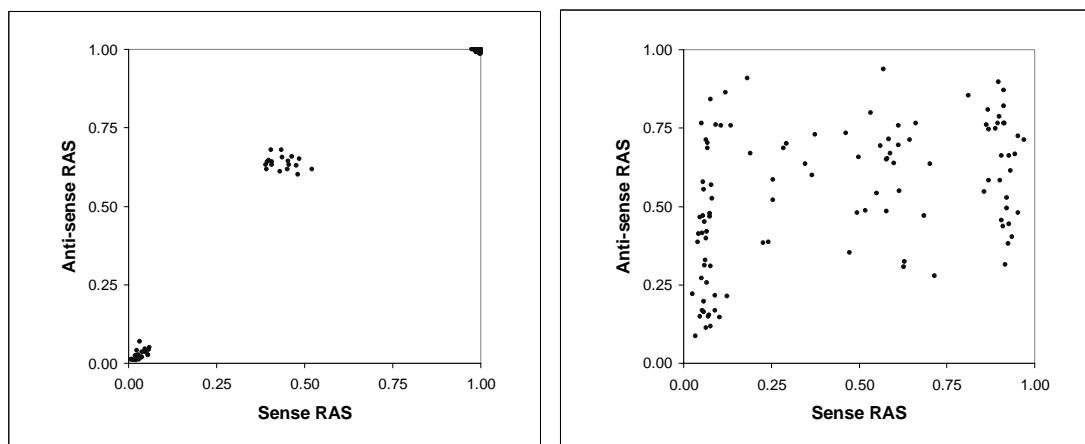
The biochemical fractionation method we devised, called “GeneChip Mapping Assay,” is shown in Figure 1.3. Total genomic DNA is digested with one of several restriction enzymes and ligated to adaptors recognizing the cohesive four base overhangs. All fragments resulting from restriction enzyme digestion, regardless of size, are substrates for adaptor ligation. A generic primer, which recognizes the adaptor sequence, is used to amplify ligated DNA fragments and PCR conditions are optimized to preferentially amplify fragments in the 250-1000 bp size range. The amplified DNA is labeled and hybridized to GeneChip arrays. The arrays are washed and stained on a GeneChip fluidics station and scanned on a GeneChip Scanner 3000.



**Figure 1.3**  
GeneChip Mapping Assay



Clustering is the process by which we analyze the relative allele signal (RAS) for each SNP on both strands across many DNA samples. RAS is simply the ratio of the signal of the A allele to the sum of the A and B alleles  $A/(A+B)$ . Thus, AA genotypes show RAS values approaching 1, AB genotypes approach RAS values of 0.5, and BB genotypes show RAS values approaching 0. Figure 1.4 shows clustering properties of a good SNP (left) and a bad SNP (right). For SNPs with sufficient minor allele frequencies, we observe all three possible genotypes (only SNPs with three observed clusters were included in the product). We derived a quantitative measure of clustering quality, the silhouette score ( $s$ ), whose value ranges from 0 to 1. SNPs with  $s$  values closer to 1 show clusters that are tight and well separated, while poorly clustering SNPs show lower  $s$  values.



**Figure 1.4**  
Clustering properties of a good and bad SNP

This process for selection of SNPs on the Mapping 10K is described in *SNP Selection Criteria for the GeneChip Human Mapping 10K Array Xba 131* Technical Note. Briefly, it includes selection based on such criteria as clustering properties, call rates, accuracy, heterozygosity, reproducibility and Mendelian inheritance. SNPs with poor clustering, call rates, and other criteria were omitted from the Mapping 10K Array.

Most of the SNPs on the GeneChip Mapping 10K 2.0 Array were on the GeneChip Mapping 10K Array. For more information on the SNPs chosen

for the Mapping 10K 2.0 Array, please see the GeneChip® Human Mapping 10K Array Xba 142 2.0 Data Sheet.

The biochemical approach we use relies on the position of restriction endonuclease sites in the genome and the position of resulting 250 to 1000 bp size fragments; therefore, the genome-wide distribution of SNPs in the GeneChip Mapping 10K 2.0 Array is random, but not completely uniform. Gaps are most frequently associated with telomeres and centromeres due to the paucity of SNPs discovered by The SNP Consortium (TSC) in these regions.

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## Chapter 2



## ➤ IMPORTANT

*Please read this chapter before planning any experiments.*

The GeneChip® Mapping Assay has been optimized to reliably and accurately detect greater than 10,000 SNPs in each sample. The assay reduces the complexity of the genome by preferentially amplifying 250-1000 basepair Xba I fragments using a single PCR primer from only 250 ng DNA. As with all genotyping applications care should be taken to minimize possible sources of contamination that would reduce genotyping accuracy, call rate, and/or genetic power.

To reduce the possibility of cross-contamination, Affymetrix recommends maintaining a single direction workflow. To facilitate this, the GeneChip® Mapping 10K Xba Assay Kit has been subdivided into three boxes (see Figure 2.1).

## ➤ IMPORTANT

*The most likely potential source of contamination for the GeneChip Mapping Assay is previously amplified PCR product.*

Steps should be taken to minimize the possibility of contaminating pre-PCR steps with amplified PCR product. Suggested precautions include:

- Store reagents in proper room according to the box label and manual instructions.
- Restrict movements through labs containing amplified DNA.
- Use proper gowning procedures.
- Use dedicated equipment (e.g., pipets, tips, thermocyclers, etc.) for pre-PCR stages.
- Print separate copies of the protocol for each room.

In addition, the following areas should be used when performing this assay: **pre-PCR Clean Room, PCR Staging Room, and Main Lab.**

**➤ IMPORTANT**

*Once a high copy area has been entered, a low copy area should not be entered until the researcher has showered and changed into freshly laundered clothing.*

A summary of the areas is shown in Figure 2.1.

## Pre-PCR Clean Room

---

The Pre-PCR Clean Room (or dedicated area such as a biosafety hood) should be free of DNA template and PCR amplicons. The master stocks of PCR primer and adapter should be stored here, with aliquots taken for use in the PCR Staging Room (or area). Reagent preparation tasks, such as preparing Master Mixes, should be done in this room. The use of gowns, booties, and gloves is strongly recommended to prevent PCR carryover and will minimize the risk of trace levels of contaminants being brought into the Pre-PCR Clean Area.

## PCR Staging Room

---

The PCR Staging Room (or Area) is a low copy template lab which should be free from any PCR product (amplicons). It is the area where non-amplified template (genomic DNA) should be handled. The digestion and ligation reactions should be conducted in this area. The PCR reactions should be prepared in this area. The use of gowns and gloves is recommended to prevent PCR carryover.

## Main Lab

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The Main Lab has airborne contamination with PCR product and template. After entering the main lab it is inadvisable to re-enter the Pre-PCR Clean Area or the PCR Staging Area without first showering and changing into freshly laundered clothes.







**💡 TIP**

*To support this workflow, the GeneChip® Mapping 10K Xba Assay Kit has been subdivided into three boxes. Each box should be stored in a separate room, as illustrated in Figure 2.1.*

**➤ IMPORTANT**

*Each room should contain dedicated equipment such as thermocyclers, microfuges, pipets, tips, etc.*



|  | Template (Genomic DNA)  | PCR Product   |
|--|---|---|
| <p><b>Pre-PCR Clean Room</b></p> <p><b>Assay Steps:</b><br/>           Step 1: Reagent Preparation</p> <p>Store GeneChip® Mapping 10K Xba Assay Kit Box 1</p>  |  |  |
| <p><b>PCR Staging Room</b></p> <p><b>Assay Steps:</b><br/>           Step 2: Enzyme Digestion<br/>           Step 3: Ligation<br/>           Step 4: PCR (set up only)</p> <p>Store GeneChip® Mapping 10K Xba Assay Kit Box 2</p>  |  |  |
| <p><b>Main Lab</b></p> <p><b>Assay Steps:</b><br/>           Step 4: PCR Thermal Cycling<br/>           Step 5: PCR Clean up<br/>           Step 6: Fragmentation<br/>           Step 7: Labeling<br/>           Step 8: Hybridization<br/>           Step 9: Wash and Stain</p> <p>Store GeneChip® Mapping 10K Xba Assay Kit Box 3</p> <p>See page 126 for detailed information on the GeneChip® Mapping 10K Xba Assay Kit.</p> <p><b>Figure 2.1</b><br/>           A summary of rooms required to perform the GeneChip® Mapping Assay.</p> |  |  |

## Safety Precautions

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The GeneChip Mapping 10K Xba Assay Kit and the GeneChip Human Mapping 10K Array Xba 142 2.0 are for research use only.

All blood and other potentially infectious materials should be handled as if capable of transmitting infection and disposed of with proper precautions in accordance with federal, state, and local regulations.

 **WARNING**

*Some components required for this assay may pose significant health risks. Follow prudent laboratory practices when handling and disposing of carcinogens and toxics. Refer to the manufacturer's Material Safety Data Sheet for additional information.*

Wear appropriate personal protective equipment when performing this assay. At a minimum, safety glasses and chemical resistant gloves should be worn.



# Chapter 3





# *Genomic DNA Preparation*

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*This chapter describes the general requirements for genomic DNA sources and extraction methods. A genomic DNA control (Reference Genomic DNA, 103) is provided in the GeneChip® Mapping 10K Xba Assay Kit. This control DNA meets the requirements outlined below and should be used as a routine experimental positive control and for troubleshooting. Assay performance may vary for genomic DNA samples that do not meet the general requirements outlined below; however, the reliability of any given result should be assessed in the context of overall experimental design and goals.*

## General Requirements for Human Genomic DNA

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1. **DNA must be double-stranded (not single-stranded).** This requirement relates to the restriction enzyme digestion step in the protocol.
2. **DNA must be free of PCR inhibitors.** Examples of inhibitors include high concentrations of heme (from blood) and high concentrations of chelating agents (i.e., EDTA). The genomic DNA extraction/purification method should render DNA that is generally salt-free because high concentrations of certain salts can also inhibit PCR and other enzyme reactions. DNA should be prepared as described in Chapter 4, *GeneChip<sup>®</sup> Mapping 10K Assay Protocol*.
3. **DNA must not be contaminated with other human genomic DNA sources, or with genomic DNA from other organisms.** PCR amplification of the ligated genomic DNA is not human specific, so sufficient quantities of non-human DNA may also be amplified and could potentially result in compromised genotype calls. Contaminated or mixed DNA may manifest as high detection rates and low call rates (see *Contaminated Genomic DNA* on page 107).
4. **DNA must not be highly degraded.** For any particular SNP, the genomic DNA fragment containing the SNP must have both Xba I restriction sites intact on the same fragment so that ligation can occur on both ends of the fragment and PCR can be successful. The approximate average size of genomic DNA may be assessed on a 1% agarose gel using an appropriate size standard control. High quality genomic DNA will run as a major band at approximately 10-20 kb on the gel; assay performance may vary with DNA that is substantially more degraded.
5. **Genomic DNA that has been pre-amplified** (i.e., PEP) or pre-digested with other restriction enzymes (other than Xba I) have not been tested by Affymetrix with this assay. Customer feedback suggests that certain methods (e.g., Phi 29) (Paez, *et al.* 2004) may give reasonable results, whereas other methods (e.g., PEP) do not. You should plan your own experiments to evaluate pre-amplification with this assay.

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## Sources of Human Genomic DNA

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The following sources of human genomic DNA have been successfully tested in the laboratories at Affymetrix for DNA that meets the requirements described in the section *General Requirements for Human Genomic DNA* on page 20.

- blood
- cell line
- buccal (cells from the mouth)\* (Lench, *et al.* 1988; Heath, *et al.* 2001; Feigelson, *et al.* 2001; King, *et al.* 2002)

\* when prepared to be free of contaminating microbial DNA.

Success with other types of samples such as formalin fixed paraffin-embedded tissue will depend on quality (degree of degradation, degree of inhibitors present, etc.), quantity of genomic DNA extracted, and purity of these types of samples, as described in the section *General Requirements for Human Genomic DNA* on page 20.

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## Genomic DNA Extraction / Purification Methods

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Genomic DNA extraction and purification methods that meet the general requirements for genomic DNA outlined above should yield successful results. Methods that include boiling or strong denaturants are not acceptable, because the DNA would be rendered single-stranded. Genomic DNA extracted using the following methods have been tested in the laboratories at Affymetrix:

1. SDS/ProK digestion, phenol-chloroform extraction, Microcon® or Centricon® (Millipore®) ultrapurification and concentration.
2. QIAGEN; QIAamp DNA Blood Maxi Kit.

## DNA Cleanup

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If you suspect your genomic DNA preparation has inhibitors, you may wish to use the following procedure for cleanup.

1. Add 0.5 volumes of 7.5 M  $\text{NH}_4\text{OAc}$ , 2.5 volumes of absolute ethanol (stored at  $-20^\circ\text{C}$ ), and 0.5  $\mu\text{L}$  of glycogen (5 mg/mL) to 250 ng genomic DNA.
2. Vortex and incubate at  $-20^\circ\text{C}$  for 1 hour.
3. Centrifuge at 12,000 x g in a microcentrifuge at room temperature for 20 minutes.
4. Remove supernatant and wash pellet with 0.5 mL of 80% ethanol.
5. Centrifuge at 12,000 x g at room temperature for 5 minutes.
6. Remove the 80% ethanol and repeat the 80% ethanol wash one more time.
7. Re-suspend the pellet in TE (10 mM Tris, pH 8.0, 0.1mM EDTA, pH 8.0).

## References

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- Feigelson, H.S., Rodriguez, C., Robertson, A.S., Jacobs, E.J., Calle, E.E., Reid, Y.A., Thun, M.J. Determinants of DNA yield and quality from buccal cell samples collected with mouthwash. *Cancer Epidemiol Biomarkers Prev.* **10**(9), 1005-8 (2001).
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- King, I.B., Satia-Abouta, J., Thornquist, M.D., Bigler, J., Patterson, R.E., Kristal, A.R., Shattuck, A.L., Potter, J.D., White, E., Abouta, J.S. Buccal cell DNA yield, quality, and collection costs: comparison of methods for large-scale studies. *Cancer Epidemiol Biomarkers Prev.* **11**(10 Pt 1), 1130-3 (2002).
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Paez J.G., Lin M., Beroukhim R., Lee J.C., Zhao X., Richter D.J., Gabriel S., Herman P., Sasaki H., Altshuler D., Li C., Meyerson M., Sellers W.R.  
Genome coverage and sequence fidelity of phi29 polymerase-based multiple strand displacement whole genome amplification. *Nucleic Acids Research* 32(9); e 71 (2004).





# Chapter 4





# *GeneChip® Mapping 10K Assay Protocol*

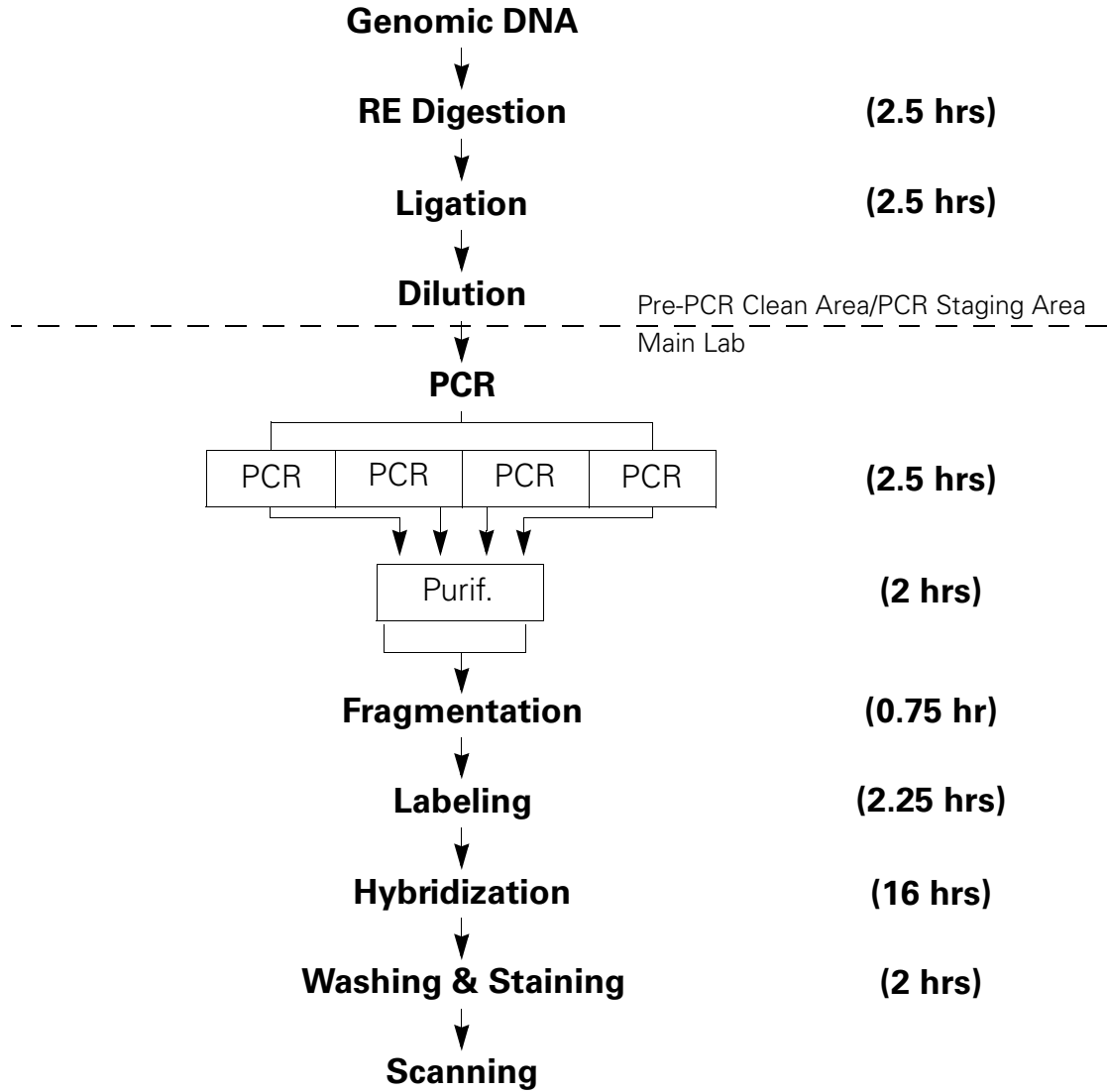
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## Introduction

The Affymetrix GeneChip® Mapping Assay, in conjunction with the GeneChip Human Mapping 10K Array Xba 142 2.0, is designed to detect Single Nucleotide Polymorphisms (SNPs) in samples of genomic DNA. The protocol starts with 250 ng of genomic DNA and will generate SNP genotype calls for more than 10,000 SNPs. An overview of the assay is shown in Figure 4.1. The assay utilizes a strategy that reduces the complexity of human genomic DNA up to 50 fold by first digesting the genomic DNA with the Xba I restriction enzyme and then ligating sequences onto the DNA fragments. The complexity is reduced by a PCR procedure optimized for short fragments. Following the complexity reduction step the PCR products (amplicons) are fragmented, end-labeled, and hybridized to a GeneChip array.

**Assay Overview**

GeneChip® Mapping Assay Overview



**Figure 4.1**  
Assay overview

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## Genomic DNA Preparation

### Preparation of Genomic DNA

The concentration of the genomic DNA should be determined and the working stocks diluted to 50 ng/ $\mu$ L using reduced EDTA TE buffer (0.1 mM EDTA, 10 mM Tris HCl, pH 8.0).

#### IMPORTANT

*An elevated EDTA level is not recommended as it may interfere with subsequent reactions.*

For high throughput assays, aliquot 5  $\mu$ L (50 ng/ $\mu$ L) of each diluted genomic DNA into each well of a 96-well plate. Make multiple replicates of the plates if needed.

### Reagents

- Reduced EDTA TE Buffer (10 mM Tris HCl, pH 8.0, 0.1mM EDTA, pH 8.0) TEKnova P/N T0223

## STEP 1: Reagent Preparation and Storage

The reagents necessary for the restriction digestion, ligation and PCR steps should be stored to minimize cross contamination between samples. Affymetrix recommends storing these reagents in the pre-PCR clean room as described in Chapter 2 (an area free of DNA template and free of PCR product). To avoid re-entering the pre-PCR clean room after entering either the PCR-Staging Room or the Main Lab, Affymetrix recommends aliquoting each of the reagents in the pre-PCR clean room before starting the rest of the experiment.

#### IMPORTANT

*The GeneChip Mapping 10K Assay kits are designed to allow only a 5% excess in master mix volumes. If you need greater quantities in your master mix, additional reagent kits may be needed.*

## STEP 2: Restriction Enzyme Digestion

### Reagents and Equipment

- Genomic DNA working stock: 50 ng/μL
- Xba I (20,000 U/mL): New England Biolab (NEB); P/N R0145L containing:
  - NE Buffer 2: New England Biolab (NEB); P/N B7002S
  - BSA (Bovine Serum Albumin): New England Biolab (NEB); P/N B9001S
- H<sub>2</sub>O (Molecular Biology Water): BioWhittaker Molecular Applications/Cambrex; P/N 51200
- 96-well plate: MJ Research; P/N MLP-9601; or Applied Biosystems; P/N 403083
- 96-well PLT. Clear Adhesive Films: Applied Biosystems; P/N 4306311
- Thermal cycler (any Pre-PCR Clean Room thermocycler)

#### ▲ WARNING

*PCR tubes must be compatible and qualified with either MJ Research, MJ DNA Engine Tetrad, or ABI GeneAmp 9700. For Example, Individual Tubes: MJ Research P/N TWI-0201.*

*Using incompatible tubes with either the MJ Research® Tetrad or the GeneAmp 9700 could cause tubes to crush and lead to loss of sample.*

#### ✓ Note

*The BSA is supplied as 100X (10 mg/mL), and needs to be diluted 1/10 with molecular biology grade water before use.*

#### ✓ Note

*Reference Genomic DNA, 103 is supplied in Box 2 of the GeneChip® Mapping 10K Xba Assay Kit, P/N 900441. This DNA can be used as a positive control.*

#### ✓ Note

*A process negative control can be included at the beginning of the assay to assess the presence of contamination. Refer to Chapter 2 and Chapter 7 for more information.*

#### ! CAUTION

*Reference Genomic DNA 103 tubes should be kept with the original kit to avoid contamination.*



## Digestion Procedure

### Pre-PCR Clean Area

1. Prepare the following Digestion Master Mix ON ICE (for multiple samples make 5% excess):

| Reagent Stock       | 1 Sample     | Final Conc. in Sample |
|---------------------|--------------|-----------------------|
| H <sub>2</sub> O    | 10.5 µL      |                       |
| NE buffer 2 (10X)   | 2 µL         | 1X                    |
| BSA (10X (1 mg/mL)) | 2 µL         | 1X                    |
| Xba I (20 U/µL)     | 0.5 µL       | 0.5 U/µL              |
| <b>Total</b>        | <b>15 µL</b> |                       |

### PCR Staging Area

2. Add 5 µL genomic DNA (50 ng/µL) to each well of 96-well plate. Total amount of genomic DNA is 250 ng for each sample.
3. Aliquot 15 µL of the Digestion Master Mix to each well of the 96-well plate containing genomic DNA.

| Reagent Stock          | Volume/Sample |
|------------------------|---------------|
| Genomic DNA (50 ng/µL) | 5 µL          |
| Digestion Master Mix   | 15 µL         |
| <b>Total Volume</b>    | <b>20 µL</b>  |

#### TIP

*To expedite the aliquoting, the master mix can be first divided into 8 or 12 microwell strips and then dispensed into the plate with an 8-channel or 12-channel pipette. Pipet up and down for several times to mix the genomic DNA and digestion mix.*

4. Cover the plate with a plate cover and seal tightly, VORTEX, and spin briefly at 2,000 rpm for 1 minute.

5. Place the plate in a thermal cycler and run the following program:

| Temperature | Time        |
|-------------|-------------|
| 37°C        | 120 minutes |
| 70°C        | 20 minutes  |
| 4°C         | Hold        |



*Store samples at -20°C if not proceeding to the next step.*

## STEP 3: Ligation

---

### Reagents and Equipment

- T4 DNA Ligase: New England Biolab (NEB); P/N M0202L containing:
  - T4 DNA Ligase Buffer: New England Biolab (NEB); P/N B0202S
- H<sub>2</sub>O (Molecular Biology Water): BioWhittaker Molecular Applications/Cambrex; P/N 51200
- Adaptor Xba (5 µM); Affymetrix; P/N 900410, available in Box 1 of the GeneChip® Mapping 10K Xba Assay Kit, P/N 900441
- Thermal cycler (any Pre-PCR Clean Room thermocycler)



*Avoid multiple freeze-thaw cycles with Ligase Buffer, according to vendor's instructions.*

## Ligation Procedure

### Pre-PCR Clean Area

#### ▶ IMPORTANT

*Ligase Buffer contains ATP and should be defrosted and held at 4°C. Mix ligase buffer thoroughly before use to ensure precipitate is resuspended. Avoid multiple freeze-thaw cycles.*

1. Prepare the following Ligation Master Mix ON ICE (for multiple samples make 5% excess):

| Reagent                      | 1 Sample       | Final Conc. in Sample |
|------------------------------|----------------|-----------------------|
| Adaptor Xba (5 µM)           | 1.25 µL        | 0.25 µM               |
| T4 DNA Ligase buffer (10X) * | 2.5 µL         | 1X                    |
| <b>Total</b>                 | <b>3.75 µL</b> |                       |

\* Contains ATP and DTT. Keep on ice.

### PCR Staging Area

2. Aliquot 3.75 µL of the Ligation Master Mix into each digested DNA samples.

#### 💡 TIP

*To expedite the aliquoting, the Ligation Master Mix can be first divided into 8 or 12 microwell strips and then dispensed into the wells of the plate with an 8-channel or 12-channel pipette. Pipet up and down for several times to mix.*

3. Add 1.25 µL of T4 DNA Ligase to each digested DNA sample. Final reaction volume for each reaction is following:

| Reagent       | Volume/ Sample |
|---------------|----------------|
| Digested DNA  | 20.00 µL       |
| Ligation mix* | 3.75 µL        |
| T4 DNA Ligase | 1.25 µL        |
| <b>Total</b>  | <b>25.0 µL</b> |

**TIP**

*To expedite the aliquoting, the ligase can be first divided into 8 or 12 microwell strips and then dispensed into the wells of the plate with an 8-channel or 12-channel pipette. Pipet up and down several times to mix.*

4. Cover the plate with plate cover and seal tightly, vortex at medium speed for 2 seconds, and spin briefly at 2,000 rpm for 1 minute.
5. Place the plate in a thermal cycler and run the following program:

| Temperature | Time        |
|-------------|-------------|
| 16°C        | 120 minutes |
| 70°C        | 20 minutes  |
| 4°C         | Hold        |

**✓ Note**

*Store samples at -20°C if not proceeding to the next step within 60 minutes.*

6. Dilute each DNA ligation reaction by adding 75  $\mu$ L of molecular biology-grade H<sub>2</sub>O as described below:

| Reagent          | Volume/ Sample               |
|------------------|------------------------------|
| Ligated DNA      | 25 $\mu$ L                   |
| H <sub>2</sub> O | 75 $\mu$ L                   |
| <b>Total</b>     | <b>100 <math>\mu</math>L</b> |

**➔ IMPORTANT**

*It is crucial to dilute the ligated DNA with Molecular Biology Water prior to PCR.*

---

## STEP 4: PCR

---

### Reagents and Equipment

- H<sub>2</sub>O (Molecular Biology Water): BioWhittaker Molecular Applications/Cambrex; P/N 51200
- dNTP (2.5 mM each): Panvera Takara; P/N 4030, or Fisher Scientific; P/N TAK 4030<sup>1</sup>
- PCR Primer, 001 (10 µM): Affymetrix; P/N 900409, available in Box 1 of the GeneChip® Mapping 10K Xba Assay Kit, P/N 900441 (see page 126 for sequence)
- QIAquick PCR Purification Kit: QIAGEN; P/N 28106
- AmpliTaq Gold PCR Kit: Applied Biosystems; P/N N808-0249 containing:
  - MgCl<sub>2</sub>: Applied Biosystems; P/N N808-0249
  - PCR Buffer II: Applied Biosystems; P/N N808-0249
- 2% TBE Gel: BMA Reliant precast (2% SeaKem Gold); P/N 54939
- All Purpose Hi-Lo DNA Marker: Bionexus, Inc.; P/N BN2050
- Gel Loading Solution: Sigma; P/N G2526
- Tubes:
  - Individual tubes: MJ Research; P/N TWI-0201
  - 8-Tube Strips, thin-wall (0.2 mL): MJ Research, P/N TBS-0201; Strip of 8 caps: MJ Research, P/N TCS-0801
- Plate:
  - 96-well plate: MJ Research; P/N MLP-9601
- 96-well PLT. Clear Adhesive Films: Applied Biosystems; P/N 4306311
- PCR Thermal Cycler (this assay has only been optimized for the following two thermal cyclers) (see footnote page 128):
  - GeneAmp PCR System 9700, Applied Biosystems, or
  - DNA Engine Tetrad PTC-225, MJ Research

---

1. dNTPs from Invitrogen (P/N R72501) have been tested on a limited basis with similar results. You should test in your own lab prior to full scale production.

**IMPORTANT**

- Program the thermal cycler in advance.
- Make sure the ligated DNA from the ligation step was diluted to 100  $\mu\text{L}$  with water.
- Prepare PCR Master Mix in Pre-PCR Clean room.
- Set up PCRs in PCR Staging Area.
- Prepare 4 PCRs for each sample (4 PCRs are required for each array).

**PCR Procedure****Pre-PCR Clean Room**

1. Prepare the following PCR Master Mix ON ICE and vortex at medium speed for 2 seconds. (For multiple samples make 5% excess.)

| Stock Reagent                        | 1 PCR                              | 4 PCR                               | Final Conc. in Sample    |
|--------------------------------------|------------------------------------|-------------------------------------|--------------------------|
| PCR buffer (10X)                     | 10 $\mu\text{L}$                   | 40 $\mu\text{L}$                    | 1X                       |
| dNTP (2.5 mM each)                   | 10 $\mu\text{L}$                   | 40 $\mu\text{L}$                    | 250 $\mu\text{M}$ (each) |
| MgCl <sub>2</sub> (25 mM)            | 10 $\mu\text{L}$                   | 40 $\mu\text{L}$                    | 2.5 mM                   |
| PCR Primer Xba (10 $\mu\text{M}$ )   | 7.5 $\mu\text{L}$                  | 30 $\mu\text{L}$                    | 0.75 $\mu\text{M}$       |
| AmpliTaq Gold® (5 U/ $\mu\text{L}$ ) | 2 $\mu\text{L}$                    | 8 $\mu\text{L}$                     | 0.1 U/ $\mu\text{L}$     |
| H <sub>2</sub> O                     | 50.5 $\mu\text{L}$                 | 202 $\mu\text{L}$                   |                          |
| <b>Total</b>                         | <b>90 <math>\mu\text{L}</math></b> | <b>360 <math>\mu\text{L}</math></b> |                          |

**✓ Note**

*A PCR negative control can be included in the experiment to assess the presence of contamination. Refer to Chapter 2 and Chapter 7 for more information.*

**IMPORTANT**

*Prepare fresh PCR Master Mix just prior to use.*

## PCR Staging Area

- Transfer 10  $\mu\text{L}$  of each of the diluted ligated DNA, from 96-well plate, into corresponding wells in four new PCR plates using an 8- or 12-channel pipette.
- Add 90  $\mu\text{L}$  PCR Master mix to obtain a total volume of 100  $\mu\text{L}$ . Final volume for each PCR is listed in the following table.

### TIP

*It is convenient to dispense the PCR Master Mix with a repetitive dispenser (such as Gilson Distriman®, available from Ranin) or pipet the PCR Master Mix from a solution basin (Labcor Products, Inc., Cat. No. 730-014; available from PGC Scientifics) or with an 8-channel or 12-channel pipette.*

| Reagent                                  | Volume/PCR                          |
|--|-------------------------------------|
| PCR Master Mix                           | 90 $\mu\text{L}$                    |
| Diluted ligated DNA (from Ligation step) | 10 $\mu\text{L}$                    |
| <b>Total</b>                             | <b>100 <math>\mu\text{L}</math></b> |

### ✓ Note

*Four PCR reactions are needed to produce sufficient product for hybridization to one array (each reaction = 100  $\mu\text{L}$ ).*

- Seal the plate with plate cover, vortex at medium speed for 2 seconds, and spin briefly at 2,000 rpm for 1 minute.

## Main Lab

- Run the following PCR thermal cycles on an MJ DNA Engine Tetrad or GeneAmp PCR System 9700.

### ➔ IMPORTANT

*PCR protocols for MJ and PE thermal cyclers are different as listed below.*

**MJ DNA Engine Program:**

**Use Heated Lid and Calculated Temperature.** Program the thermal cycler in advance with the following protocol:

| Temperature | Time       | Cycles |
|-------------|------------|--------|
| 95°C        | 3 minutes  | 1X     |
| 95°C        | 20 seconds | 35X    |
| 59°C        | 15 seconds |        |
| 72°C        | 15 seconds |        |
| 72°C        | 7 minutes  | 1X     |
| 4°C         | HOLD       |        |

**Gene Amp PCR System 9700 Program:**

Specify **100 µL volume** and **maximum mode**.

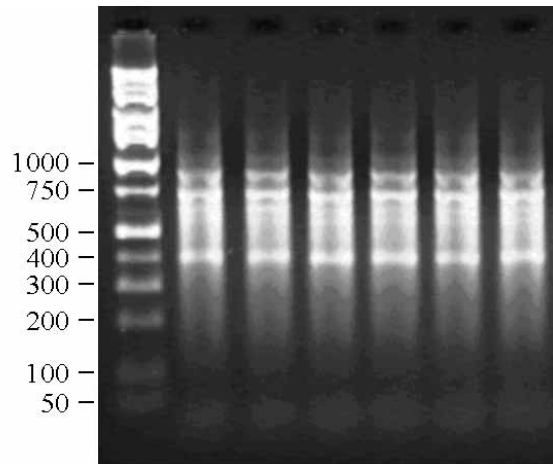
| Temperature | Time       | Cycles |
|-------------|------------|--------|
| 95°C        | 3 minutes  | 1X     |
| 95°C        | 30 seconds | 35X    |
| 59°C        | 30 seconds |        |
| 72°C        | 30 seconds |        |
| 72°C        | 7 minutes  | 1X     |
| 4°C         | HOLD       |        |

- Run 3 µL of each PCR product mixed with 3 µL 2X Gel Loading Dye on 2% TBE gel at 120V for 1 hour.

 **Note**

*PCR products can be stored at -20°C if not proceeding to the next step within 60 minutes.*





**Figure 4.2**  
Example of PCR products run on 2% TBE agarose gel at 120V for 1 hour.

---

## STEP 5: PCR Purification and Elution with QIAGEN MinElute 96 UF PCR Purification Plate

---

### Reagents and Equipment

- Manifold - QIAvac multiwell unit: QIAGEN P/N 9014579
- MinElute 96 UF PCR Purification Kit: QIAGEN P/N 28051 (four plates), or P/N 28053 (24 plates)
- Buffer EB (1000 mL): QIAGEN P/N120002
- Biomek Seal and Sample Aluminum Foil Lids: Beckman P/N 538619
- Jitterbug 115 VAC: Boekel Scientific P/N 130000
- Vacuum Regulator for use during the PCR clean up step. QIAGEN Vacuum Regulator (use with QIAvac manifolds): QIAGEN; P/N 19530\*  
\* The QIAGEN protocol requires ~800mb vacuum. If your lab does not have an internally regulated vacuum source, this vacuum regulator is strongly suggested

Follow the steps as outlined below. Consult the QIAGEN MinElute Handbook for the general procedure and ordering information.

1. Connect a vacuum manifold to a suitable vacuum source able to maintain ~800 mbar, e.g., QIAvac Multiwell Unit (QIAGEN). Place a waste tray inside the base of the manifold.
2. Place a MinElute 96 UF PCR Purification Plate on top of the manifold. Cover wells that are not needed with PCR plate cover.

#### Note

*To cover the unneeded wells, a PCR plate cover or an aluminum foil lid can be placed on top of the MinElute plate. Apply pressure to make the cover stick to the plate. Then using a razor, cut between the needed and unneeded wells. Remove the portion that covers the needed wells.*

3. Consolidate the four PCR reactions for each sample into one well of the MinElute plate.

**✓ Note**

*For PCR samples prepared in four 96-well PCR plates, an 8- or 12-channel pipette can be used to transfer each row of 12 samples in the PCR plates to the corresponding row of the MinElute plate. With the vacuum on, the four PCR reactions for each sample (400 µL) can be combined into one well of the MinElute plate.*

**! CAUTION**

*When solution is pipetted into the well of the MinElute plate, be careful not to pierce the membrane.*

**! CAUTION**

*Make sure the orientations of PCR plates are consistent. Use a method of distinguishing between used and unused wells on the plate.*

4. Apply a vacuum and maintain a ~800 mbar vacuum until the wells are completely dry. It takes about 90 minutes to dry 400 µL PCR samples.
5. Wash the PCR products by adding 50 µL molecular biology water and dry the wells completely (approximately 20 minutes). **Repeat this step 2 times** for a total number of 3 water washes.
6. Switch off vacuum source and release the vacuum.
7. Carefully remove the MinElute plate from the vacuum manifold.
8. Gently tap the MinElute plate on a stack of clean absorbent paper to remove any liquid that might remain on the bottom of the plate.
9. Add 40 µL EB buffer to each well. Cover the plate with PCR plate cover film.
10. Moderately shake the MinElute plate on a plate shaker, e.g., Jitterbug (Boekel Scientific, model 130000), for 5 minutes.
11. Recover the purified PCR product by pipetting the eluate out of each well. For easier recovery of the eluates, the plate can be held at a slight angle.

## STEP 6: Quantification of Purified PCR Product

---

1. Use spectrophotometric analysis to determine the purified PCR product yield. If available, a plate reader is preferred for efficient DNA concentration determination.
2. Add 4  $\mu\text{L}$  of purified PCR product to 156  $\mu\text{L}$  molecular water (40-fold dilution) and MIX WELL.
3. Read the absorbance at 260 nm. Ensure that the reading is in the quantitative range of the instrument (generally 0.2 to 0.8 OD).
4. Apply the convention\* that 1 absorbance unit at 260 nm equals 50  $\mu\text{g}/\text{mL}$  for double-stranded PCR product.  
\*This convention assumes a path length of 1 cm. Consult your spectrophotometer handbook for further information.
5. Normalize the DNA concentration to 20  $\mu\text{g}$  of PCR product per 45  $\mu\text{L}$  solution by adding EB buffer (10mM Tris-HCl, pH 8.5).
6. Transfer 45  $\mu\text{L}$  (20  $\mu\text{g}$ ) of each of the purified DNA to corresponding wells of a new plate for fragmentation.

**➤ IMPORTANT**

*If 20  $\mu\text{g}$  of PCR product has a volume less than 45  $\mu\text{L}$ , make up the volume to 45  $\mu\text{L}$  by adding EB Buffer (10 mM Tris-Cl, pH 8.5).*

**➤ IMPORTANT**

*The spectrophotometer should be regularly calibrated to ensure correct readings.*

## STEP 7: Fragmentation

### Reagents and Equipment

- Fragmentation Reagent (DNase I): Affymetrix, P/N 900131, available in Box 3 of the GeneChip® Mapping 10K Xba Assay Kit, P/N 900441
- 10 X Fragmentation Buffer: Affymetrix, P/N 900422, available in Box 3 of the GeneChip® Mapping 10K Xba Assay Kit, P/N 900441
- Molecular Biology Water: Bio Whittaker Molecular Applications/Cambrex, P/N 51200
- 4% TBE Gel: BMA Reliant precast (4% NuSieve 3:1 Plus Agarose); P/N 54929
- All Purpose Hi-Lo DNA Marker: Bionexus, Inc.; 50 - 10000bp; P/N BN2050
- Gel Loading Solution: Sigma; P/N G2526
- 96-well plate: MJ Research; P/N MLP-9601
- 96-well PLT. Clear Adhesive Films: Applied Biosystems; P/N 4306311
- Thermal cycler
  - DNA Engine Tetrad: MJ Research
  - Gene Amp PCR System 9700

### Main Lab

#### ➤ IMPORTANT

*Fragmentation of PCR product before hybridization onto GeneChip probe arrays has been shown to be critical in obtaining optimal assay performance. Due to the sensitive nature of the Fragmentation Reagent (DNase I), these general rules need to be followed to ensure the success of the step:*

- *Store the Fragmentation Reagent stock at -20°C until ready for use.*
- *Make sure the purified PCR products are in EB buffer with proper volume (45 µL).*
- *Pre-heat the thermal cycler at 37°C before setting up the fragmentation reaction.*
- *Prepare the Fragmentation Reagent dilution immediately prior to use.*
- *Prepare diluted Fragmentation Reagent in excess to avoid errors in taking a small volume of Fragmentation Reagent.*
- *Perform all the dilutions, addition and mixing steps ON ICE.*
- *Perform all the steps AS QUICKLY AS POSSIBLE.*
- *Discard remaining Fragmentation Reagent after use.*

### Fragmentation Procedure

1. Pre-heat thermal cycler to 37°C.
2. Add 5  $\mu\text{L}$  10X Fragmentation Buffer to each sample on the fragmentation plate ON ICE and vortex at medium speed for 2 seconds. Place back on ice.

| Reagent  | Volume/ Sample                     |
|--|------------------------------------|
| Purified PCR product (20 $\mu\text{g}$ in EB buffer) | 45 $\mu\text{L}$                   |
| 10X Fragmentation Buffer                             | 5 $\mu\text{L}$                    |
| <b>Total</b>   | <b>50 <math>\mu\text{L}</math></b> |

3. Examine the label of the GeneChip Fragmentation Reagent tube for U/ $\mu\text{L}$  definition, and calculate dilution.

**Y** = number of  $\mu\text{L}$  of stock Fragmentation Reagent

**X** = number of U of stock Fragmentation Reagent per  $\mu\text{L}$  (see label on tube)

**0.048 U/ $\mu\text{L}$**  = final concentration of diluted Fragmentation Reagent

**125  $\mu\text{L}$**  = final volume of diluted Fragmentation Reagent\*

$$Y = \frac{0.048 \text{ U}/\mu\text{L} * 125 \mu\text{L}}{X \text{ U}/\mu\text{L}}$$

\* enough for 20 reactions

#### Note

*As the concentration of stock Fragmentation Reagent (U/ $\mu\text{L}$ ) may vary from lot to lot, it is essential to check the concentration before conducting the dilution. Do calculations prior to diluting sample and Fragmentation Reagent.*

- Dilute the stock of Fragmentation Reagent to **0.048 U/μL** using Fragmentation Buffer and Molecular Biology Water ON ICE and vortex at medium speed for 2 seconds.

Two **examples** of dilution are listed below for two different concentrations of Fragmentation Reagent.

| Reagent                  | 2 units/μL    | 3 units/μL    |
|--------------------------|---------------|---------------|
| Fragmentation Reagent    | 3 μL          | 2 μL          |
| 10X Fragmentation Buffer | 12.5 μL       | 12.5 μL       |
| H <sub>2</sub> O         | 109.5 μL      | 110.5 μL      |
| <b>Total</b>             | <b>125 μL</b> | <b>125 μL</b> |

### ✓ Note

*The Fragmentation Reagent is viscous so it is important that it is mixed well.*

If the concentration on your tube is not shown in the table above, use the formula provided in step 3.

- Divide the diluted Fragmentation Reagent into 8 or 12 microwell strips ON ICE.
- Add 5 μL of diluted Fragmentation Reagent (0.048 U/μL) with an 8- or 12-channel pipette to the fragmentation plate containing Fragmentation Mix ON ICE. Pipet up and down several times to mix. The total volume for each sample is listed below.

| Reagent                                   | Volume/Sample |
|---|---------------|
| Fragmentation Mix                         | 50 μL         |
| Diluted Fragmentation Reagent (0.048U/μL) | 5 μL          |
| <b>Total</b>                              | <b>55 μL</b>  |

### ➔ IMPORTANT

*For 20 μg of purified PCR product, a total of 0.24 U of Fragmentation Reagent is needed in a final reaction volume of 55 μL.*

- Cover the fragmentation plate with a plate cover and seal tightly.

8. Vortex the fragmentation plate at medium speed for 2 seconds, and spin briefly at 2,000 rpm for 1 minute.
9. Place the fragmentation plate in pre-heated thermal cycler (37°C) as quickly as possible.

**➔ IMPORTANT**

*Make sure the reaction tubes are securely sealed prior to running this program in order to minimize solution loss due to evaporation at the DNase I inactivation step (95°C).*

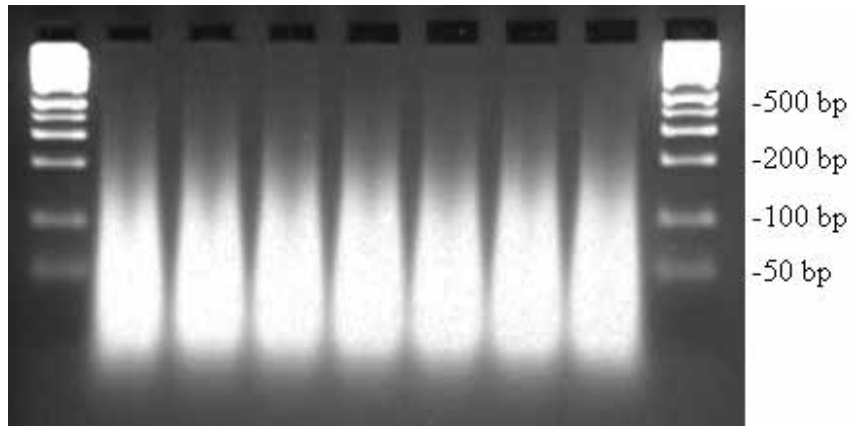
*Press the tubes to make sure all reaction tubes fit snugly into the wells of the heating block. Do not use a low-quality substitute of the 96-well plate that do not fit with the thermal cycler.*

10. Run the following program:

| Temperature | Time       |
|-------------|------------|
| 37°C        | 30 minutes |
| 95°C        | 15 minutes |
| 4°C         | Hold       |

11. Spin the plate briefly after fragmentation reaction.
12. Dilute 4 µL of fragmented PCR product with 4 µL gel loading dye and run on 4% TBE gel at 120V for 30 minutes to 1 hour. (See Figure 4.3.)
13. Proceed immediately to Labeling step, if your gel matches the example in Figure 4.3.





**Figure 4.3**

Example of fragmented PCR products run on 4% TBE agarose gel at 120V for 30 minutes to 1 hour.

## STEP 8: Labeling

### Reagents

- GeneChip DNA Labeling Reagent: Affymetrix; P/N 900430, available in Box 3 of the GeneChip® Mapping 10K Xba Assay Kit, P/N 900441
- Terminal Deoxynucleotidyl Transferase (30 U/μL): Affymetrix; P/N 900426, available in Box 3 of the GeneChip® Mapping 10K Xba Assay Kit, P/N 900441
- 5X Terminal Deoxynucleotidyl Transferase Buffer: Affymetrix; P/N 900425, available in Box 3 of the GeneChip® Mapping 10K Xba Assay Kit, P/N 900441

### Labeling Procedure

#### Main Lab

1. Prepare Labeling Mix as master mix ON ICE and vortex at medium speed for 2 seconds (for multiple samples, make 5% excess).

| Reagent                              | 1X             | Final Conc. in Sample |
|--------------------------------------|----------------|-----------------------|
| 5X TdT Buffer                        | 14 μL          | 1X                    |
| GeneChip DNA Labeling Reagent (5 mM) | 2 μL           | 0.143 mM              |
| TdT (30 U/μL)                        | 3.4 μL         | 1.5 U/μL              |
| <b>Total</b>                         | <b>19.4 μL</b> |                       |

2. Aliquot 19.4 μL of Labeling Master Mix into the fragmentation plate containing 50.6 μL of fragmented DNA samples as follows:

| Reagent                                  | Volume/ Rx   |   |
|--|--------------|---|
| Fragmented DNA (from Fragmentation step) | 50.6 μL      | Remaining fragmented DNA is used for gel analysis |
| Labeling Mix                             | 19.4 μL      |   |
| <b>Total</b>                             | <b>70 μL</b> |   |

**TIP**

*To expedite the aliquoting, the Labeling Master Mix can be first divided into 8 or 12 microwell strips and then dispensed into the wells of the plate with an 8- or 12-channel pipette. Pipet up and down several times to mix.*

3. Seal the plate tightly with a plate cover.
4. Vortex the plate at medium speed for 2 seconds, and briefly spin the plate at 2,000 rpm for 1 minute.
5. Run the following program:

| Temperature | Time       |
|-------------|------------|
| 37°C        | 2 hours    |
| 95°C        | 15 minutes |
| 4°C         | Hold       |

**IMPORTANT**

*Make sure the reaction tubes are securely sealed prior to running this program in order to minimize solution loss due to evaporation at denaturation step (95°C).*

6. Briefly spin the plate at 2,000 rpm for 1 minute after the labeling reaction.

**Note**

*Samples can be stored at -20°C if not immediately proceeding to next step.*

## STEP 9: Target Hybridization

---

### Reagents

- 5M TMACL (Tetramethyl Ammonium Chloride): Sigma; P/N T3411
- 10% Tween-20: Pierce; P/N 28320 (Surfactamps); diluted to 3% in molecular biology grade water
- MES hydrate: Sigma; P/N M5287
- MES Sodium Salt: Sigma; P/N M5057
- DMSO: Sigma; P/N D5879
- EDTA: Ambion; P/N 9260G
- Denhardt's Solution: Sigma; P/N D2532
- HSDNA (Herring Sperm DNA): Promega; P/N D1815
- Human Cot-1: Invitrogen; P/N 15279-011
- Oligonucleotide Control Reagent: Affymetrix; P/N 900440, available in Box 3 of the GeneChip® Mapping 10K Xba Assay Kit, P/N 900441

### Reagent Preparation

#### 12 X MES Stock

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



**IMPORTANT**

*Do not autoclave. Store between 2°C and 8°C, and shield from light. Discard solution if it turns yellow.*

#### **For 1000 mL:**

70.4 g MES Hydrate

193.3 g MES Sodium Salt

800 mL 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.

## Hybridization Procedure

### ▲ WARNING

*Gloves, safety glasses, and lab coats must be worn when preparing the hybridization cocktail. Please consult the appropriate MSDS for reagent storage and handling requirements.*

### ➔ IMPORTANT

*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.*

1. Prepare the following Hybridization Cocktail Master Mix (for multiple samples make 5% excess):

| Reagent                   | 1X                           | Final Conc. in Sample |
|---------------------------|------------------------------|-----------------------|
| MES (12X; 1.22 M)         | 12 $\mu$ L                   | 0.056 M               |
| DMSO (100%)               | 13 $\mu$ L                   | 5.0%                  |
| Denhardt's Solution (50X) | 13 $\mu$ L                   | 2.50                  |
| EDTA (0.5 M)              | 3 $\mu$ L                    | 5.77 mM               |
| HSDNA (10 mg/mL)          | 3 $\mu$ L                    | 0.115 mg/mL           |
| Oligonucleotide Control   | 2 $\mu$ L                    | 1X                    |
| Human Cot-1 (1 mg/mL)     | 3 $\mu$ L                    | 11.5 $\mu$ g/mL       |
| Tween-20 (3%)             | 1 $\mu$ L                    | 0.0115%               |
| TMACL (5M)                | 140 $\mu$ L                  | 2.69 M                |
| <b>Total</b>              | <b>190 <math>\mu</math>L</b> |                       |

Mix well.

- Transfer each of the labeled sample from the plate to a 1.5 mL Eppendorf tube. Aliquot 190  $\mu\text{L}$  of the Hybridization Cocktail Master Mix into the 70  $\mu\text{L}$  of labeled DNA samples as follows:

| Reagent           | Volume/ Rx                          |
|-------------------|-------------------------------------|
| Labeled DNA       | 70 $\mu\text{L}$                    |
| Hybridization Mix | 190 $\mu\text{L}$                   |
| <b>Total</b>      | <b>260 <math>\mu\text{L}</math></b> |

**Note**

*This Hybridization Cocktail Master Mix can stored at -20°C before proceeding to the next step.*

- Heat the 260  $\mu\text{L}$  of hybridization mix and labeled DNA at 95°C in a heat block for 10 minutes to denature.
- Cool down on crushed ice for 10 seconds.

**Note**

*Do not leave on ice for longer than 10 seconds.*

- Spin briefly at 2,000 rpm for 1 minute in a microfuge to collect any condensate.

**Note**

*If there is anything that has come out of the solution, pipette briefly to resuspend before adding solution to the array.*

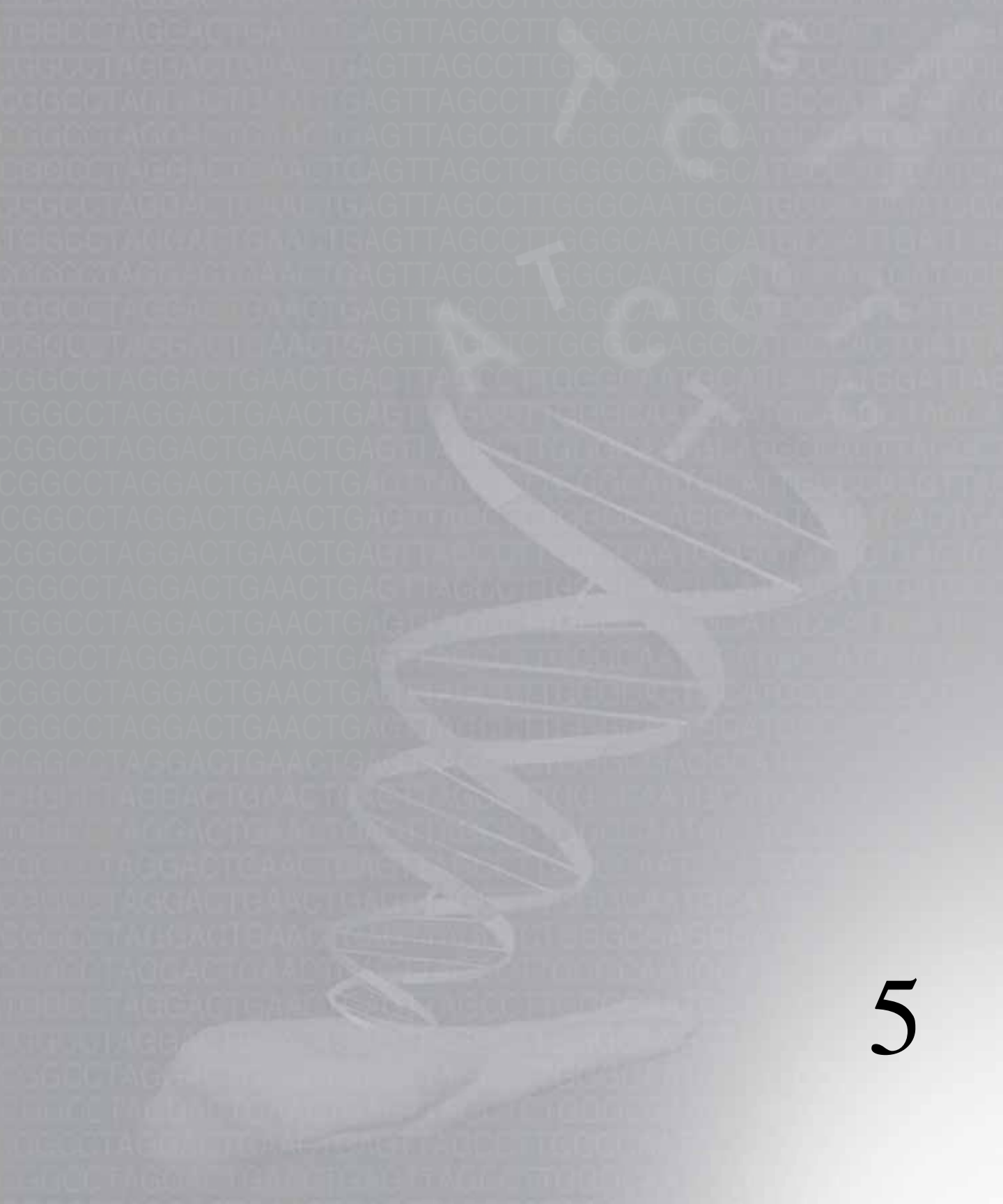
- Place the tubes at 48°C for 2 minutes.
- Inject 80  $\mu\text{L}$  denatured hybridization mix into the array.
- Hybridize at 48°C for 16 to 18 hours at 60 rpm.

**Note**

*The remaining hybridization mix can be stored at -20°C for future use.*

**Note**

*The hybridization temperature is 48°C. This is different from the GeneChip expression assay.*



# Chapter 5







# *Mapping Arrays: Washing, Staining, and Scanning*

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*This chapter contains instructions for using the Fluidics Station 450/250 to automate the washing and staining of GeneChip® Mapping probe arrays, and instructions for scanning probe arrays using 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 Chapter 7.*

## Reagents and Materials Required

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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, Chapter 10 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. Chapter 10 contains a master list of all reagents required for the GeneChip Mapping Assay protocol.

- 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
- 20X SSPE (3M NaCl, 0.2M NaH<sub>2</sub>PO<sub>4</sub>, 0.02 M EDTA), BioWhittaker Molecular Applications / Cambrex, P/N 51214
- Anti-streptavidin antibody (goat), biotinylated, Vector Laboratories, P/N BA-0500
- R-Phycoerythrin Streptavidin, Molecular Probes, P/N S-866
- 10% surfact-Amps20 (Tween-20), Pierce Chemical, P/N 28320
- Bleach (5.25% Sodium Hypochlorite), VWR Scientific, P/N 21899-504 (or equivalent)
- Denhardt's Solution, 50X concentrate: Sigma; P/N D2532
- MES hydrate, Sigma-Aldrich, P/N M5287
- MES Sodium Salt, Sigma-Aldrich, P/N M5057
- 5M NaCl, RNase-free, DNase-free, Ambion, P/N 9760G

**Miscellaneous Supplies**

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

## Reagent Preparation

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### **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  $\mu$ m filter.

Store at room temperature.

### **Wash B: Stringent Wash Buffer**

(0.6X SSPE, 0.01% Tween 20)

#### **For 1000 mL:**

30 mL of 20X SSPE

1.0 mL of 10% Tween-20

969 mL of water

Filter through a 0.2  $\mu$ m filter.

Store at room temperature.

### **0.5 mg/mL Anti-Streptavidin Antibody**

Resuspend 0.5 mg in 1 mL of water.

Store at 4°C.

### 12X MES Stock Buffer

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

**For 1,000 mL:**

70.4g of MES hydrate

193.3g of MES Sodium Salt

800 mL of Molecular Biology Grade water

Mix and adjust volume to 1,000 mL.

The pH should be between 6.5 and 6.7.

Filter through a 0.2 µm filter.

**▶ IMPORTANT**

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

### 1X Array Holding Buffer

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

**For 100 mL:**

8.3 mL of 12X MES Stock Buffer

18.5 mL of 5M NaCl

0.1 mL of 10% Tween-20

73.1 mL of water

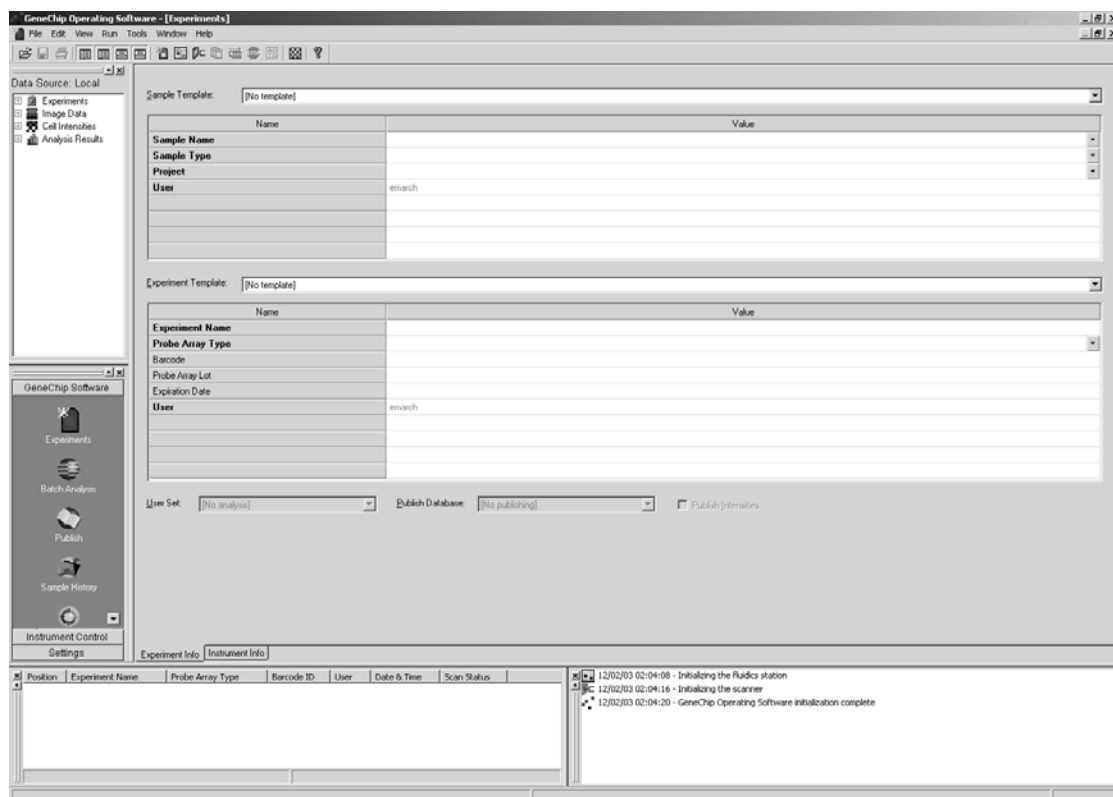
Store at 2°C to 8°C, and shield from light

## Experiment and Fluidics Station Setup

The following instructions are for GeneChip Operating Software (GCOS) 1.1.1 with Patch 5 or GeneChip Operating Software Server (GCOS Server).

### Step 1: Registering a New Experiment in GCOS

1. From the **File** menu click **New Experiment**.
  - ⇒ The New Experiment window appears in the display pane (Figure 5.1).
  - The top half of the display pane refers to the sample and the bottom half refers to the experiment.



**Figure 5.1**  
GCOS Sample Entry Pane

2. Enter information into the appropriate boxes.
  - Fields that are highlighted in bold require an entry.
  - Drop-down menus are available for Sample/Project information (default information can be used or new information can be entered).
  - The **Experiment Name** must be unique.
  - Library files must be installed for a probe array to appear in the drop-down menu.
3. From the **File** menu click **Save As**, or click the **Save** icon on the tool bar to register the experiment into the database.

**TIP**

*The Sample Information fields can be customized. See the GCOS User's Guide for further information.*

## Step 2: Preparing the Fluidics Station

The Fluidics Station 400, 450 or 250 is used to wash and stain the probe arrays; it is operated using GeneChip Operating Software.

### 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 in GCOS.
  - ⇒ The Fluidics Station dialog box appears with a drop-down list for selecting the experiment name for each of the fluidics station modules. A second drop-down list is accessed for choosing the Protocol for each of the fluidics station modules. Use the radio buttons to access each module.

**Note**

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

### Priming the Fluidics Station

Priming ensures the lines of the fluidics station are filled with the appropriate buffers and the fluidics station is ready to run the 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.
  5. Follow LCD instructions.
  6. Time out.

#### IMPORTANT

*The wash and staining buffers are different from the GeneChip expression buffers.*

#### Note

*All modules can be selected by selecting the **All Modules** button in the fluidics dialog box.*



## Probe Array Wash and Stain

The Affymetrix staining protocol for Mapping arrays is a three stage process consisting of a Streptavidin Phycoerythrin (SAPE) stain, followed by an antibody amplification step and final stain with Streptavidin Phycoerythrin (SAPE). Following staining, the array is filled with Array Holding Buffer prior to scanning as outlined in Table 5.5.

1. After 16 hours of hybridization, remove the hybridization cocktail from the probe array and set it aside in a microcentrifuge vial. Store on ice during the procedure or at -80°C for long-term storage.
2. Fill the probe array completely with 80 µL of Array Holding Buffer.

### ✓ Note

*If necessary, the probe array can be stored in the Array Holding Buffer 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.*

## Preparing the Staining Reagents

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

### *Stain Buffer*

**Table 5.1**  
Stain Buffer

| Components          | 1X            | Final Concentration |
|---------------------|---------------|---------------------|
| H <sub>2</sub> O    | 666.7 µL      |                     |
| SSPE (20X)          | 300 µL        | 6X                  |
| Tween-20 (3%)       | 3.3           | 0.01%               |
| Denhardt's (50X)    | 20            | 1X                  |
| <b>Subtotal</b>     | <b>990 µL</b> |                     |
| <b>Subtotal / 2</b> | <b>495 µL</b> |                     |

### SAPE Stain Solution

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

**Table 5.2**  
SAPE Solution Mix

| Components                                | Volume                       | Final Concentration |
|---|------------------------------|---------------------|
| Stain Buffer                              | 495 $\mu$ L                  | 1X                  |
| 1 mg/mL Streptavidin Phycoerythrin (SAPE) | 5.0 $\mu$ L                  | 10 $\mu$ g/mL       |
| <b>Total</b>                              | <b>500 <math>\mu</math>L</b> |                     |

Mix well.

 **Note**

**FS450 Users:** A vial containing SAPE Stain Solution must be placed in sample holder 1 for each module used.

**FS400 Users:** A vial containing SAPE Stain Solution must be used for the first and third stains.

## Antibody Stain Solution

**Table 5.3**  
Antibody Solution Mix

| Components                      | Volume                       | Final Concentration |
|---------------------------------|------------------------------|---------------------|
| Stain Buffer                    | 495 $\mu$ L                  | 1X                  |
| 0.5 mg/mL biotinylated antibody | 5 $\mu$ L                    | 5 $\mu$ g/mL        |
| <b>Total</b>                    | <b>500 <math>\mu</math>L</b> |                     |

Mix well.

### ✓ Note

**FS450 Users:** A vial containing Antibody Stain Solution must be placed in sample holder 2 for each module used.

**FS400 Users:** A vial containing Antibody Stain Solution must be used for the second stain.

## Array Holding Buffer

**Table 5.4**  
Array Holding Buffer

| Components             | Volume        |
|------------------------|---------------|
| MES Stock Buffer (12X) | 8.3 mL        |
| 5M NaCl                | 18.5 mL       |
| Tween-20 (10%)         | 0.1 mL        |
| Water                  | 73.1 mL       |
| <b>Total</b>           | <b>100 mL</b> |

Add 800  $\mu$ L of Array Holding Buffer to each microcentrifuge tube. One tube is needed per module used.

### ✓ Note

**FS450 Users:** A vial containing Array Holding Buffer must be placed in sample holder 3 for each module used.

**FS400 Users:** A vial containing Array Holding Buffer must be used at the final step in the fluidics protocol. This will ensure that the array is filled with Array Holding Buffer when the fluidics protocol is complete.

**Table 5.5**

Fluidics Protocol - Antibody Amplification for Mapping Targets  
 (protocols for the Fluidics Station 450/250 will have \_450 as a suffix)

| <b>169 Format</b><br>Mini_Mapping10Kv1_450 |  |
|--|--|
| <b>Post Hyb Wash #1</b>                    | 6 cycles of 5 mixes/cycle with Wash Buffer A at 25°C                                   |
| <b>Post Hyb Wash #2</b>                    | 6 cycles of 5 mixes/cycle with Wash Buffer B at 45°C                                   |
| <b>Stain</b>                               | Stain the probe array for 10 minutes in SAPE at 25°C                                   |
| <b>Post Stain Wash</b>                     | 6 cycles of 5 mixes/cycle with Wash Buffer A at 25°C                                   |
| <b>2nd Stain</b>                           | Stain the probe array for 10 minutes in antibody solution at 25°C                      |
| <b>3rd Stain</b>                           | Stain the probe array for 10 minutes in SAPE solution at 25°C                          |
| <b>Final Wash</b>                          | 10 cycles of 6 mixes/cycle with Wash Buffer A at 30°C. The holding temperature is 25°C |
| <b>Filling Array</b>                       | Fill the array with Array Holding Buffer.  |

Wash Buffer A = non-stringent wash buffer

Wash Buffer B = stringent wash buffer

## Washing and Staining the Probe Array Using FS-450/250

### FS-450

#### If you are using the Fluidics Station 450/250:

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 **Mini\_Mapping10Kv1\_450**, to control the washing and staining of the probe array.
3. Choose **Run** in the Fluidics Station dialog box to begin the washing and staining. Follow the instructions in the LCD window on the fluidics station.

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

4. Insert the appropriate probe array into the designated module of the fluidics station while the cartridge lever is in the down, or eject, position. When finished, verify that the cartridge lever is returned to the up or engaged position.
5. Remove any microcentrifuge vials remaining in the sample holders of the fluidics station module(s) being used.
6. When prompted to "Load Vials 1-2-3," place the three experiment sample vials (the microcentrifuge vials) into the sample holders 1, 2 and 3 on the fluidics station.
  - Place one vial containing 500  $\mu$ L Streptavidin Phycoerythrin (SAPE) stain solution mix in sample holder 1.
  - Place one vial containing 500  $\mu$ L anti-streptavidin biotinylated antibody stain solution in sample holder 2.
  - Place one vial containing 800  $\mu$ L Array Holding Buffer in sample holder 3.
  - Press down on the needle lever to snap needles into position and to start the run.

Once these steps are complete, the fluidics protocols begin. The Fluidics Station dialog box at the workstation terminal and the LCD window displays the status of the washing and staining steps.

7. When staining is finished, remove the microcentrifuge vials containing stain and replace with three empty microcentrifuge vials as prompted.
8. Remove the probe arrays from the fluidics station modules by first pressing down the cartridge lever to the eject position.
9. Check the probe array window for large bubbles or air pockets.
  - If bubbles are present, the probe array should be filled with Array Holding Buffer manually, using a pipette. Take out one-half of the solution and then manually fill the probe array with Array Holding Buffer.
  - If the probe array has no large bubbles, it is ready to scan on the GeneChip Scanner 3000. Pull up on the cartridge lever to engage wash block and proceed to *Probe Array Scan* on page 71.

**➤ IMPORTANT**

*If a bubble is present, do not return the probe array to the probe array holder. The probe array must be filled manually with Array Holding Buffer.*

If you can not scan the arrays right away, keep the probe arrays at 4°C and in the dark until ready for scanning. Scan within 24 hours.

If no more samples require washing and staining, shut down the fluidics station following the procedure outlined in the section, *Shutting Down the Fluidics Station* on page 74.

**✓ Note**

*For proper cleaning and maintenance of the fluidics station, including the bleach protocol, please refer to Chapter 6, Fluidics Station Maintenance Procedures.*

**➤ IMPORTANT**

*The wash and staining buffers are different from the GeneChip expression buffers.*

## Washing and Staining the Probe Array Using FS-400

### FS-400

#### If you are using the Fluidics Station 400:

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 **Mini\_Mapping10Kv1**, to control the washing and staining of the probe array.
3. Choose **Run** in the Fluidics Station dialog box to begin the washing and staining. Follow the instructions on the LCD window on the fluidics station.
4. If you are unfamiliar with inserting and removing probe arrays from the fluidics station modules, please refer to the Fluidics Station 400 User's Guide, or Quick Reference Card (P/N 08-0072).
5. Insert the appropriate probe array into the designated module of the fluidics station while the cartridge lever is in the **EJECT** position. When finished, verify the cartridge lever is returned to the **ENGAGE** position.
6. Remove any microcentrifuge vials remaining in the sample holders of the fluidics station module(s) being used.
7. When the LCD window indicates, place the microcentrifuge vial containing 500  $\mu$ L Streptavidin Phycoerythrin (SAPE) stain solution into the sample holder. Verify that the metal sampling needle is in the vial with its tip near the bottom.
8. When the LCD window indicates, replace the microcentrifuge vial containing the SAPE stain solution with a microcentrifuge vial containing antibody stain solution into the sample holder, making sure that the metal sampling needle is in the vial with its tip near the bottom.
9. When the LCD window indicates, replace the microcentrifuge vial containing the antibody stain solution with the first microcentrifuge vial containing 500  $\mu$ L Streptavidin Phycoerythrin (SAPE) stain solution into the sample holder. Verify the metal sampling needle is in the vial with its tip near the bottom.

10. When the LCD window indicates, replace the microcentrifuge vial containing the SAPE solution with a microcentrifuge vial containing 800  $\mu$ L Array Holding Buffer.
  - ⇒ 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.
11. Remove microcentrifuge vial containing Array Holding Buffer and replace with an empty microcentrifuge vial.
12. Remove the probe arrays from the fluidics station modules by first moving the probe array holder lever to the **EJECT** position.
13. Check the probe array window for large bubbles or air pockets.
  - If bubbles are present, the probe array should be filled with Array Holding Buffer manually, using a pipette. Take out one-half of the solution and then manually fill the probe array with Array Holding Buffer.
  - If the probe array has no large bubbles, it is ready to scan on the GeneChip Scanner 3000. **ENGAGE** wash block and proceed to *Probe Array Scan* on page 71.

**IMPORTANT**

*If a bubble is present, do not return the probe array to the probe array holder. The probe array must be filled manually with Array Holding Buffer.*

If you can not scan the arrays right away, keep the probe arrays at 4°C and in the dark until ready for scanning. Scan within 24 hours.

If there are no more samples to wash and stain, shut down the fluidics station following the procedure outlined in the section, *Shutting Down the Fluidics Station* on page 74.

**✓ Note**

*For proper cleaning and maintenance of the fluidics station including the bleach protocol, please refer to Chapter 6, Fluidics Station Maintenance Procedures.*

**IMPORTANT**

*The wash and staining buffers are different from the GeneChip expression buffers.*



## Probe Array Scan

The scanner is also controlled by Affymetrix GeneChip Operating Software 1.1.1 with Patch 5, or higher. The probe array is scanned after the wash protocols are complete. Make sure the laser is warmed up prior to scanning by turning the laser on at least 10 minutes before use. If the probe array was stored at 4°C, allow to warm to room temperature before scanning.

### ✓ Note

*Refer to the GCOS 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.*

*You must have read and be familiar with the operation of the scanner before attempting to scan a probe array. Please refer to the GeneChip Scanner 3000 Quick Reference Card (P/N 08-0075).*

## Handling the GeneChip® Probe Array

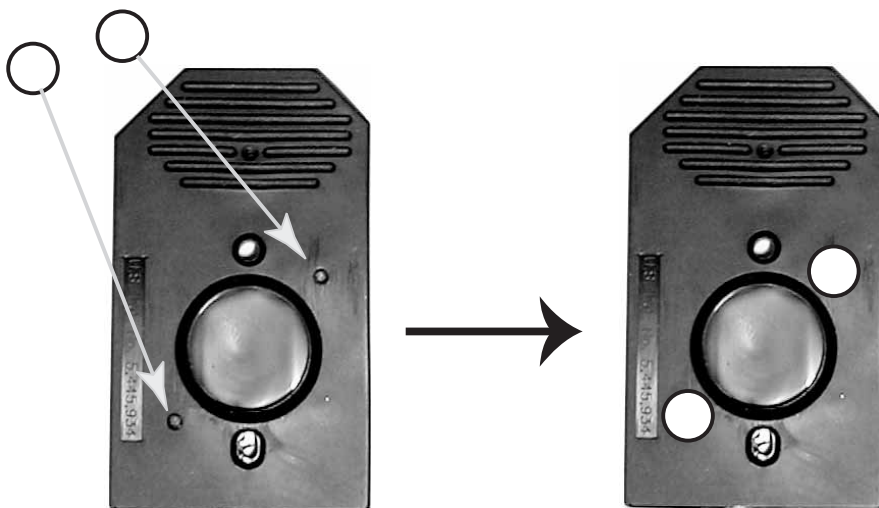
Before you scan the probe array, follow the directions in this section on handling the probe array. If necessary, clean the glass surface of the probe array with a non-abrasive towel or tissue before scanning. Do not use alcohol to clean the glass.

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

### ➔ IMPORTANT

*Apply the spots just before scanning. Do not use them in the washing and staining process.*

1. On the back of the probe array cartridge, clean excess fluid from around the septa.
2. Carefully apply one Tough-Spot to each of the two septa. Press to ensure 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 5.2.



**Figure 5.2**  
Applying Tough-Spots™ to the probe array cartridge

---

3. Insert the cartridge into the scanner and test the autofocus to ensure 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 all unscanned experiments.
2. Select the experiment name that corresponds to the probe array you wish to scan.

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.

### ✓ Note

*If you do not see your experiment name, open the workflow monitor, right-click your experiment, and select **Advance to Scan**. Refer to the GCOS manual for further information.*

3. Click the **Load/Eject** button and place the array in the scanner. Only one scan is required for the GeneChip Scanner 3000.

### ▲ WARNING

*Please do not attempt to manually open or close the GeneChip Scanner 3000 scanner door as you may damage the scanner.*

4. Once the experiment has been selected, click the **Start** button.
  - ⇒ A dialog box prompts you to load the array into the scanner.
5. Pixel resolution and wavelength for the GeneChip Scanner 3000 are preset and cannot be changed.
6. Open the sample door of the scanner and insert the probe array into the holder. Do not force the probe array into the holder. The door of the GeneChip Scanner 3000 closes automatically.

### ⇒ IMPORTANT

*Do not force the probe array into the holder.*

7. Click **OK** in the Start Scanner dialog box.
  - ⇒ The scanner begins scanning the probe array. 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 wash block by pushing up on the cartridge lever to the **ENGAGE** position.  
If you are using the FS-450/250, 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 processing is 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 appropriate *Fluidics Station User's Guide* for more information.
7. After Shutdown protocol is complete, flip the ON/OFF switch of the fluidics station to the OFF position.

### ➤ IMPORTANT

*To maintain the cleanliness of the fluidics station and obtain the highest quality image and data possible, a weekly bleach protocol and a monthly decontamination protocol are highly recommended. Please refer to Chapter 6, Fluidics Station Maintenance Procedures.*

## 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 GeneChip Operating Software 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 5.6.

**Table 5.6**  
Valid Ranges for Wash/Stain Parameters

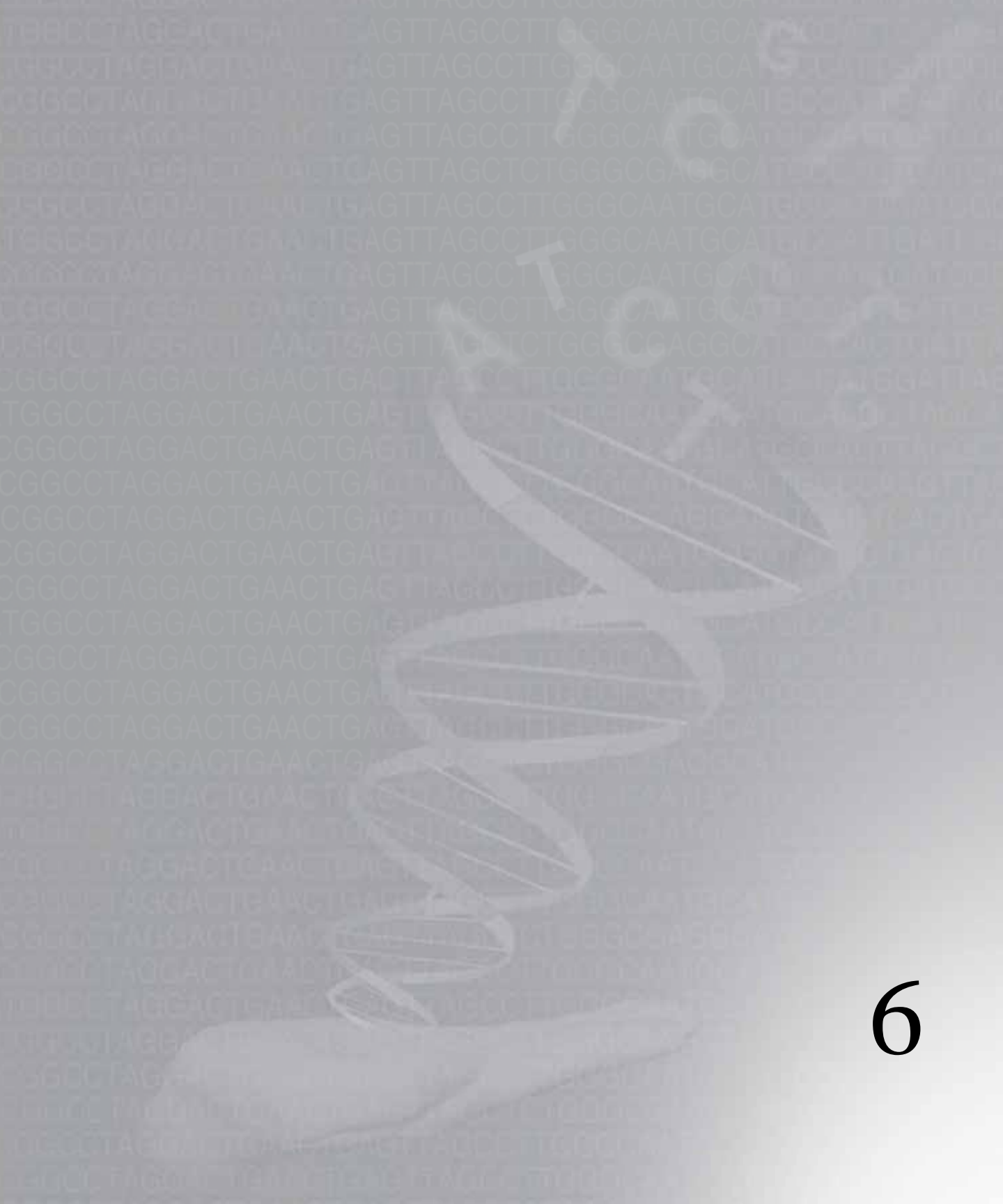
| Parameter                                  | Valid Range |
|--|-------------|
| Wash Temperature for A1, B, A2, or A3 (°C) | 15 to 50    |
| Number of Wash Cycles for A1, B, A2, or A3 | 0 to 99     |
| Mixes / Wash cycle for A1, B, A2, or A3    | 15 to 50    |
| Stain Time (seconds)               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

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.

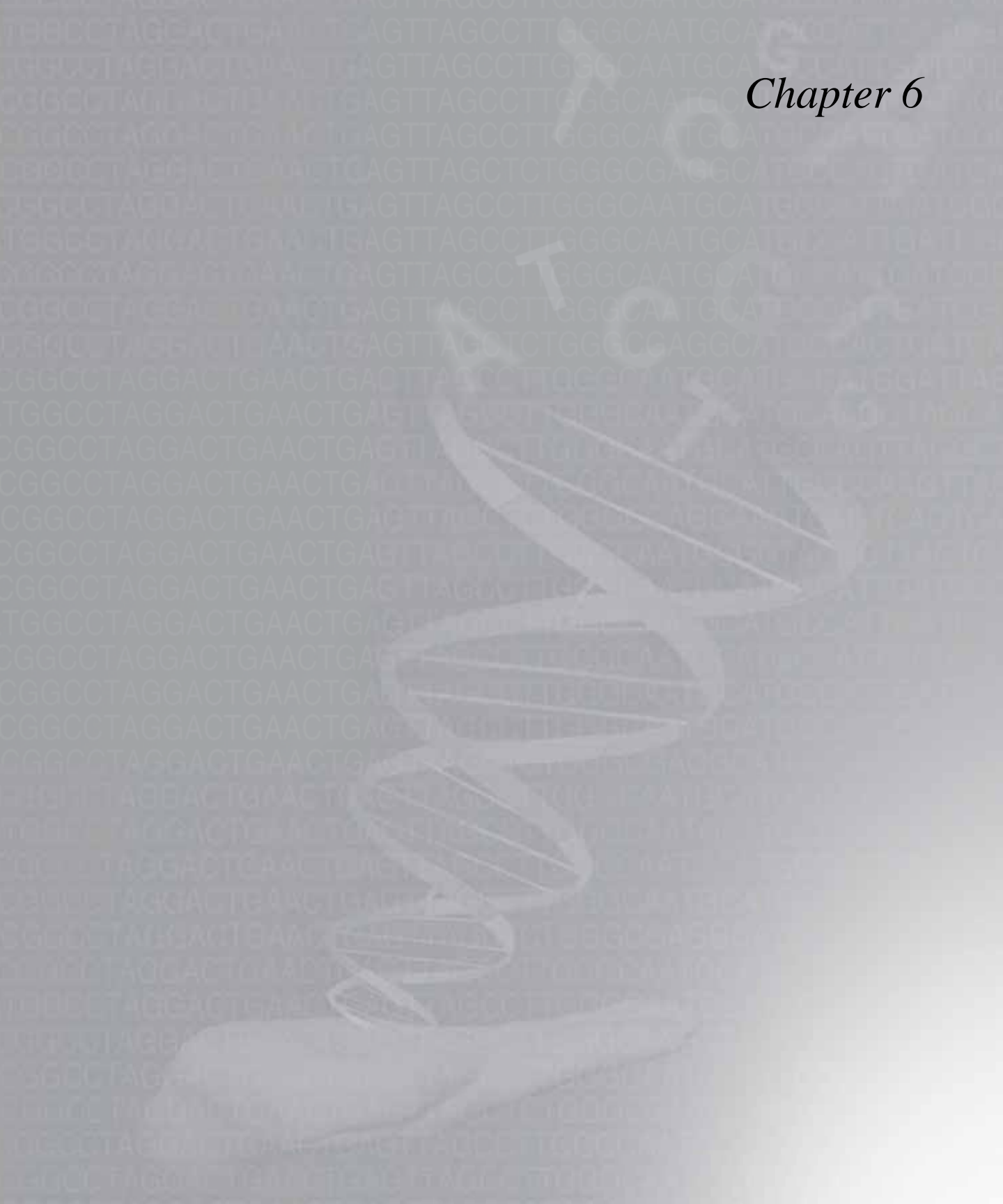
### ! CAUTION

*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.



# Chapter 6







# Fluidics Station Maintenance Procedures

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## Weekly Fluidics Station Cleanout

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A cleaning protocol is recommended for fluidics station maintenance if the antibody staining procedure is used. Choose **Bleach** or **Bleach\_450** for all modules from the drop-down list in the Fluidics Station dialog box. Click the **Run** button for all modules and follow LCD instructions.

### Bleach Protocol

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

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

#### Note

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

2. Cut tubing.

#### FS-450

### If you are using the Fluidics Station 450/250:

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

## FS-400

**If you are using the Fluidics Station 400:**

Cut four pieces of tubing at least 2.5 to 3 feet in length.

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

**✓ Note**

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

4. Choose **Fluidics** from the **Run** menu. Alternatively, click the down arrow Protocol list on the toolbar.
5. Choose **Bleach** or **Bleach\_450** for the respective modules in the **Protocol** drop-down list.
6. Disengage the washblock for each module by pressing down on the cartridge lever.



Washblocks disengaged with cartridge levers down

**Figure 6.1**

Disengaged washblocks showing cartridge levers in the down position.

**✓ Note**

*Temperature will ramp up to 50°C.*

7. Connect tubing to needles.

**FS-450****If you are using the Fluidics Station 450/250:**

Connect one end of the plastic tubing to each of the three needles. The proper technique is to press down on the cartridge lever until the needles extend a convenient distance from the module, then slip the tube on as you hold the cartridge lever down with the free hand as shown in Figure 6.2.

**✓ Note**

*Take care not to bend or break the needles.*

**FS-400****If you are using the Fluidics Station 400:**

Connect one end of the plastic tubing to each needle at the bottom of each module.



**Figure 6.2**

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

8. Insert the other ends into 0.525% sodium hypochlorite solution (at least 500 mL for all four modules) as shown in Figure 6.3.

✓ Note

*Remove cartridges before you start the bleach protocol.*



**Figure 6.3**  
The tubes extending from the modules to the bleach bottle. Note that the probe array cartridges must be removed before the protocol can begin.

---

Ensure that all the tube ends remain immersed in the bleach solution by tamping down on the tubes using a dowel or similar object as shown in Figure 6.4.



**Figure 6.4**

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

✓ **Note**

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

9. Remove any probe array cartridges and engage the washblock as shown in Figure 6.5. The fluidics station will begin the protocol, begin to empty the lines, and perform three cleaning cycles of 10 rinses each using bleach solution.



**Figure 6.5**

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

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



*Do not pull the tube out, as this may damage the needle in the process.*

11. Load empty microcentrifuge vials onto each module.  
The fluidics station will empty the lines and run three cycles with three rinses each. In addition, the fluidics station will rinse the needle 20 times, twice using distilled water, then bring the temperature back to 25°C and drain the lines with air.
12. The LCD display will read **CLEANING DONE**.

## Monthly Fluidics Station Decontamination Protocol

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

This protocol ensures that all of the tubing associated with the station is kept clean. Keeping this tubing as clean as possible ensures high-quality results.

1. Prepare 2 liters of 0.525% sodium hypochlorite solution using distilled water. Mix well.
2. Place all three wash lines of the fluidics station in 1 liter of 0.525% sodium hypochlorite solution.
3. Run the **Prime** protocol (Fluidics Station 400) or the **Prime\_450** protocol (Fluidics Station 450/250) on all four modules with wash lines in 0.525% sodium hypochlorite solution (instead of wash buffers A and B).
4. Run the **Shutdown** protocol (Fluidics Station 400) or the **Shutdown\_450** (Fluidics Station 450/250) on all four modules with wash lines in 0.525% sodium hypochlorite solution (instead of distilled water).
5. Follow Bleach Protocol (as described on page 79) with the following change in Step 3: place the three wash lines of the fluidics station in 1 liter of 0.525% sodium hypochlorite solution instead of distilled water.
6. Change intake tubing and peristaltic tubing, if required (as described in the *Fluidics Station 400 User's Guide* or the *Fluidics Station 450/250 User's Guide*).
7. Run the **Bleach** protocol (Fluidics Station 400) or the **Bleach\_450** protocol (Fluidics Station 450/250) with three wash lines of the fluidics station in distilled water.
8. Run the **Prime** protocol (Fluidics Station 400) or the **Prime\_450** protocol (Fluidics Station 450/250) with wash lines in distilled water (instead of wash buffers A and B).



9. Run the **Shutdown** protocol (Fluidics Station 400) or the **Shutdown\_450** protocol (Fluidics Station 450/250) with wash lines in distilled water.
10. Run the **Prime** protocol (Fluidics Station 400) or the **Prime\_450** protocol (Fluidics Station 450/250) with wash lines in distilled water (instead of wash buffers A and B).
11. Run the **Shutdown** protocol (Fluidics Station 400) or the **Shutdown\_450** protocol (Fluidics Station 450/250) with distilled water.

 **Note**

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





# Chapter 7





# Data Analysis

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*The purpose of this chapter is to outline the necessary steps to analyze data following the scanning of GeneChip® Human Mapping 10K Array Xba 142 2.0 and to present some guidelines to assess the quality of the data. This chapter is designed as a supplement to the information sources listed below and does not replace them:*

- GeneChip Operating Software (GCOS) User's Guide
- GeneChip DNA Analysis Software (GDAS) User's Guide

## Software Requirements

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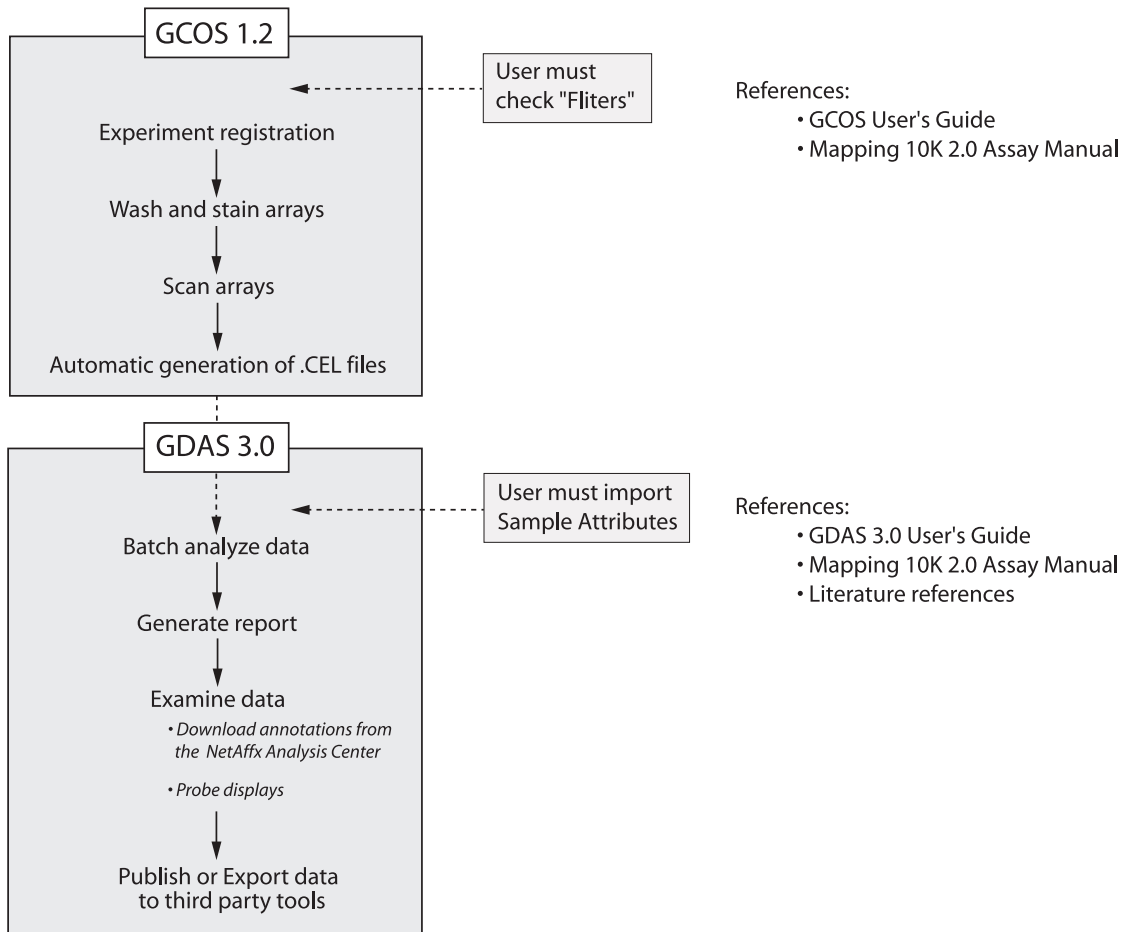
- GeneChip Operating Software (GCOS) 1.1.1 with Patch 5 or higher
- GeneChip DNA Analysis Software (GDAS) 2.0 or higher
- GeneChip Mapping 10K library files: Mapping10K\_Xba142



*All of the above must be installed on the same workstation.*

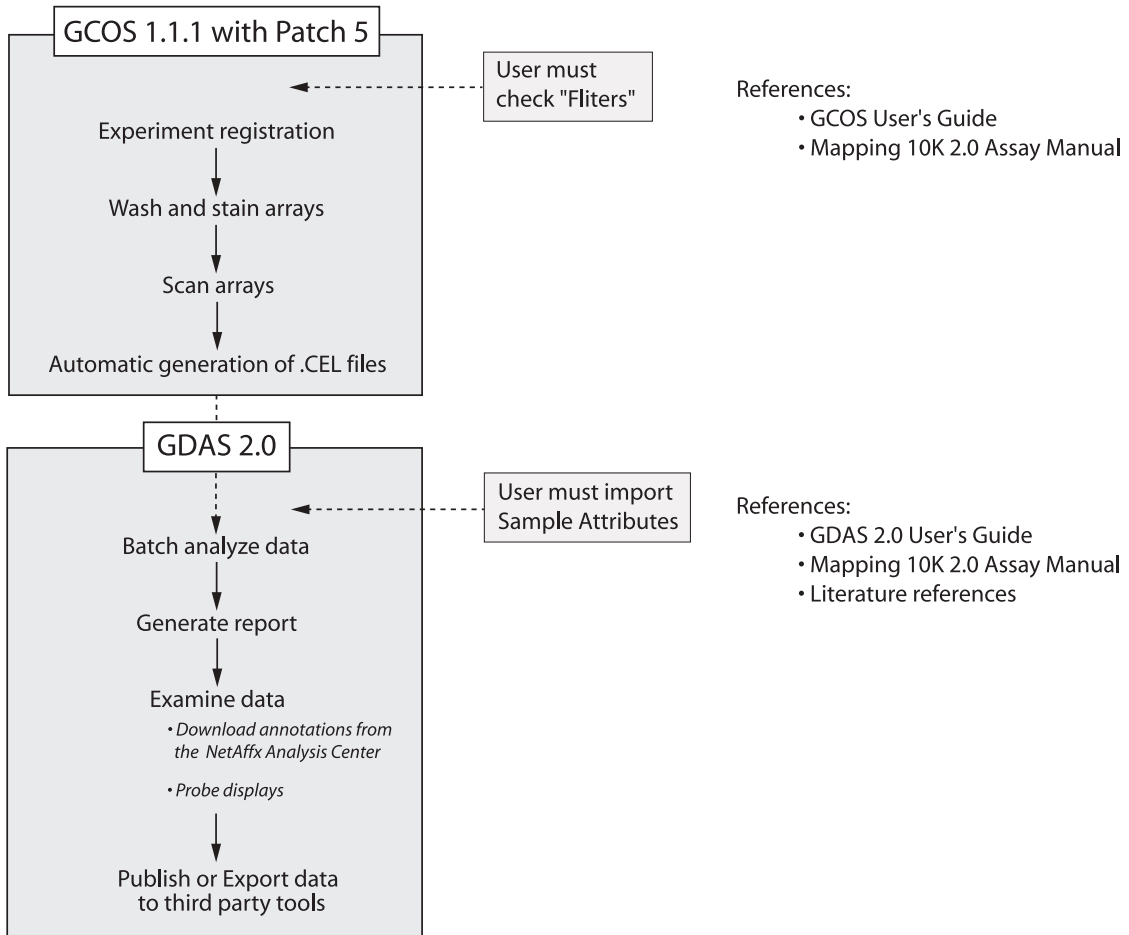
## Analysis Workflow

This section describes the methods for acquiring data using the GeneChip Operating Software (GCOS) and then analyzing the data using GeneChip DNA Analysis Software (GDAS). The outline of the data analysis schemes are shown in Figure 7.1, Figure 7.2, and Figure 7.3. Different data analysis workflows must be used depending on the workstation software configuration.

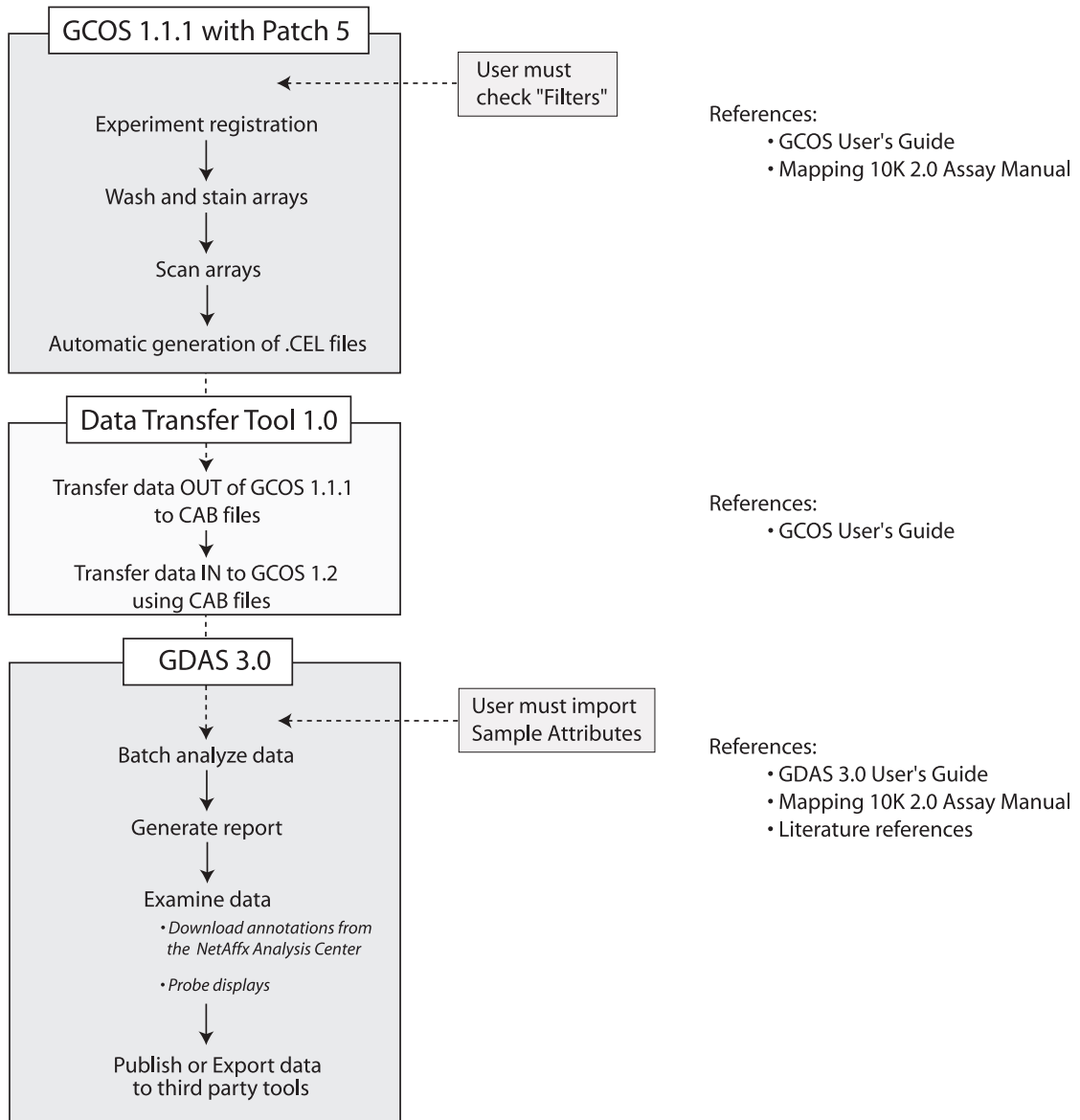


**Figure 7.1**

Data analysis flow for GeneChip® Human Mapping 10K Array Xba 142 2.0 when using GCOS 1.2 and GDAS 3.0

**Figure 7.2**

Data analysis flow for GeneChip® Human Mapping 10K Array Xba 142 2.0 when using GCOS 1.1.1 with Patch 5 and GDAS 2.0



**Figure 73**  
Data analysis flow for GeneChip® Human Mapping 10K Array Xba 142 2.0 when using GCOS 1.1.1 with Patch 5, Data Transfer Tool 1.0, and GDAS 3.0



## Analysis

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The analysis of the data files takes place using GDAS 2.0 or higher. Note that GDAS 3.0 can only be used in conjunction with GCOS 1.2 or higher. Also, GCOS and GDAS must be installed on the same workstation. The following is an overview of the steps necessary to analyze data using GDAS. Further information can be found in the GDAS Software User's Guide.

1. The default settings can be found by selecting **Mapping Analysis Settings** from the **Tools** menu.  
These settings have been chosen because they provide accurate genotype calls. Discussion about these settings can be found in the GDAS Software User's Guide.
2. Open the Batch Analysis window. Then, select the appropriate .cel files and drag the files into the Batch Analysis window.

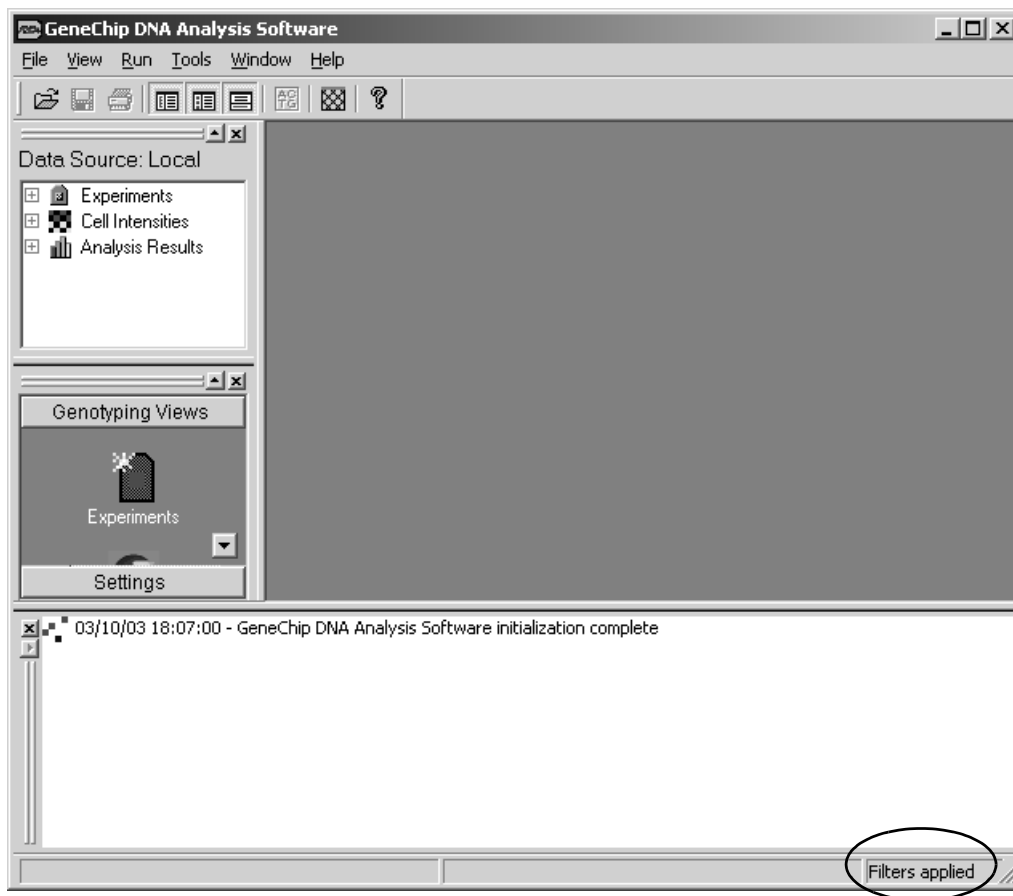
 **Note**

*In order to analyze data with GDAS 3.0, .cel files that were generated using GCOS 1.1.1 with Patch 5 must first be saved as a .cab file using the Data Transfer Tool. Then, using the same tool, that data must be transferred into GCOS 1.2. Refer to Figure 7.3 for an illustration of that workflow.*

**TIP**

If no files can be seen in the file tree, check to see if any filters are applied, this is shown by the “Filter Applied” message in the bottom right of the window (Figure 7.4). The filter settings can be adjusted by selecting the filter options in the Tools menu bar.

3. Click **Analyze** and the files will be analyzed.
4. Following analysis a report will be displayed summarizing data from the samples.



**Figure 7.4**  
GDAS main window - Filter Applied message

## Report

A Mapping Algorithm report is automatically generated when you analyze cell intensity data from Mapping arrays. The report is automatically displayed when you use the Batch Analysis window to analyze data.

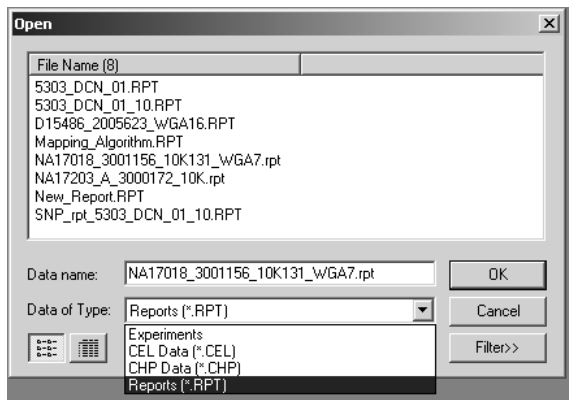
| Mapping Array Report   |                   |               |             |                  |         |         |         |
|--|-------------------|---------------|-------------|------------------|---------|---------|---------|
| Report File Name - C:\GeneChip\Affy_Data\Data\NA17203_X_mini10K_4001825_IVT1.RPT |                   |               |             |                  |         |         |         |
| Date:  | 06/01/04 13:55:23 |               |             |                  |         |         |         |
| [REDACTED]   |                   |               |             |                  |         |         |         |
| Total number of SNPs:  | 10204             |               |             |                  |         |         |         |
| Total number of QC Probes:   | 4                 |               |             |                  |         |         |         |
| Probe array type:  | Mapping10K_Xba142 |               |             |                  |         |         |         |
| SNP Performance  |                   |               |             |                  |         |         |         |
| CEL Data   | Gender            | Called Gender | SNP Call    | Signal Detection | AA Call | AB Call | BB Call |
| NA17203_X_mini10K_4001825_IVT1   | Unknown           | M             | 99.42%      | 99.97%           | 33.12%  | 34.22%  | 32.66%  |
| NA17203_X_mini10K_4001825_IVT2   | Unknown           | M             | 99.22%      | 99.98%           | 33.21%  | 34.03%  | 32.76%  |
| NA17203_X_mini10K_4001825_TAK1   | Unknown           | M             | 99.15%      | 99.99%           | 33.18%  | 34.08%  | 32.74%  |
| NA17203_X_mini10K_4001825_TAK2   | Unknown           | M             | 99.11%      | 99.95%           | 33.22%  | 34.08%  | 32.69%  |
| QC Performance   |                   |               |             |                  |         |         |         |
| CEL Data   | AFFX-5Q-123       | AFFX-5Q-456   | AFFX-5Q-789 | AFFX-5Q-ABC      |         |         |         |
| NA17203_X_mini10K_4001825_IVT1   | 1160.5            | 368.0         | 1645.5      | 3302.0           |         |         |         |
| NA17203_X_mini10K_4001825_IVT2   | 957.5             | 296.0         | 1336.0      | 2541.5           |         |         |         |
| NA17203_X_mini10K_4001825_TAK1   | 1295.0            | 421.0         | 1901.5      | 3588.0           |         |         |         |
| NA17203_X_mini10K_4001825_TAK2   | 859.0             | 357.5         | 1254.0      | 2276.0           |         |         |         |

**Figure 7.5**

The Mapping Algorithm report

You can also open a Mapping Algorithm saved report by:

1. Clicking the **Open** button in the main toolbar, or selecting **File** → **Open** from the main menu.  
⇒ The Open dialog box appears.



**Figure 7.6**  
Open dialog box

2. From the **Data of Type** drop-down list, select **Reports (\*.RPT)**.
3. Select the Mapping Algorithm report from the displayed list.
4. Click **OK** to open the report.

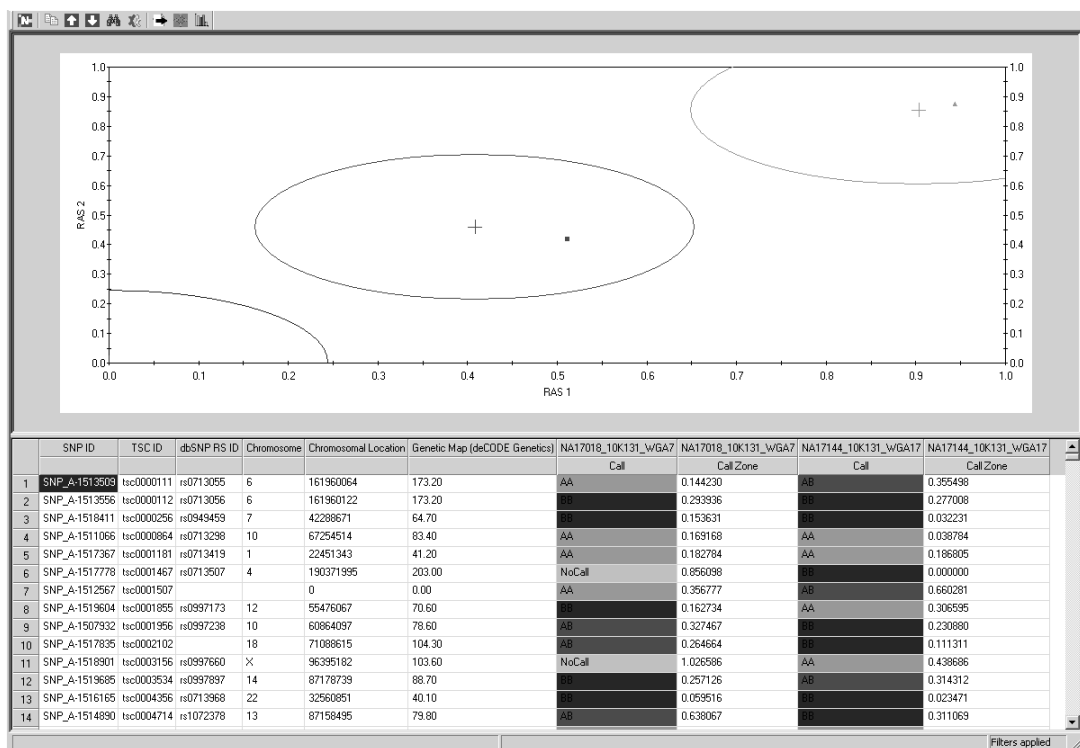
The report contains information that can be used to assess the performance of the sample, including:

- **Call rate:** The percentage of SNPs called on the array.
- **Signal Detection Rate:** The percentage of SNPs that have passed the discrimination filter.
- **AA%:** Percentage of SNPs that have a AA call.
- **AB%:** Percentage of SNPs that have a AB call.
- **BB%:** Percentage of SNPs that have a BB call.

Also, the QC probe metrics are reported. You can learn more about reports in the GDAS Software User's Guide.

## Output

Following analysis .chp files are generated for each sample. The .chp files can be opened by double-clicking on the files in the GDAS data file tree. Multiple .chp files can be opened simultaneously. Once opened the .chp files will display a RAS scatter plot in the upper portion of the window and a data table in the lower portion of the window as shown in Figure 7.7. In the data table, a genotype call for each SNP along with a call zone score is displayed. Double-clicking on a SNP will change the RAS scatter plot.



**Figure 7.7**  
GDAS 2.0 Table View



**TIP**

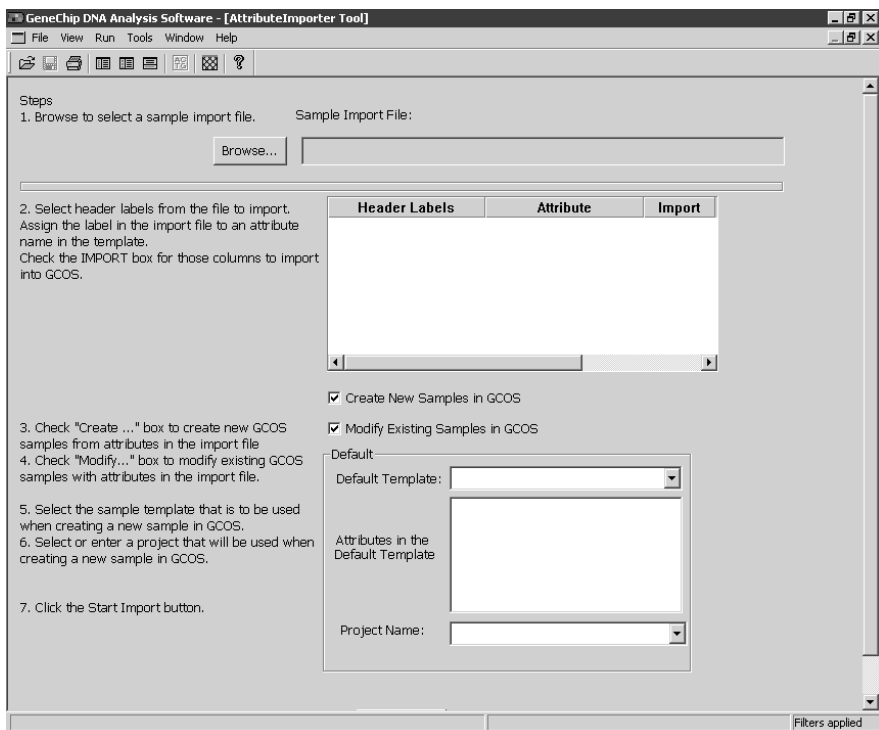
*If you do not see the .chp files that you have generated in the data file tree, right-click the "Analysis Results" bar and click "Refresh all."*

## Additional Functionality

GDAS provides additional functionality including the ability to drill down to raw probe intensity data, updating SNP annotation from the NetAffx Analysis Center, and data export. Refer to the GDAS Software User's Guide for more information.

## Import Sample Attributes

Pedigree information associated with the sample can be imported into the GCOS Process database by using the AttributeImporter Tool. Attributes can be imported from multiple samples in batch from files or excel spreadsheets. Attributes includes information like father, mother, family and diseased state and must be imported before checking Mendelian Inheritance or importing into Merlin or GeneHunter.



**Figure 7.8**  
GDAS AttributeImporter Tool

## Examining the Raw Probe Data

The raw probe intensity information can be obtained by selecting a SNP identifier and clicking on the “Probe Intensity Window” item in the “Run” menu. Select the appropriate .cel file and the probe intensity data will be displayed in bar or line graph format. Refer to the GDAS Software User’s Guide for more information.

## Export

SNP call data can be exported for use in third party software products as either a tab delimited file; in Merlin format, GeneHunter format, or Microsoft Excel format. To export data:

1. Open the .chp files you would like to export.
2. Select **Export** and **Table** in the menu bar.
3. Specify the location and format of the export data.

**Note**

*The data set displayed in the table is exported.*

## NetAffx™ SNP Annotation

The NetAffx™ Analysis Center provides regularly updated information about the SNPs on the Mapping arrays. You can download the annotation data from the NetAffx Analysis Center and display it in the SNP table of the analysis window.

Default annotations (provided with the software) include:

- The SNP Consortium (TSC) IDs
- dbSNP Database IDs
- Chromosome position of the SNP
- Genetic map position of the SNP

You can also download additional annotations, including:

- Flanking microsatellite markers
- Nearest genes

**Note**

*Some annotations are provided by default. These annotations are automatically installed on your computer and displayed when the Mapping window opens.*

 **Note**

*You should download updates frequently from the NetAffx Analysis Center to ensure that you have the most up-to-date information.*

The default NetAffx annotations are displayed automatically when you display analysis results in the Mapping window.

 **Note**

*You may need to initialize NetAffx access to obtain certain types of annotations to download annotations in the future.*

*Detailed information for initializing the default settings, and downloading and selecting the annotations can be found in the “NetAffx Annotations” chapter in the GDAS 3.0 (or higher) Software User’s Guide.*

To use the NetAffx annotation option for the first time:

1. Initialize the NetAffx default settings.
2. Download the NetAffx annotations you wish to display.
3. Select the NetAffx annotations to display.



## How Do I Know That My Experiment Has Worked?

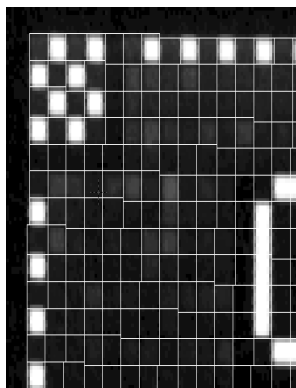
---

The purpose of this section is to help researchers establish quality control processes for Mapping 10K 2.0 experiments. The following are a series of parameters associated with the assay and hybridization that can be monitored and used to flag potential outlier samples. It is important to create a **running log** of these parameters for each project. Evaluation of a particular sample should be based on the examination of all sample and array performance metrics.

### B2 Oligo Performance

The B2 oligo is a component of the Oligonucleotide Control Reagent (OCR). It is spiked into each hybridization cocktail and is highlighted on the image by the following:

- The alternating pattern of intensities on the border
- The checkerboard pattern at each corner (Figure 7.9)
- The array name, located in the upper-left or upper-middle of the array (Figure 7.10)



**Figure 7.9**  
Example of corner checkerboard pattern.



**Figure 7.10**  
Array name.

B2 Oligo serves as a positive hybridization control and is used by the software to place a grid over the image. Variation in B2 hybridization intensities across the array is normal and does not indicate variation in hybridization efficiency.

### **Oligonucleotide Controls**

The oligonucleotide control reagent (GeneChip Mapping 10K Xba Assay Kit Box 3) in addition to oligonucleotide B2 contains 4 hybridization control oligonucleotides. Each control oligonucleotide is present at determined concentrations ranging from 2.5 pM to 75 pM. The signals generated from the control oligos are reported in the .rpt files. The relative intensities for each oligonucleotide should correlate with the respective spike concentration. The exact signal value reported for each oligonucleotide is not significant. But each oligo should be detected and the signals reported should be in linear order relative to the spike in controls. If they are not detected or do not form a roughly linear response in the array, this indicates a problem in the washing and staining procedures. Consult Chapter 8, *Troubleshooting* for further information.

## Call Rate

The Call Rate is displayed in the Mapping Algorithm report which is automatically generated every time Mapping Cell Intensity data is analyzed by GDAS.

It is a good indicator of the overall performance of the assay. A call rate in excess of 92% indicates all steps, from DNA isolation to scanning, have worked well. An error in any of the steps or low quality sample may be reflected in a reduced call rate. A sample with a call rate below 85% indicates that this sample should be evaluated further before including the data in downstream analysis. The low call rate should serve as an indicator to consider additional information before accepting the sample. Some of the factors that can lead to lower call rates include:

- Genomic DNA quality
- Deviation from assay protocol
- Contaminated DNA

The Reference Genomic DNA 103 is a process control that will give call rates of > 92% when the assay is performed correctly.

| Mapping Array Report           |   |               |             |                  |         |         |         |
|--------------------------------|---|---------------|-------------|------------------|---------|---------|---------|
| Report File Name -             | C:\GeneChip\Affy_Data\Data\NA17203_X_mini10K_4001825_IVT1.RPT |               |             |                  |         |         |         |
| Date:                          | 06/01/04 13:55:23   |               |             |                  |         |         |         |
| Total number of SNPs:          | 10204   |               |             |                  |         |         |         |
| Total number of QC Probes:     | 4   |               |             |                  |         |         |         |
| Probe array type:              | Mapping10K_Xba142   |               |             |                  |         |         |         |
| SNP Performance                |   |               |             |                  |         |         |         |
| CEL Data                       | Gender  | Called Gender | SNP Call    | Signal Detection | AA Call | AB Call | BB Call |
| NA17203_X_mini10K_4001825_IVT1 | Unknown   | M             | 99.42%      | 99.97%           | 33.12%  | 34.22%  | 32.66%  |
| NA17203_X_mini10K_4001825_IVT2 | Unknown   | M             | 99.22%      | 99.98%           | 33.21%  | 34.03%  | 32.76%  |
| NA17203_X_mini10K_4001825_TAK1 | Unknown   | M             | 99.15%      | 99.99%           | 33.18%  | 34.08%  | 32.74%  |
| NA17203_X_mini10K_4001825_TAK2 | Unknown   | M             | 99.11%      | 99.95%           | 33.22%  | 34.08%  | 32.69%  |
| QC Performance                 |   |               |             |                  |         |         |         |
| CEL Data                       | AFFX-5Q-123   | AFFX-5Q-456   | AFFX-5Q-789 | AFFX-5Q-ABC      |         |         |         |
| NA17203_X_mini10K_4001825_IVT1 | 1160.5  | 368.0         | 1645.5      | 3302.0           |         |         |         |
| NA17203_X_mini10K_4001825_IVT2 | 957.5   | 296.0         | 1336.0      | 2541.5           |         |         |         |
| NA17203_X_mini10K_4001825_TAK1 | 1295.0  | 421.0         | 1901.5      | 3588.0           |         |         |         |
| NA17203_X_mini10K_4001825_TAK2 | 859.0   | 357.5         | 1254.0      | 2276.0           |         |         |         |

**Figure 7.11**  
Mapping Algorithm report

## Genomic DNA Quality

Genomic DNA should be prepared following the guidelines in Chapter 3 of this manual; DNA prepared outside these guidelines (e.g., degraded DNA, nicked DNA or DNA with inhibitors) may produce lower call rates without necessarily reducing accuracy. A gel image of the DNA before restriction digestion can be used to evaluate DNA quality. It is highly recommended if an alternate genomic DNA preparation method is used, a small pilot experiment should be conducted to evaluate reproducibility and accuracy of genotype calls.

## Deviation from Assay Protocol

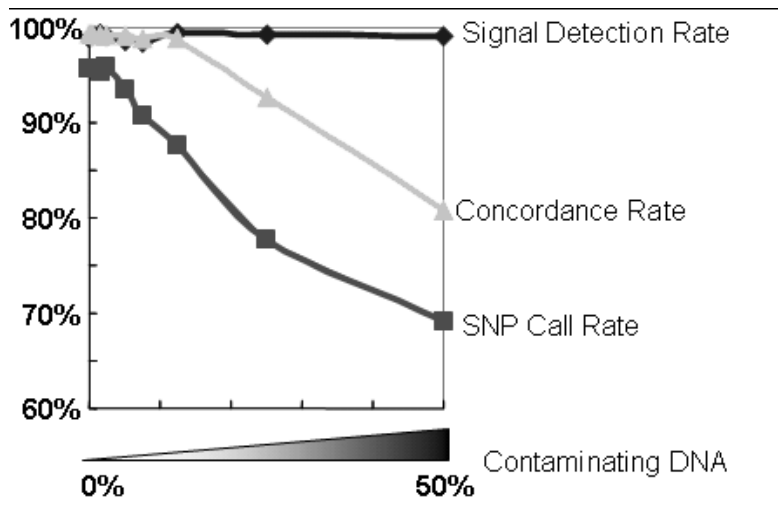
A problem in any step of the assay may lead to a decreased call rate. The gel images produced before DNA digestion, before PCR cleanup, the PCR yield after cleanup, and a the gel image after fragmentation can be used to identify problematic steps. Consult Chapter 8, *Troubleshooting* for further information.

At a minimum, PCR negative control (water instead of DNA template) should be incorporated into each group of samples processed.

### Contaminated Genomic DNA

If the genomic DNA sample is contaminated with other DNA, a decrease in the call rate is observed. An indicator of this is when the Signal Detection Rate remains high while the SNP Call Rate decreases. The SNP Call Rate and Signal Detection Rate can be seen in the GDAS Mapping Array Report (Figure 7.11). The results, illustrated in Figure 7.12, show that as the SNP Call Rate and Concordance decrease the Signal Detection Rate remains unchanged.

If you suspect a sample has DNA contamination, you may want to consider using a fresh sample of DNA, and/or testing it with microsatellite markers.



**Figure 7.12**

The effect of genomic DNA contamination on Signal Detection Rate, Concordance Rate and SNP Call Rate

### Concordance with Reference Genotypes on Reference Genomic DNA 103

A simple test that can be used to determine if the assay is performing to specifications is to measure the concordance between two samples. The Reference Genomic DNA 103, included in the GeneChip Mapping 10K Xba Assay Kit, can be used for this purpose. The calls generated from the reference DNA should be > 99% concordant with the reference calls. The following method allows users to quickly assess the concordance of their data with the reference calls.

1. Open the reference Genotype calls in Microsoft Excel.
  - a. Sort the calls in ascending order on the SNP ID.
2. Copy and paste the calls from GDAS from the reference DNA and paste into Microsoft Excel.
3. Check that the SNP IDs match.

Columns used to calculate concordance

| Excel Column | Contents             |
|--------------|----------------------|
| A            | Reference DNA SNP ID |
| B            | Reference DNA call   |
| C            | Test DNA call        |
| D            | Concordance check    |
| E            | No call              |
| F            | Valid concordant SNP |

4. Create column called “concordance check”. This column will contain a 1 if the two genotype calls match and a 0 if they do not.
  - a. Enter the following formula “=IF(B2=C2,1,0)”.
  - b. Auto fill down.
5. Create a second Column Called “No Call”. This column will contain “TRUE” if either call column contains a “NC”.
  - a. Enter the following formula “=OR(B2="NC",C2="NC")”.
  - b. Auto fill the column down.

6. Create a third column called “Valid Concordant SNP”. This column will contain a “TRUE” if the SNP calls match and neither is a “NC”.
  - a. Enter the following formula “=AND(D2=1,E2=FALSE)”.
    - b. Auto fill down.
7. Count the number of SNPs that had a genotype call in both experiments.
  - a. At the base of the “NO Call” column enter the following formula “=COUNTIF(E2:E10044,"FALSE")”.
    - b. Press return.
8. Count the number of SNPs that have a genotype call in both experiments and are concordant.
  - a. At the base of the “Valid Concordant SNP” column enter the following formula “=COUNTIF(F2:F10044,"TRUE")”.
    - b. Press return.

 **Note**

*The concordance rate should always be greater than 99%. Reasons for a decline in the concordance rate include:*

- *Selecting the wrong data set to compare to the reference DNA.*
- *Contamination at some step in the assay.*
- *Poor sample preparation.*

## Downstream Analysis Considerations

### Data clean up

For many genotyping applications, loss of accuracy can result in a significant decrease in genetic power. Genotyping errors can be caused by SNPs that give systematic errors, or by sporadic errors that occur due to stochastic, sample or experimental factors. SNPs that systematically give errors have been removed through a SNP selection process<sup>1</sup> (e.g., out of Hardy Weinberg equilibrium, or Mendel errors). Overall accuracy has been shown to be > 99.5% on the Mapping 10K 2.0 Array.

---

1. See *SNP Selection Criteria for the GeneChip Human Mapping 10K Array Xba 131* Technical Note.

Prior to downstream analysis, the user should consider taking steps to identify and eliminate sporadic genotype errors. Steps may include eliminating SNPs out of Hardy Weinberg equilibrium in control samples from the population, or eliminating genotypes showing Mendel inconsistency or unlikely genotypes. GDAS 3.0 has functionality to identify SNPs showing Mendelian errors in user defined samples (see Chapter 8 in the GDAS 3.0 Software User's Guide).





# Chapter 8



## Assay Recommendations

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Genotyping applications require very high accuracy to achieve maximum power. Therefore, great care should be taken to avoid possible sources of cross contamination that would lead to genotyping errors. As with any assay using PCR, the GeneChip® Mapping Assay has an inherent risk of carryover contamination with PCR product from previous reactions. In Chapter 2, we recommend a workflow to minimize the risk of cross contamination during the assay procedure. It is essential to adhere to workflow recommendations. PCR reactions should only be carried out in the main laboratory and personnel should not re-enter the Pre-PCR Clean and PCR staging areas following potential exposure to PCR product without first showering and changing into clean clothes.

It is essential to carefully read and follow the protocol as written. This assay has been validated using the reagents and suppliers listed, substitution of reagents and shortcuts are not recommended as they could result in suboptimal results.

Additional recommendations are listed below:

1. Think ahead to ensure that reagents and equipment you require, including designated pipettes, are in the correct work location. This will make workflow easier and prevent contamination risks.
2. Check that your spectrophotometer is accurately calibrated and ensure readings are in dynamic range (~ 0.2 to 0.8 OD). This will ensure you use the correct amount of genomic DNA, necessary for fragmentation reaction efficiency and identifying good PCR yields. Adding the same amount of labeled target to the arrays will help obtain reproducible call rates.

3. Pay particular attention to the storage and handling of reagents. This is especially important for enzymes such as DNA Ligase and GeneChip Fragmentation Reagent (DNase I) which are sensitive to temperatures exceeding  $-20^{\circ}\text{C}$ . To prevent loss of enzyme activity when the enzymes are removed from the freezer use a bench top freeze block and return to  $-20^{\circ}\text{C}$  directly after use. Take care when pipetting enzymes stored in glycerol, which is viscous. Do not store at  $-80^{\circ}\text{C}$ .
4. Fragmentation Reagent (DNase I) activity can decline over time after dilution on ice, and should be added to samples as quickly as possible.
5. The use of master mixes prepared with a 5% excess ensures consistency in reagent preparation by minimizing pipetting errors and reducing handling time of temperature sensitive reagents. The success of this assay depends on the accurate pipetting and subsequent thorough mixing of small volumes of reagents.
6. The PCR reaction for this assay has been validated using one of the specified thermal cyclers. These thermal cyclers were chosen because of their ramping times. Take care programming your thermal cycler and use the thin walled reaction tubes recommended. Thicker walled tubes may result in reduced PCR efficiency and lower yields.
7. It is essential to run gels to monitor both the PCR reaction and the fragmentation reaction.

For the PCR reaction, individual PCR products are run on a 2% agarose gel – product (bands) should be visible in the 250 to 1000 bp size range. See Figure A.2 on page 145.

Following fragmentation, run samples on a 4% agarose gel. Successful fragmentation is confirmed by the presence of a smear the darkest region of which corresponds to  $\sim 50$  bp. See Figure A.5 on page 153.

8. Run controls in parallel with each group of samples.  
Substitute molecular biology grade water for DNA at the PCR step as a negative control. The absence of bands on your PCR gel for this control confirms no previously amplified PCR product has contaminated your samples. The Reference Genomic DNA (103) is supplied as a positive control in the assay kits. This is an effective troubleshooting tool confirming all individual steps have been successfully completed.

9. Oligonucleotide controls are included in the assay kit, these are added to the target samples prior to hybridization and act to confirm successful hybridization and sensitivity of the array. The oligonucleotide control reagents contain oligo B2 which is used for grid alignment.

### **Important Differences Between GeneChip Mapping 10K Arrays and GeneChip Expression Arrays**

10. For laboratories that also run GeneChip Expression arrays it is important to check the temperature setting on the hybridization oven – for the GeneChip® Human Mapping 10K Array Xba 142 2.0, this should be set to 48°C. **The temperature for hybridization on expression arrays is 45°C.**
11. Buffer B is different for the expression and DNA arrays. Using the MES based buffer B from the Expression protocol will result in substantially reduced call rates for the GeneChip Mapping 10K Arrays. Care should be taken to ensure the fluidics station is properly maintained and primed with the correct buffers prior to use.
12. Both protocols use the same stain reagents for each staining step. However, after the last wash the Mapping 10K Array is filled with Array Holding Buffer. Stain buffers should be prepared fresh each day, as SAPE is light sensitive it should be prepared in amber tubes.
13. GeneChip Mapping 10K 2.0 Arrays are scanned on a GeneChip Scanner 3000 Scanner.

## Troubleshooting Guide for the GeneChip Mapping Assay

| <b>Problem</b>                               | <b>Likely Cause</b>  | <b>Solution</b>   |
|--|--|---|
| <b>Faint/absent bands on PCR gel</b>         |  |   |
| Both samples & positive control affected.    | Problem with master mixes or individual reagents.                                  | Ensure all reagents added to master mixes and enzymes are stored at -20°C. Work quickly with enzymes and return to -20°C directly after use to prevent loss of activity.        |
|  | Failed restriction digest.   | Run gel to confirm restriction enzyme activity.   |
|  | Failed adaptor ligation reaction.  | Confirm enzyme activity.<br><br>Ligase buffer contains ATP and should be defrosted/ held at 4°C. Mix ligase buffer thoroughly before use to ensure precipitate is re-suspended. |
|  | Reduced adaptor ligation efficiency due to adaptor self-ligation, DNA re-ligation. | To prevent self-ligation of adaptor work rapidly and add DNA ligase last.   |
|  | Failed PCR reaction.   | Check PCR reagents. Take care with preparation of master mixes and ensure accurate pipetting and thorough mixing.   |
|  | Reduced PCR reaction yield – non optimal PCR conditions.                           | Use a validated thermal cycler, check PCR programs. Use recommended thin walled reaction tubes.   |
|  | Ligation mix not diluted prior to PCR reaction.                                    | Ligation mixture diluted 1:4 with molecular grade to remove potential inhibitors and maintain optimal pH and salt concentration.  |
|  | Incorrect concentration of nucleotides.  | Check dNTP stock concentration and vendor (2.5 mM for each dNTP).   |
| Samples affected (but positive controls OK). | Non-optimal reaction conditions.   | Use master mixes and include a positive control to eliminate reagents and assay problems as detailed above.   |
|  | Sample DNA contains enzymatic or chemical inhibitors.                              | Ensure genomic DNA is purified and diluted in Low EDTA (0.1mM) TE buffer.<br><br>Ethanol precipitate genomic DNA to remove inhibitors.  |
|  | Degraded sample DNA.   | Confirm quality of genomic DNA sample.  |

| <b>Problem</b>   | <b>Likely Cause</b>   | <b>Solution</b>   |
|--|---|---|
| <b>Bright bands present in negative control on PCR gel</b>   |   |   |
|  | Contamination during assay is most likely due to amplified PCR product from assays run previously. Sample results prepared in parallel are likely contaminated and unreliable. Reagents and/or pipettors are likely contaminated. | Ensure lab personnel strictly adhere to one way workflow and refrain from entering Pre-PCR clean area and PCR staging area after being in the main laboratory areas.<br>Decontaminate Pre-PCR clean area and PCR staging area including all equipment.<br>Use new reagents and new pipettors. |
| <b>Low PCR yield</b>   |   |   |
| Gel image shows PCR product but following purification spectrophotometer measurements at 260nm indicate low PCR yield. | Sufficient PCR product is present but spectrophotometer is out of calibration.  | Calibrate spectrophotometer. Proceed with assay.  |
|  | PCR product was eluted with incorrect volume of EB buffer.  | PCR reactions are purified using QIAGEN Ququick columns follow the QIAGEN protocol up to the elution step. All four PCR reactions per sample are pooled into a total volume of 55 $\mu$ L EB buffer. This EB buffer is added step wise to each of the four QIAGEN columns in turn.            |
|  | PCR product is lost during purification due to improperly diluted or expired buffers.   | Follow QIAGEN manual. Buffer PE must be diluted with 100% Ethanol (molecular biology grade) and sealed tightly to avoid evaporation. Replace expired buffers.   |
|  | PCR product is lost due to reduced temperature.   | All purification steps must be carried out at 25 to 35°C including centrifugation.  |
|  | Pipetman used for quantitation not calibrated.  | Calibrate Pipetman. Proceed with assay.   |
| <b>Fragmented PCR product is not ~ 50 bp</b>   |   |   |
| PCR product is still visible in 250-1000 bp size region  | Incomplete fragmentation due to underestimated DNA concentration.   | Ensure spectrophotometer is properly calibrated so only 20 $\mu$ g of DNA is added to the fragmentation reaction.   |
|  | Failed or incomplete fragmentation due to reduced DNase activity.   | Check you have entered the correct activity of DNase into the formula (page 151) for calculating amount of DNase to add to fragmentation reaction.  |
|  |   | Ensure fragmentation reagent (DNASE) is kept at -20°C. Do not reuse diluted working stock.  |

| <b>Problem</b>   | <b>Likely Cause</b>  | <b>Solution</b>   |
|--|--|---|
| <b>.CEL file can not be generated</b>  |  |   |
| GCOS 1.1.1 with Patch 5 is unable to align grid.   | Unable to place a grid on the .DAT file due to the absence of B2 signal.                             | Hybridization controls including oligo B2 must be added to hybridization cocktail for grid alignment.   |
| .CEL image is dim.   | Insufficient signal intensity or staining failure.   | Make fresh stain buffers.   |
|  | Incorrect wash buffers used on fluidics station.   | Prime the fluidics station with the correct buffers prior to running the assay. Incorrect wash buffers will disrupt hybridization of the labeled, fragmented DNA.   |
| <b>Low SNP call rates</b>  |  |   |
| Gel images and spectrophotometric quantitation indicate successful PCR reaction.                         | Over fragmentation of DNA sample due to incorrect dilution of Fragmentation Reagent (DNase I) stock. | Use correct concentration of Fragmentation Reagent (DNase I). Check U/ $\mu$ L on the label and check dilution formula on page 151. Work quickly and on ice, transfer reaction tubes to pre-heated thermal cycler (37°C). |
| Extremely low call rate<br>Sample hybridization is absent on .CEL and .DAT images but B2 grid is bright. | Labeling reaction suboptimal.  | Use a new vial of Terminal Dideoxy Transferase. Verify the labeling reagents and repeat labeling.   |
| Positive control has good call rates but samples are lower than expected.                                | Genomic DNA not optimal.   | Ensure DNA samples are of high quality, use positive control sample as a reference guide for assay procedures. Prepare master mixes for samples and controls.   |
| SNP detection rate high, SNP call rate low.  | Mixed or contaminated genomic DNA sample (page 107).   | Use uncontaminated stock of DNA sample.   |





# Chapter 9





# *Affymetrix Technical Support*

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## Affymetrix Technical Support

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

**Affymetrix Inc.**

3380 Central Expressway  
Santa Clara, CA 95051  
USA

**Tel:** 1-888-362-2447 (1-888-DNA-CHIP)

**Fax:** 1-408-731-5441

**E-mail:** [support@affymetrix.com](mailto:support@affymetrix.com)

**Affymetrix UK Ltd**

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

**Tel:** +44 (0)1628 552550

**Fax:** +44 (0)1628 552585

**E-mail:** [supporteurope@affymetrix.com](mailto:supporteurope@affymetrix.com)

**Affymetrix Japan, K. K.**

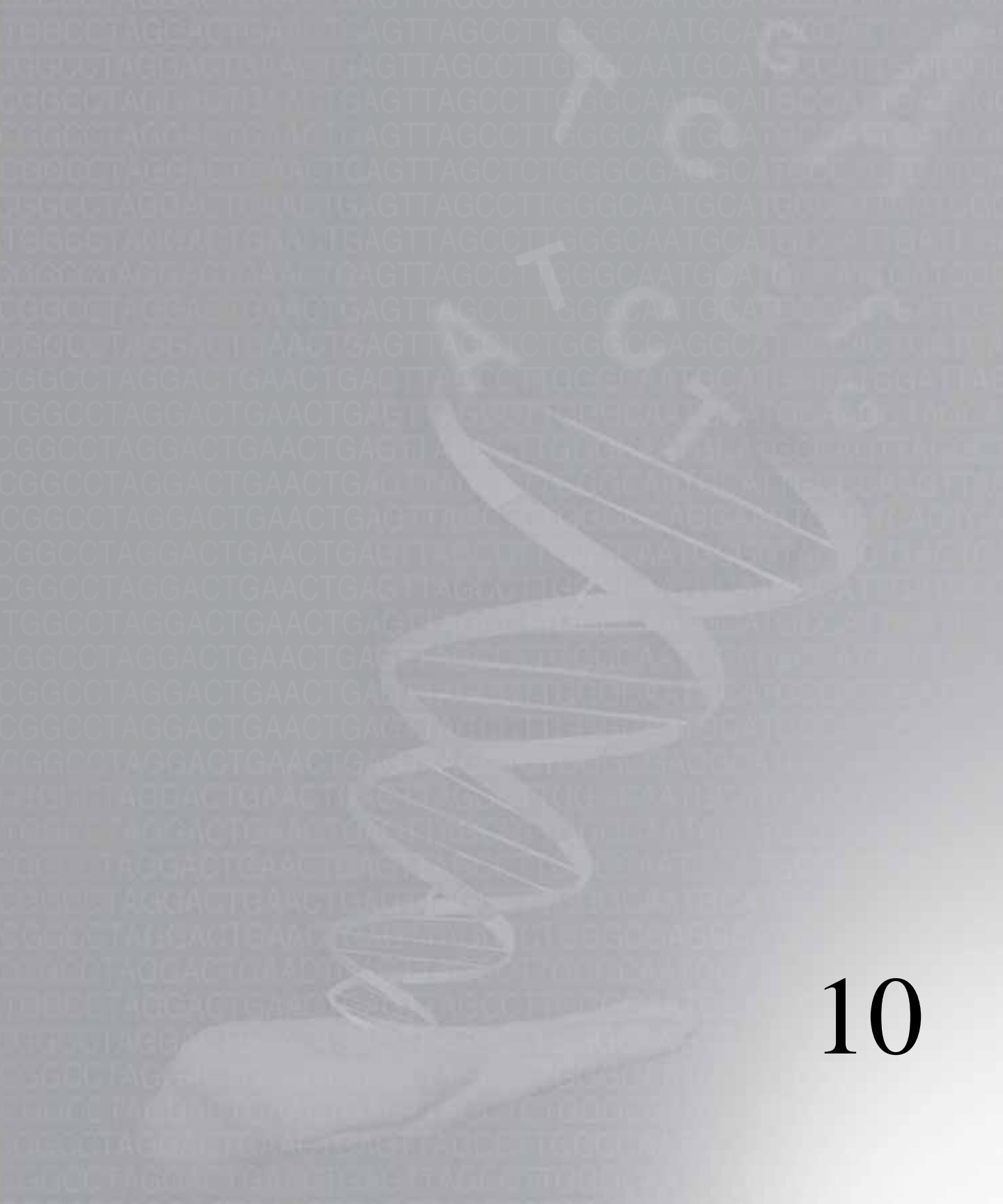
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# Chapter 10





# Reagents, Instruments & Supplies

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*This section describes the reagents, supplies and equipment required to implement the GeneChip Mapping Assay. The first section below describes the Affymetrix GeneChip® Mapping 10K Xba Assay Kit (P/N 900441), which contains critical reagents and reagent components specifically developed and/or optimized for the GeneChip Mapping Assay, and controls that are integrated for use with the GeneChip® Human Mapping 10K Array Xba 142 2.0. This section also indicates the Affymetrix equipment - scanner, fluidic station, hybridization oven - and GeneChip® Human Mapping 10K Array Xba 142 2.0 required for this assay. The second section describes the reagents, supplies and instruments that are required, but not supplied directly by Affymetrix. The products and vendors listed in this section have been tested at Affymetrix during the development of the product - other products and vendors have not been verified at Affymetrix. In some cases, we have seen lower performance when using non-recommended vendors.*

## Required Reagents and Instruments

The following reagents and equipment are required to process the GeneChip® Human Mapping 10K Array Xba 142 2.0.

Reagents Supplied by Affymetrix

| <b>Affymetrix GeneChip® Mapping 10K Xba Assay Kit P/N 900441</b> |               |                        |   |              |
|--|---------------|------------------------|---|--------------|
| This kit includes sufficient reagent for 30 arrays               |               |                        |   |              |
| <b>Component</b>   | <b>Volume</b> | <b>Concentration</b>   | <b>Description</b>  | <b>Box #</b> |
| Adaptor, Xba   | 38 µL         | 5 µM                   | Two annealed oligonucleotides (see below*), 1 specific for ligation to Xba restriction site.  | 1            |
| PCR Primer, 001  | 1125 µL       | 10 µM                  | PCR primer (see below*), to amplify ligated genomic DNA   | 1            |
| Reference Genomic DNA, 103                                       | 25 µL         | 50 ng/µL               | Human genomic DNA (single source)   | 2            |
| GeneChip Fragmentation Reagent                                   | 20 µL         | See label on tube      | DNase I enzyme, formulated to fragment purified PCR amplicons   | 3            |
| 10X Fragmentation Buffer   | 225 µL        | 10X                    | Buffer for fragmentation reaction   | 3            |
| GeneChip DNA Labeling Reagent                                    | 60 µL         | 5 mM                   | Biotin-labeled reagent for end-labeling fragmented PCR amplicons  | 3            |
| Terminal Deoxynucleotidyl Transferase                            | 105 µL        | 30 U/µL                | Enzyme used to end-label fragmented PCR amplicons with the GeneChip DNA Labeling Reagent  | 3            |
| 5X Terminal Deoxynucleotidyl Transferase Buffer                  | 420 µL        | 5X                     | Buffer for labeling reaction  | 3            |
| Oligonucleotide Control Reagent                                  | 60 µL         | See protocol Chapter 4 | Mixture of five biotin-labeled oligonucleotides, which hybridize to control regions (gridding controls and array controls) on the GeneChip Mapping 10K Array. | 3            |

\* 'Adaptor, Xba' is 'PCR Primer, 001' annealed to '001-Xba5'

|                 |   |
|-----------------|---|
| PCR Primer, 001 | 5' ATTATGAGCACGACAGACGCCTGATCT 3'       |
| 001-Xba5        | 3' AATACTCGTGCTGTCTGCGGACTAGAGATCp*T 5' |



**Note**

*PCR Primer, 001 is available as a separate component (P/N 900534).*



## Equipment and Software Supplied by Affymetrix

| Item  | Part Number       |
|---|-------------------|
| GeneChip Fluidics Station 400 or GeneChip Fluidics Station 450/250* | 900116 or 00-0079 |
| GeneChip Hybridization Oven*  | 800139            |
| GeneChip Scanner 3000*  | 00-0074           |
| Affymetrix GeneChip Operating Software 1.1.1 with Patch 5 or 1.2*   | 690031            |
| Affymetrix GeneChip DNA Analysis Software 2.0 or 3.0*               | 690030            |
| GeneChip Human Mapping 10K Array Xba 142 2.0*                       | 900446            |

## Reagents, Equipment and Supplies Not Supplied by Affymetrix

| Assay Step                   | Reagents  | Equipment  |
|------------------------------|---|--|
| Genomic DNA Preparation      | <ul style="list-style-type: none"> <li>■ Reduced EDTA TE Buffer (10 mM Tris HCl, pH 8.0, 0.1mM EDTA, pH 8.0) TEKnova P/N T0223</li> <li>■ <b>Reference Genomic DNA, 103 (50 ng/μL) Affymetrix*, available in Box 1 of the GeneChip® Mapping 10K Xba Assay Kit, P/N 900441</b></li> </ul>  |  |
| Restriction Enzyme Digestion | <ul style="list-style-type: none"> <li>■ Genomic DNA stock: 50 ng/μL</li> <li>■ <b>Xba I (20,000 U/mL): New England Biolab (NEB)*; P/N R0145L containing:</b> <ul style="list-style-type: none"> <li>■ <b>10X NE Buffer 2: New England Biolab (NEB)*</b></li> <li>■ <b>100X BSA (Bovine Serum Albumin): New England Biolab (NEB)*</b></li> </ul> </li> <li>■ H<sub>2</sub>O (Molecular Biology Water): BioWhittaker Molecular Applications/Cambrex; P/N 51200</li> <li>■ 8-Tube Strips, thin-wall (0.2 mL): MJ Research, P/N TBS-0201; Strip of 8 caps: MJ Research, P/N TCS-0801</li> <li>■ 96-well plate: MJ Research; P/N MLP-9601; or Applied Biosystems; P/N 403083</li> <li>■ 96-well PLT. Clear Adhesive Films: Applied Biosystems; P/N 4306311</li> </ul> | <ul style="list-style-type: none"> <li>■ Thermal Cycler</li> </ul> |
| Ligation                     | <ul style="list-style-type: none"> <li>■ <b>T4 DNA Ligase (400,000 U/mL): New England Biolab (NEB)*; P/N M0202L containing:</b> <ul style="list-style-type: none"> <li>■ <b>T4 Ligase Buffer: New England Biolab (NEB)*</b></li> </ul> </li> <li>■ H<sub>2</sub>O (Molecular Biology Water): BioWhittaker Molecular Applications/Cambrex; P/N 51200</li> <li>■ <b>Adaptor, Xba, Conc: 5 μM: Affymetrix; P/N 900410*, available in Box 1 of the GeneChip® Mapping 10K Xba Assay Kit, P/N 900441</b></li> </ul>   | <ul style="list-style-type: none"> <li>■ Thermal Cycler</li> </ul> |

\* Denotes critical reagents, equipment or supplies. Formulations or vendors not listed here have not been tested and verified at Affymetrix. In some cases, lower performance has been demonstrated by reagents from non-qualified vendors.

Reagents, Equipment and Supplies Not Supplied by Affymetrix

| Assay Step | Reagents   | Equipment   |
|------------|--|---|
| PCR        | <ul style="list-style-type: none"> <li>■ H<sub>2</sub>O (Molecular Biology Water): Cambrex; P/N 51200</li> <li>■ <b>dNTP mixture (2.5 mM each): Takara; P/N 4030 (Available in US from Fisher or PanVera as P/N TAK4030; in Europe this product is distributed by Cambrex)<sup>a*</sup></b></li> <li>■ <b>PCR Primer Xba (10 µM): Affymetrix; P/N 900409*, available in Box 1 of the GeneChip Mapping 10K Xba Assay Kit, P/N 900441</b></li> <li>■ <b>AmpliTaq Gold PCR Kit: Applied Biosystems*;</b><br/><b>P/N N808-0249 containing:</b> <ul style="list-style-type: none"> <li>■ <b>MgCl<sub>2</sub>: Applied Biosystems*</b></li> <li>■ <b>PCR Buffer II: Applied Biosystems*</b></li> </ul> </li> <li>■ 2% TBE Gel: (2% SeaKem Gold precast Reliant Gel System); Cambrex P/N 54939</li> <li>■ All Purpose Hi-Lo DNA Marker: Bionexus, Inc.; P/N BN2050, or DirectLoad wide range DNA Marker (Sigma P/N D7058)</li> <li>■ Gel Loading Solution: Sigma; P/N G2526</li> <li>■ <b>PCR Tubes*</b> (must be compatible and qualified with MJ Research Tetrad, or ABI GenAmp PCR System) For example:           <ul style="list-style-type: none"> <li>■ Individual tubes: MJ Research; P/N TWI-0201</li> <li>■ 8-Tube Strips, thin-wall (0.2 mL): MJ Research, P/N TBS-0201; Strip of 8 caps: MJ Research, P/N TCS-0801</li> <li>■ 96-well plate: MJ Research; P/N MLP-9601; or Applied Biosystems; P/N 403083</li> <li>■ 96-well PLT. Clear Adhesive Films: Applied Biosystems; P/N 4306311</li> </ul> </li> </ul> | <ul style="list-style-type: none"> <li>■ <b>PCR Thermal Cycler<sup>b*</sup></b><br/>(Ramp speeds on these models are critical - assay protocols have been optimized for these two thermal cycler models only):           <ul style="list-style-type: none"> <li>■ <b>MJ Tetrad (PTC-225) 96 well block, or</b></li> <li>■ <b>ABI GeneAmp PCR System 9700 - 96 well block</b></li> </ul> </li> <li>■ gel apparatus</li> <li>■ <b>Jitterbug: Boekel Scientific, model 130000</b></li> </ul> |

**\* Denotes critical reagents, equipment or supplies. Formulations or vendors not listed here have not been tested and verified at Affymetrix. In some cases, lower performance has been demonstrated by reagents from non-qualified vendors.**

- a. dNTPs from Invitrogen (P/N R72501) have been tested on a limited basis with similar results. You should test in your own lab prior to full scale production.
- b. The PCR process is covered by patents owned by Roche Molecular Systems, Inc. and F. Hoffmann-LaRoche Ltd ("Roche"). A license to use the PCR process for certain research and development activities accompanies the purchase of certain reagents from licensed suppliers when used in conjunction with an authorized thermal cycler. If you are using an MJ Research thermal cycler, your thermal cycler may not be an authorized thermal cycler and it may be necessary for you to obtain authorization from Roche or ABI (see PCR licensing information in the MJ Research User Manual). For information about obtaining a license contact The Director of Licensing at Applied Biosystems, 850 Lincoln Center Drive, Foster City, CA 94404 or the Licensing Department, Roche Molecular Systems, Inc., 1145 Atlantic Avenue, Alameda, CA 94501.

| Assay Step                   | Reagents  | Equipment   |
|------------------------------|---|---|
| PCR Purification and Elution | <ul style="list-style-type: none"> <li>■ <b>QIAquick PCR Purification Kit (single column): QIAGEN; P/N 28106 for 250, P/N 28104 for 50*</b></li> <li>■ <b>Manifold - QIAvac multiwell unit: QIAGEN P/N 9014579*</b></li> <li>■ <b>MinElute 96 UF PCR Purification Kit: QIAGEN P/N 28051 (four plates), or P/N 28053 (24 plates)*</b></li> <li>■ <b>Buffer EB (1000 mL): QIAGEN P/N120002</b></li> <li>■ Biomek Seal and Sample Aluminum Foil Lids: Beckman P/N 538619</li> </ul>  | <ul style="list-style-type: none"> <li>■ Vacuum Regulator for use during the PCR clean up step. QIAGEN Vacuum Regulator (use with QIAvac manifolds): QIAGEN; P/N 19530*</li> </ul>  |
| Fragmentation and Labeling   | <ul style="list-style-type: none"> <li>■ <b>GeneChip Fragmentation Reagent (DNase I): Affymetrix; P/N 900131*, available in Box 3 of the GeneChip® Mapping 10K Xba Assay Kit, P/N 900441</b></li> <li>■ <b>10 X Fragmentation Buffer: Affymetrix, P/N 900422*, available in Box 3 of the GeneChip Mapping 10K Xba Assay Kit, P/N 900441</b></li> <li>■ Molecular Biology Water: Bio Whittaker Molecular Applications/ Cambrex, P/N 51200</li> <li>■ 4% TBE Gel: (4% NuSieve 3:1 precast Reliant Gel System); Cambrex P/N 54929</li> <li>■ All Purpose Hi-Lo DNA Marker: Bionexus, Inc.; 50 - 10000bp; P/N BN2050, or DirectLoad wide range DNA Marker (Sigma P/N D7058)</li> <li>■ Gel Loading Solution: Sigma; P/N G2526</li> <li>■ 8-Tube Strips, thin-wall (0.2 mL): MJ Research, P/N TBS-0201; Strip of 8 caps: MJ Research, P/N TCS-0801</li> <li>■ 96-well plate: MJ Research; P/N MLP-9601</li> <li>■ 96-well PLT. Clear Adhesive Films: Applied Biosystems; P/N 4306311</li> <li>■ <b>GeneChip DNA Labeling Reagent: Affymetrix; P/N 900430*, available in Box 3 of the GeneChip Mapping 10K Xba Assay Kit, P/N 900441</b></li> <li>■ <b>Terminal Deoxynucleotidyl Transferase (30 U/μL): Affymetrix; P/N 900426*, available in Box 3 of the GeneChip Mapping 10K Xba Assay Kit, P/N 900441</b></li> <li>■ <b>5X Terminal Deoxynucleotidyl Transferase Buffer: Affymetrix; P/N 900425*, available in Box 3 of the GeneChip Mapping 10K Xba Assay Kit, P/N 900441</b></li> </ul> | <ul style="list-style-type: none"> <li>■ <b>PCR Thermal Cycler<sup>b*</sup></b> (Ramp speeds on these models are critical - assay protocols have been optimized for these two thermal cycler models only):</li> <li>■ <b>MJ Tetrad (PTC-225) 96 well block, or</b></li> <li>■ <b>ABI GeneAmp PCR System 9700 - 96 well block</b></li> </ul> |

\* Denotes critical reagents, equipment or supplies. Formulations or vendors not listed here have not been tested and verified at Affymetrix. In some cases, lower performance has been demonstrated by reagents from non-qualified vendors.

| Assay Step           | Reagents  | Equipment  |
|----------------------|---|--|
| Target Hybridization | <ul style="list-style-type: none"> <li>■ 5M TMACL (Tetramethyl Ammonium Chloride): Sigma; P/N T3411</li> <li>■ 10% Tween-20: Pierce; P/N 28320 (Surfactamps)</li> <li>■ MES hydrate: Sigma; P/N M5287</li> <li>■ MES Sodium Salt: Sigma; P/N M5057</li> <li>■ DMSO: Sigma; P/N D5879</li> <li>■ 0.5M EDTA, pH 8.0: Ambion; P/N 9260G</li> <li>■ Denhardt's Solution, 50X concentrate: Sigma; P/N D2532</li> <li>■ HSDNA (Herring Sperm DNA) 10 mg/mL: Promega; P/N D1815</li> <li>■ Human Cot-1: Invitrogen; P/N 15279-011</li> <li>■ <b>Oligonucleotide Control Reagent: Affymetrix; P/N 900440*, available in Box 3 of the GeneChip Mapping 10K Xba Assay Kit, P/N 900441</b></li> </ul>  | <ul style="list-style-type: none"> <li>■ GeneChip Hybridization Oven</li> </ul>  |
| Wash and Staining    | <ul style="list-style-type: none"> <li>■ 20X SSPE: BioWhittaker Molecular Applications/Cambrex; P/N 51214</li> <li>■ Denhardt's Solution, 50X concentrate: Sigma; P/N D2532</li> <li>■ 10% Tween-20: Pierce; P/N 28320 (Surfactamps)</li> <li>■ Molecular Biology Grade water: BioWhittaker Molecular Applications/Cambrex; P/N 51200</li> <li>■ <b>SAPE (Streptavidin, R-phycoerythrin conjugate): Molecular Probes; P/N S866, 1 mg/mL*</b></li> <li>■ <b>Ab (Biotinylated Anti-Streptavidin): Vector; P/N BA-0500, 0.5mg;*</b><br/><b>reconstitute according to product instructions</b></li> <li>■ Distilled water, Invitrogen Life Technologies, P/N 15230147</li> <li>■ Acetylated Bovine Serum Albumin (BSA) solution (50 mg/mL), Invitrogen Life Technologies, P/N 15561-020</li> <li>■ Bleach (5.25% Sodium Hypochlorite), VWR Scientific, P/N 21899-504 (or equivalent)</li> </ul> | <ul style="list-style-type: none"> <li>■ GeneChip Fluidics Station 400 or GeneChip Fluidics Station 450/250</li> </ul> |
| Scanning             |   | <ul style="list-style-type: none"> <li>■ GeneChip Scanner 3000</li> </ul>  |

**\* Denotes critical reagents, equipment or supplies. Formulations or vendors not listed here have not been tested and verified at Affymetrix. In some cases, lower performance has been demonstrated by reagents from non-qualified vendors.**

## Supplier Contact List

| <b>Supplier</b>              | <b>Web Site</b>  |
|------------------------------|--|
| Ambion                       | <a href="http://www.ambion.com">www.ambion.com</a>   |
| Applied Biosystems           | <a href="http://home.appliedbiosystems.com">home.appliedbiosystems.com</a>                         |
| Bionexus Inc.                | <a href="http://www.bionexus.net">www.bionexus.net</a>   |
| Boekel Scientific            | <a href="http://www.boekelsci.com/">www.boekelsci.com/</a>   |
| Cambrex                      | <a href="http://www.cambrex.com">www.cambrex.com</a>   |
| Fisher Scientific            | <a href="http://www.fisherscientific.com/">www.fisherscientific.com/</a>                           |
| Invitrogen Life Technologies | <a href="http://www.invitrogen.com">www.invitrogen.com</a>   |
| MJ Research                  | <a href="http://www.mjr.com">www.mjr.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>   |
| Operon Technologies          | <a href="http://www.operon.com">www.operon.com</a>   |
| PanVera                      | <a href="http://www.panvera.com">www.panvera.com</a>   |
| PerkinElmer Life Sciences    | <a href="http://lifesciences.perkinelmer.com/">lifesciences.perkinelmer.com/</a>                   |
| Pierce Chemical              | <a href="http://www.piercenet.com">www.piercenet.com</a>   |
| Promega                      | <a href="http://www.promega.com">www.promega.com</a>   |
| QIAGEN                       | <a href="http://www.qiagen.com">www.qiagen.com</a>   |
| Sigma-Aldrich                | <a href="http://www.sigma-aldrich.com">www.sigma-aldrich.com</a>                                   |
| TaKaRa Bio Inc.              | <a href="http://www.takara-bio.co.jp/english/index.htm">www.takara-bio.co.jp/english/index.htm</a> |
| TEKnova                      | <a href="http://www.teknova.com/">www.teknova.com/</a>   |
| Vector Laboratories          | <a href="http://www.vectorlabs.com">www.vectorlabs.com</a>   |





**A**

# Appendix A







# GeneChip® Mapping 10K Assay Protocol - Column Clean Up

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*In the first release of the Mapping 10K, Affymetrix offered two different clean up options following PCR, using QIAquick columns or the QIAGEN MiniElute system. Over time, we have seen both better product performance and greater customer satisfaction with the QIAGEN MiniElute protocol. This appendix has the QIAquick column version of the protocol for customers who wish to continue using this method.*

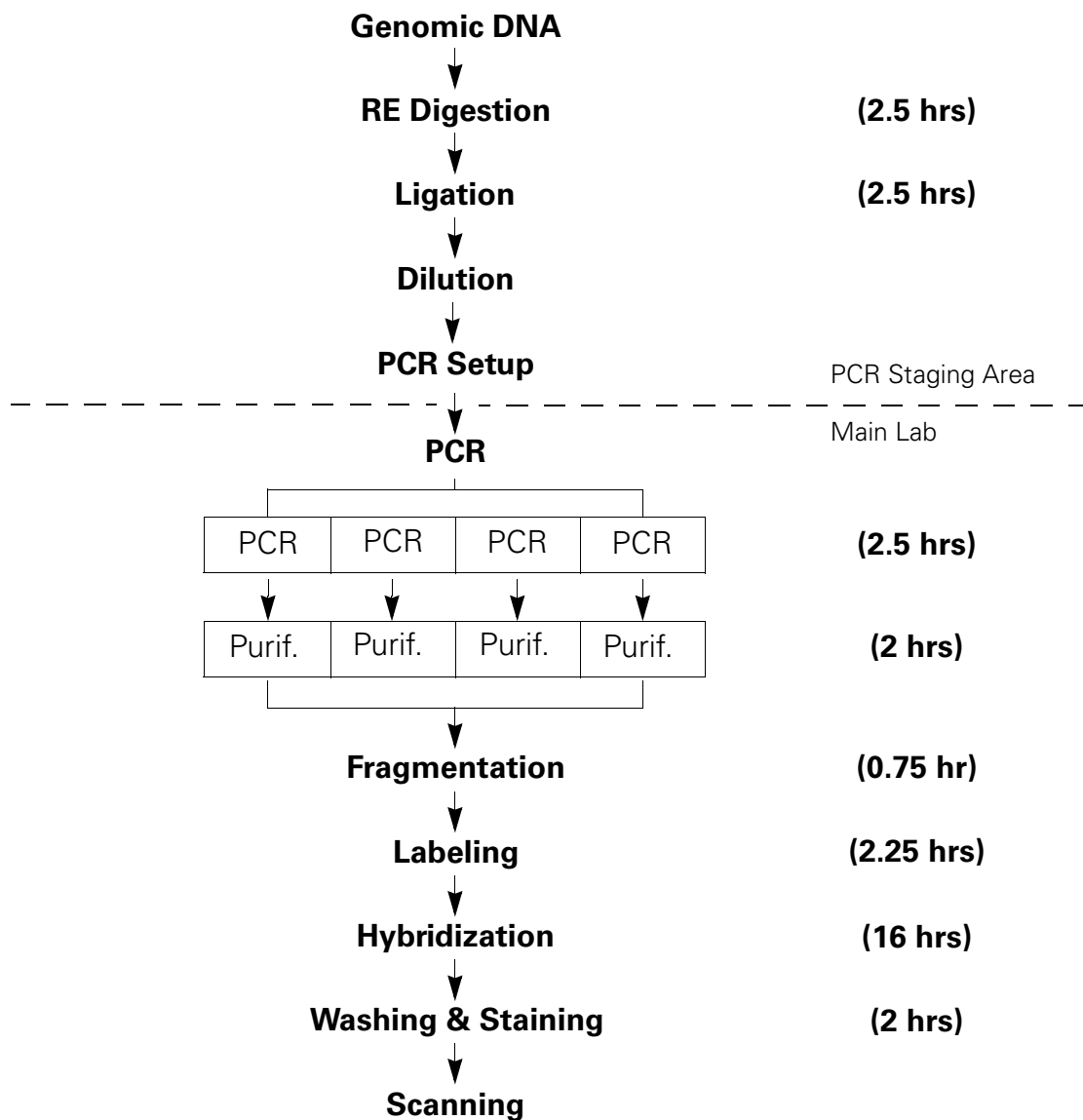
## Introduction

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The Affymetrix GeneChip® Mapping Assay, in conjunction with the GeneChip Human Mapping 10K Array Xba 142 2.0, is designed to detect Single Nucleotide Polymorphisms (SNPs) in samples of genomic DNA. The protocol starts with 250 ng of genomic DNA and will generate SNP genotype calls for more than 10,000 SNPs. An overview of the assay is shown in Figure A.1. The assay utilizes a strategy that reduces the complexity of human genomic DNA up to 50 fold by first digesting the genomic DNA with the Xba I restriction enzyme and then ligating sequences onto the DNA fragments. The complexity is reduced by a PCR procedure optimized for short fragments. Following the complexity reduction step the PCR products (amplicons) are fragmented, end-labeled, and hybridized to a GeneChip array.

## Assay Overview

## GeneChip® Mapping Assay Overview



**Figure A.1**  
Assay overview

## Genomic DNA Preparation

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### Preparation of Genomic DNA

The concentration of the genomic DNA should be determined and the working stocks diluted to 50 ng/μL using reduced EDTA TE buffer (0.1 mM EDTA, 10 mM Tris HCl, pH 8.0). An elevated EDTA level is not recommended as it may interfere with subsequent enzymatic reactions.

For high throughput assays, aliquot 5 μL (50 ng/μL) of each diluted genomic DNA into each well of a 96-well plate. Make multiple replicates of the plates if needed.

### Reagents

- Reduced EDTA TE Buffer (10 mM Tris HCl, pH 8.0, 0.1mM EDTA, pH 8.0) TEKnova P/N T0223

## STEP 1: Reagent Preparation and Storage

---

The reagents necessary for the restriction digestion, ligation and PCR steps should be stored to minimize cross contamination between samples. Affymetrix recommends storing these reagents in the pre-PCR clean room as described in Chapter 2 (an area free of DNA template and free of PCR product). To avoid re-entering the pre-PCR clean room after entering either the PCR-Staging Room or the Main Lab, Affymetrix recommends aliquoting each of the reagents in the pre-PCR clean room before starting the rest of the experiment.

## STEP 2: Restriction Enzyme Digestion

### ↪ IMPORTANT

*With the GeneChip Mapping Assay the most likely potential source of contamination is the amplified PCR product.*

### Reagents and Equipment

- Genomic DNA stock: 50 ng/μL
- Xba I (20,000 U/mL): New England Biolab (NEB); P/N R0145L containing:
  - NE Buffer 2: New England Biolab (NEB); P/N B7002S
  - BSA (Bovine Serum Albumin): New England Biolab (NEB); P/N B9001S
- H<sub>2</sub>O (Molecular Biology Water): BioWhittaker Molecular Applications/Cambrex; P/N 51200
- 8-Tube Strips, thin-wall (0.2 mL): MJ Research, P/N TBS-0201; Strip of 8 caps: MJ Research, P/N TCS-0801
- Thermal cycler (any)

### ✓ Note

*The BSA is supplied as 100X (10 mg/mL), and needs to be diluted 1/10 with molecular biology grade water before use.*

### ✓ Note

*Reference Genomic DNA, 103 is supplied in Box 2 of the GeneChip® Mapping 10K Xba Assay Kit, P/N 900441. This DNA can be used as a positive control.*

### Pre-PCR Clean Area

1. Prepare the following Digestion Master Mix ON ICE (for multiple samples make 5% excess):

| Reagent             | 1X           | Final Conc. in Sample |
|---------------------|--------------|-----------------------|
| H <sub>2</sub> O    | 10.5 μL      |                       |
| NE buffer 2 (10X)   | 2 μL         | 1X                    |
| BSA (10X (1 mg/mL)) | 2 μL         | 1X                    |
| Xba I (20 U/μL)     | 0.5 μL       | 0.5 U/μL              |
| <b>Total</b>        | <b>15 μL</b> |                       |

## PCR Staging Area

### Digestion Procedure

2. Add 5  $\mu\text{L}$  genomic DNA (50 ng/ $\mu\text{L}$ ) to each well of 8-Tube Strips. The total amount of genomic DNA should be 250 ng for each sample.
3. Aliquot 15  $\mu\text{L}$  of the Digestion Master Mix to each well of the 8-Tube Strip containing the genomic DNA.

| Reagent Stock                       | Volume/Sample                      |
|-------------------------------------|------------------------------------|
| Genomic DNA (50 ng/ $\mu\text{L}$ ) | 5 $\mu\text{L}$                    |
| Digestion Master Mix                | 15 $\mu\text{L}$                   |
| <b>Total Volume</b>                 | <b>20 <math>\mu\text{L}</math></b> |

4. Cap the 8-Tube Strip tightly, vortex at medium speed for 2 seconds and spin briefly at 2,000 rpm for 1 minute.
5. Place the 8-Tube Strip in a thermal cycler and run the following program:

| Temperature | Time        |
|-------------|-------------|
| 37°C        | 120 minutes |
| 70°C        | 20 minutes  |
| 4°C         | Hold        |

#### ✓ Note

*Store sample at -20°C if the ligation step can not be done after digestion.*

## STEP 3: Ligation

### Reagents and Equipment

- T4 DNA Ligase: New England Biolab (NEB); P/N M0202L containing:
  - T4 DNA Ligase Buffer: New England Biolab (NEB); P/N B0202S
- H<sub>2</sub>O (Molecular Biology Water): BioWhittaker Molecular Applications/Cambrex; P/N 51200
- Adaptor Xba (5  $\mu$ M); Affymetrix; P/N 900410, available in Box 1 of the GeneChip Mapping 10K Xba Assay Kit, P/N 900441
- Thermal cycler (any Pre-PCR Clean Room thermocycler)

### Ligation Procedure

#### PCR Staging Area



*To prevent self-ligation of the adaptor, the T4 DNA Ligase should be added last after the Ligase buffer and the adaptor are added to the digested DNA.*

1. Prepare the following Ligation Master Mix ON ICE (for multiple samples make 5% excess):

| Reagent                      | 1X                            | Final Conc. in Sample |
|------------------------------|-------------------------------|-----------------------|
| Adaptor Xba (5 $\mu$ M)      | 1.25 $\mu$ L                  | 0.25 $\mu$ M          |
| T4 DNA Ligase buffer (10X) * | 2.5 $\mu$ L                   | 1X                    |
| <b>Total</b>                 | <b>3.75 <math>\mu</math>L</b> |                       |

\* Contains ATP and DTT. Keep on ice.

2. Aliquot 3.75  $\mu$ L of the Ligation Master Mix into the 8-Tube Strip containing the digested DNA samples.

3. Add 1.25  $\mu\text{L}$  of T4 DNA Ligase to each of the digested DNA samples. Final reaction volume for each reaction is described below:

| Reagent       | Volume/Sample                        |
|---------------|--------------------------------------|
| Digested DNA  | 20.00 $\mu\text{L}$                  |
| Ligation mix* | 3.75 $\mu\text{L}$                   |
| T4 DNA Ligase | 1.25 $\mu\text{L}$                   |
| <b>Total</b>  | <b>25.0 <math>\mu\text{L}</math></b> |

\* Contains ATP and DTT. Keep on ice.

4. Cap the 8-Tube Strip tightly, vortex at medium speed for 2 seconds, and spin briefly at 2,000 rpm for 1 minute.
5. Place the 8-Tube Strip in a thermal cycler and run the following program:

| Temperature | Time        |
|-------------|-------------|
| 16°C        | 120 minutes |
| 70°C        | 20 minutes  |
| 4°C         | Hold        |



**Note**

*Store samples at -20°C if PCR can not be done after ligation.*



**IMPORTANT**

*It is crucial to dilute the ligated DNA with Molecular Biology Water prior to PCR.*

6. Dilute each DNA ligation reaction by adding 75  $\mu\text{L}$  of molecular biology-grade  $\text{H}_2\text{O}$  as described below:

| Reagent              | Volume/Sample                       |
|----------------------|-------------------------------------|
| Ligated DNA          | 25 $\mu\text{L}$                    |
| $\text{H}_2\text{O}$ | 75 $\mu\text{L}$                    |
| <b>Total</b>         | <b>100 <math>\mu\text{L}</math></b> |

## STEP 4: PCR

---

### Reagents and Equipment

- H<sub>2</sub>O (Molecular Biology Water): BioWhittaker Molecular Applications/Cambrex; P/N 51200
- dNTP (2.5 mM each): Panvera Takara; P/N 4030, or Fisher Scientific; P/N TAK 4030<sup>1</sup>
- PCR Primer, 001 (10 μM): Affymetrix; P/N 900409, available in Box 1 of the GeneChip Mapping 10K Xba Assay Kit, P/N 900441
- QIAquick PCR Purification Kit: QIAGEN; P/N 28106
- AmpliTaq Gold PCR Kit: Applied Biosystems; P/N N808-0249 containing:
  - MgCl<sub>2</sub>: Applied Biosystems; P/N N808-0249
  - PCR Buffer II: Applied Biosystems; P/N N808-0249
- 2% TBE Gel: BMA Reliant precast (2% SeaKem Gold); P/N 54939
- All Purpose Hi-Lo DNA Marker: Bionexus, Inc.; P/N BN2050
- Gel Loading Solution: Sigma; P/N G2526
- Tubes:
  - Individual tubes: MJ Research; P/N TWI-0201
  - 8-Tube Strips, thin-wall (0.2 mL): MJ Research, P/N TBS-0201; Strip of 8 caps: MJ Research, P/N TCS-0801
- Plate:
  - Multiplate 96 polypropylene v-bottom microplate (8 x 12 well array): MJ Research; P/N MLP-9601
- PCR Thermal Cycler (this assay has only been optimized for the following two thermal cyclers) (see footnote page 128):
  - GeneAmp PCR System 9700, Applied Biosystems, or
  - DNA Engine Tetrad PTC-225, MJ Research

---

1. dNTPs from Invitrogen (P/N R72501) have been tested on a limited basis with similar results. You should test in your own lab prior to full scale production.



**IMPORTANT**

- Program the thermal cycler in advance.
- Make sure the ligated DNA from the ligation step was diluted to 100  $\mu\text{L}$  with water.
- Prepare the PCR Master Mix in the Pre-PCR Clean Room.
- Set up the PCRs in the PCR Staging Area.
- Prepare 4 PCR reactions for each sample (4 PCRs are required for sufficient product to hybridize to each probe array).

**PCR Procedure****Pre-PCR Clean Room**

1. Prepare the following PCR Master Mix ON ICE with sufficient volume for 4 PCR reactions per sample and vortex at medium speed for 2 seconds (for multiple samples make 5% excess):

| Reagent                              | 1 PCR                              | 4 PCR                               | Final Conc. in Sample    |
|--------------------------------------|------------------------------------|-------------------------------------|--------------------------|
| PCR buffer (10X)                     | 10 $\mu\text{L}$                   | 40 $\mu\text{L}$                    | 1X                       |
| dNTP (2.5 mM each)                   | 10 $\mu\text{L}$                   | 40 $\mu\text{L}$                    | 250 $\mu\text{M}$ (each) |
| MgCl <sub>2</sub> (25 mM)            | 10 $\mu\text{L}$                   | 40 $\mu\text{L}$                    | 2.5 mM                   |
| PCR Primer, 001 (10 $\mu\text{M}$ )  | 7.5 $\mu\text{L}$                  | 30 $\mu\text{L}$                    | 0.75 $\mu\text{M}$       |
| AmpliTaq Gold® (5 U/ $\mu\text{L}$ ) | 2 $\mu\text{L}$                    | 8 $\mu\text{L}$                     | 0.1 U/ $\mu\text{L}$     |
| H <sub>2</sub> O                     | 50.5 $\mu\text{L}$                 | 202 $\mu\text{L}$                   |                          |
| <b>Total</b>                         | <b>90 <math>\mu\text{L}</math></b> | <b>360 <math>\mu\text{L}</math></b> |                          |

Four PCR reactions are needed for one array (each reaction = 100  $\mu\text{L}$ ).

**✓ Note**

*Do not mix PCR reactions from different samples.*

**PCR Staging Area**

2. Aliquot 90  $\mu\text{L}$  of the PCR Master Mix into each well, either in 8-Tube Strips or 96-Well PCR plates.
3. Add 10  $\mu\text{L}$  of DILUTED LIGATED DNA SAMPLE to 90  $\mu\text{L}$  PCR Master mix. Final volume for each PCR is 100  $\mu\text{L}$ .

| Reagent                                     | Volume ( $\mu\text{L}$ )/PCR        |
|---|-------------------------------------|
| PCR Master Mix                              | 90 $\mu\text{L}$                    |
| diluted ligated DNA<br>(from Ligation step) | 10 $\mu\text{L}$                    |
| <b>Total</b>                                | <b>100 <math>\mu\text{L}</math></b> |

4. Cap the 8-Tube Strips or Seal Plate, vortex at medium speed for 2 seconds and spin briefly at 2,000 rpm for 1 minute.

**Main Lab**

5. Run on MJ DNA Engine Tetrad or GeneAmp PCR System 9700.

**IMPORTANT**

*PCR protocols for MJ and GeneAmp PCR System 9700 thermal cyclers are different as listed below. The assay is sensitive to ramp time and model number. Please use recommended models for best results.*

**MJ DNA Engine Program:**

Use the **Heated Lid** and the **Calculated Temperature** features. Program the thermal cycler in advance with the following protocol:

| Temperature | Time       | Cycles |
|-------------|------------|--------|
| 95°C        | 3 minutes  | 1X     |
| 95°C        | 20 seconds |        |
| 59°C        | 15 seconds | 35X    |
| 72°C        | 15 seconds |        |
| 72°C        | 7 minutes  | 1X     |
| 4°C         | HOLD       |        |

**Gene Amp PCR System 9700 Program:**

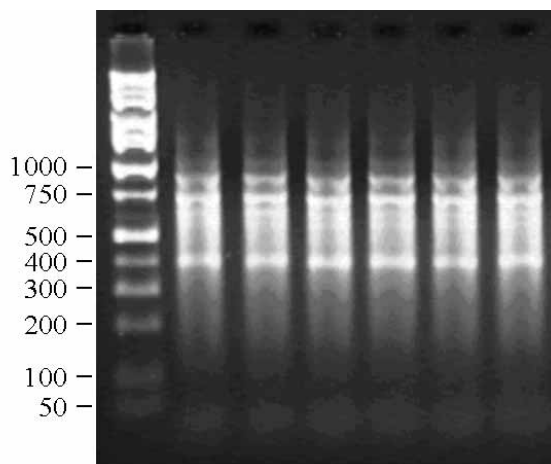
Specify **100 µL** volume and **maximum mode**.

| Temperature | Time       | Cycles |
|-------------|------------|--------|
| 95°C        | 3 minutes  | 1X     |
| 95°C        | 30 seconds |        |
| 59°C        | 30 seconds | 35X    |
| 72°C        | 30 seconds |        |
| 72°C        | 7 minutes  | 1X     |
| 4°C         | HOLD       |        |

6. Run 3 µL of each PCR product mixed with 3 µL of the 2X Gel Loading Dye on a 2% TBE gel at 120V for 1 hour. (See Figure A.2.)

**Note**

*Tubes can be stored at -20°C if not proceeding to the next step.*

**Figure A.2**

Example of PCR products run on 2% TBE agarose gel at 120V for 1 hour.

## STEP 5: QIAGEN PCR Purification and Elution

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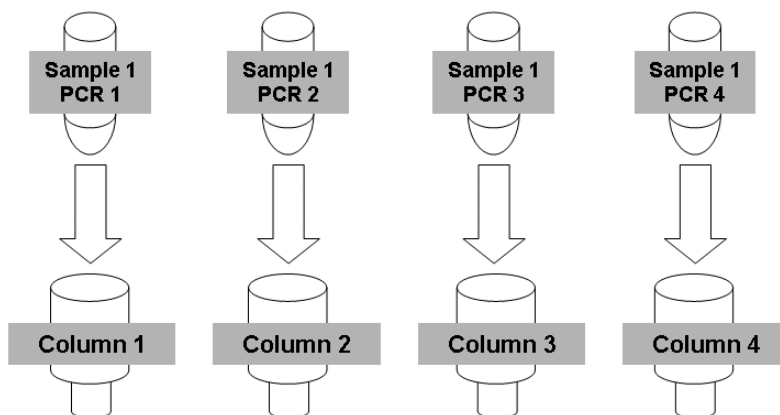
For alternative PCR cleanup protocol using QIAGEN MinElute 96 UF Purification Plate, see page 40.

### Main Lab

#### IMPORTANT

*To ensure high yield of purified PCR product, the following rules need to be followed:*

- *Store all the Buffers at 20°C to 25°C.*
- *After removing Buffer PE, centrifuge the columns at 13000 rpm for 5 minutes to dry the membrane.*
- *Add Buffer EB (25 °C) to the center of the column and let stand for 5 minutes before centrifuging.*
- *To increase the concentration of purified PCR products, it is essential to use the same Buffer EB to elute 4 columns of the PCR products. See the following elution procedure (Figure A.4).*

**Purification Procedure:****Figure A.3**

Apply each PCR product to a separate column.

**▲ WARNING**

*Do not mix PCR products from different samples.*

Steps 1 through 7: Excerpted from the *QIAquick Spin Handbook* (07/2002) with permission from QIAGEN.

1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. It is not necessary to remove mineral oil or kerosene.  
For example, add 500  $\mu\text{L}$  of Buffer PB to 100  $\mu\text{L}$  PCR sample (not including oil).
2. Place a QIAquick spin column in a provided 2 mL collection tube.
3. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30 to 60 seconds.
4. Discard flow-through. Place the QIAquick column back into the same tube.  
Collection tubes are re-used to reduce plastic waste.
5. To wash, add 750  $\mu\text{L}$  Buffer PE to the QIAquick column and centrifuge for 30 to 60 seconds.

6. Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 5 minutes.

**➤ IMPORTANT**

*Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.*

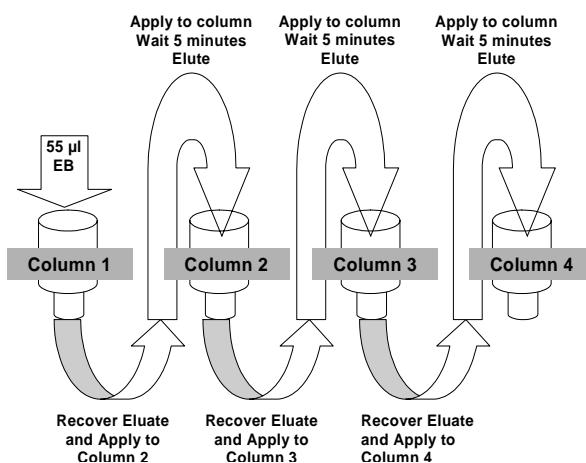
7. Place QIAquick column in a clean 1.5 mL microcentrifuge tube.

**✓ Note**

*To increase the concentration of purified PCR products, it is essential to use the same Buffer EB to elute 4 columns of the PCR products. See the following elution procedure (Figure A.4).*

8. Add 55  $\mu$ L of Buffer EB to the center of the QIAquick membrane of Column 1.
9. Let the column stand at room temperature for 5 minutes.
10. Centrifuge at 13000 rpm for 2 minutes.
11. Remove the eluate from the column and place it on the membrane of the second column.
12. Let the column stand at room temperature for 5 minutes.
13. Centrifuge at 13000 rpm for 2 minutes.
14. Use the same eluate to elute from the remaining columns as shown in Figure A.4.

## Elution Procedure



**Figure A.4**  
Elution procedure for one DNA sample

## STEP 6: Quantification of Purified PCR Product Using Spectrophotometric Analysis

### Main Lab

1. Add 4 µL of purified PCR product to 156 µL molecular biology water (40-fold dilution) and vortex at medium speed for 2 seconds.
2. Read the absorbance at 260 nm. Ensure that the reading is in the quantitative range of the instrument (generally 0.2 to 0.8 OD).
3. Apply the convention\* that 1 absorbance unit at 260 nm equals 50 µg/mL for double-stranded PCR product.  
\*This convention assumes a path length of 1 cm. Consult your spectrophotometer handbook for further information.
4. 20 µg of PCR product in a final volume of 45 µL is needed for the next fragmentation step.



**IMPORTANT**

*If 20 µg of PCR product has a volume less than 45 µL, make up the volume by adding EB Buffer (10 mM Tris-Cl, pH 8.5).*

## STEP 7: Fragmentation

### Reagents and Equipment

- Fragmentation Reagent (DNase I): Affymetrix, P/N 900131, available in Box 3 of the GeneChip® Mapping 10K Xba Assay Kit, P/N 900441
- 10 X Fragmentation Buffer: Affymetrix, P/N 900422, available in Box 3 of the GeneChip® Mapping 10K Xba Assay Kit, P/N 900441
- Molecular Biology Water: Bio Whittaker Molecular Applications/Cambrex, P/N 51200
- 4% TBE Gel: BMA Reliant precast (4% NuSieve 3:1 Plus Agarose); P/N 54929
- All Purpose Hi-Lo DNA Marker: Bionexus, Inc.; 50 - 10000bp; P/N BN2050
- Gel Loading Solution: Sigma; P/N G2526
- 8-Tube Strips, thin-wall (0.2 mL): MJ Research, P/N TBS-0201; Strip of 8 caps: MJ Research, P/N TCS-0801
- Thermal cycler:
  - DNA Engine Tetrad: MJ Research, or
  - Gene Amp PCR System 9700: Applied Biosystems

### Main Lab

#### ➔ IMPORTANT

*Fragmentation of the PCR product before hybridization onto GeneChip probe arrays has been shown to be critical in obtaining optimal assay performance. Due to the sensitive nature of the Fragmentation Reagent (DNase I), the following general rules need to be followed to ensure the success of this step:*

- *Store the Fragmentation Reagent stock at -20°C until ready for use.*
- *Make sure the purified PCR products are in EB buffer with proper volume (45 µL).*
- *Pre-heat the thermal cycler to 37°C before setting up the fragmentation reaction.*
- *Prepare the Fragmentation Reagent dilution immediately prior to use.*
- *Prepare excess diluted Fragmentation Reagent to avoid error from taking small volume of Fragmentation Reagent.*
- *Perform all the dilution, addition, and mixing steps ON ICE, AS QUICKLY AS POSSIBLE.*



## Fragmentation Procedure

1. Pre-heat the thermal cycler to 37°C.
2. Prepare the following mix ON ICE and vortex at medium speed for 2 seconds.

| Reagent                                   | Volume/Sample |
|---|---------------|
| Purified PCR Product (20 µg in EB buffer) | 45 µL         |
| 10X Fragmentation Buffer                  | 5 µL          |
| <b>Total</b>                              | <b>50 µL</b>  |

3. Aliquot 50 µL of the PCR products and buffer into 8-Tube Strip ON ICE.
4. Examine the label of the GeneChip Fragmentation Reagent tube for U/µL definition, and calculate dilution.

**Y** = number of µL of stock Fragmentation Reagent

**X** = number of U of stock Fragmentation Reagent per µL (see label on tube)

**0.048 U/µL** = final concentration of diluted Fragmentation Reagent

**125 µL** = final volume of diluted Fragmentation Reagent\*

$$Y = \frac{0.048 \text{ U/}\mu\text{L} * 125 \mu\text{L}}{X \text{ U/}\mu\text{L}}$$

\* sufficient for 20 reactions

### ✓ Note

*As the concentration of stock Fragmentation Reagent (U/µL) may vary from lot to lot, it is essential to check the concentration before conducting the dilution.*

5. Dilute the stock of Fragmentation Reagent to **0.048 U/µL** using 10X Fragmentation Buffer and Molecular Biology Water ON ICE and vortex at medium speed for 2 seconds.

Two **examples** of dilution are listed below for two different concentrations of Fragmentation Reagent.

| Reagent                  | 2 units/ $\mu\text{L}$              | 3 units/ $\mu\text{L}$              |
|--------------------------|-------------------------------------|-------------------------------------|
| Fragmentation Reagent    | 3 $\mu\text{L}$                     | 2 $\mu\text{L}$                     |
| 10X Fragmentation Buffer | 12.5 $\mu\text{L}$                  | 12.5 $\mu\text{L}$                  |
| H <sub>2</sub> O         | 109.5 $\mu\text{L}$                 | 110.5 $\mu\text{L}$                 |
| <b>Total</b>             | <b>125 <math>\mu\text{L}</math></b> | <b>125 <math>\mu\text{L}</math></b> |

If the concentration on your tube is not shown in the table above, use the formula provided in step 4.

- Add 5  $\mu\text{L}$  of the diluted Fragmentation Reagent (0.048 U/ $\mu\text{L}$ ) to 8-Tube Strip containing the PCR products ON ICE. The total volume for each sample is listed bellow.

| Reagent  | Volume/Sample                      |
|--|------------------------------------|
| PCR Products (from Step 2)                             | 50 $\mu\text{L}$                   |
| Diluted Fragmentation Reagent (0.048U/ $\mu\text{L}$ ) | 5 $\mu\text{L}$                    |
| <b>Total</b>   | <b>55 <math>\mu\text{L}</math></b> |

### ➔ IMPORTANT

*For 20  $\mu\text{g}$  of purified PCR product, a total of 0.24 U of Fragmentation Reagent is needed in a final reaction volume of 55  $\mu\text{L}$ .*

- Cap the 8-Tube Strip tightly, vortex at medium speed for 2 seconds and spin briefly at 2,000 rpm for 1 minute.
- Place the 8-Tube Strip in pre-heated thermal cycler (37°C) as quickly as possible.

### ➔ IMPORTANT

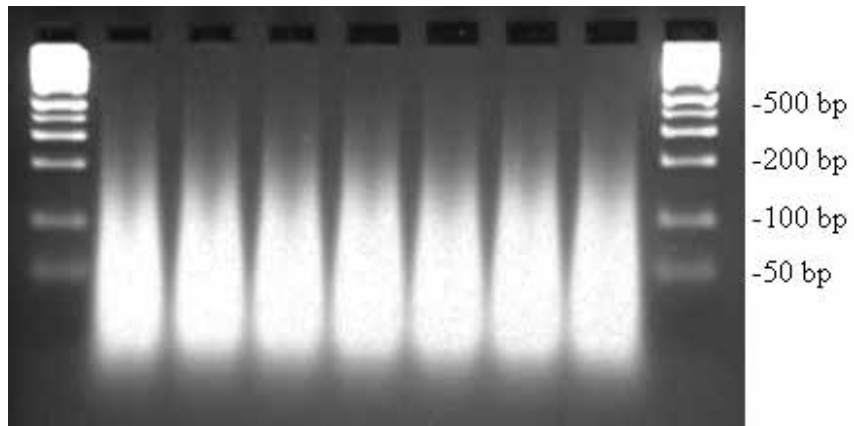
*Make sure the reaction tubes are securely sealed prior to running this program in order to minimize solution loss due to evaporation at the DNase I inactivation step (95°C).*

*Press the tubes to make sure all reaction tubes fit snugly into the wells of the heating block. Do not use low-quality substitutes of 8-Tube Strips that do not fit with the thermal cycler.*

9. Run the following program:

| Temperature | Time       |
|-------------|------------|
| 37°C        | 30 minutes |
| 95°C        | 15 minutes |
| 4°C         | Hold       |

- Spin the 8-Tube Strip briefly after the fragmentation reaction.
- Dilute 4  $\mu$ L of fragmented PCR product with 4  $\mu$ L gel loading dye and run on 4% TBE gel at 120V for 30 minutes to 1 hour. (See Figure A.5 for an example of a gel image.)
- Proceed immediately to Labeling step.



**Figure A.5**

Example of fragmented PCR products run on 4% TBE agarose gel at 120V for 30 minutes to 1 hour.

## STEP 8: Labeling

### Reagents and Equipment

- GeneChip DNA Labeling Reagent: Affymetrix; P/N 900430, available in Box 3 of the GeneChip® Mapping 10K Xba Assay Kit, P/N 900441
- Terminal Deoxynucleotidyl Transferase (30 U/μL): Affymetrix; P/N 900426, available in Box 3 of the GeneChip® Mapping 10K Xba Assay Kit, P/N 900441
- 5X Terminal Deoxynucleotidyl Transferase Buffer: Affymetrix; P/N 900425, available in Box 3 of the GeneChip® Mapping 10K Xba Assay Kit, P/N 900441
- 8-Tube Strips, thin-wall (0.2 mL): MJ Research, P/N TBS-0201; Strip of 8 caps: MJ Research, P/N TCS-0801
- Thermal cycler:
  - DNA Engine Tetrad: MJ Research, or
  - GeneAmp PCR System 9700: Applied Biosystems

### Labeling Procedure

#### Main Lab

1. Prepare Labeling Mix as master mix ON ICE and vortex at medium speed for 2 seconds (for multiple samples, make 5% excess).

| Reagent                              | 1X             | Final Conc. in Sample |
|--------------------------------------|----------------|-----------------------|
| 5X TdT Buffer                        | 14 μL          | 1X                    |
| GeneChip DNA Labeling Reagent (5 mM) | 2 μL           | 0.143 mM              |
| TdT (30 U/μL)                        | 3.4 μL         | 1.5 U/μL              |
| <b>Total</b>                         | <b>19.4 μL</b> |                       |

- Aliquot 19.4  $\mu\text{L}$  of Labeling Master Mix into the 8-Tube Strip containing 50.6  $\mu\text{L}$  of fragmented DNA samples as follows:

| Reagent                                     | Volume/Sample                      |   |
|---|------------------------------------|---|
| Fragmented DNA<br>(from Fragmentation step) | 50.6 $\mu\text{L}$                 | Remaining fragmented DNA<br>is used for gel analysis. |
| Labeling Mix                                | 19.4 $\mu\text{L}$                 |   |
| <b>Total</b>                                | <b>70 <math>\mu\text{L}</math></b> |   |

- Cap the 8-Tube Strip tightly, vortex at medium speed for 2 seconds and spin briefly at 2,000 rpm for 1 minute.
- Run the following program:

| Temperature | Time       |
|-------------|------------|
| 37°C        | 2 hours    |
| 95°C        | 15 minutes |
| 4°C         | Hold       |

### ➔ IMPORTANT

*Make sure the reaction tubes are securely sealed prior to running this program in order to minimize solution loss due to evaporation at denaturation step (95°C).*

- Briefly spin the tubes at 2,000 rpm for 1 minute after the labeling reaction.

### ✓ Note

*Tubes can be stored at -20°C if not immediately proceeding to the next step.*

## STEP 9: Target Hybridization



### Note

*The hybridization temperature is 48°C. This is different from the GeneChip expression assay.*

### Reagents

- 5M TMACL (Tetramethyl Ammonium Chloride): Sigma; P/N T3411
- 10% Tween-20: Pierce; P/N 28320 (Surfactamps); diluted to 3% in molecular biology grade water
- MES hydrate: Sigma; P/N M5287
- MES Sodium Salt: Sigma; P/N M5057
- DMSO: Sigma; P/N D5879
- EDTA: Ambion; P/N 9260G
- Denhardt's Solution: Sigma; P/N D2532
- HSDNA (Herring Sperm DNA): Promega; P/N D1815
- Human Cot-1: Invitrogen; P/N 15279-011
- Oligonucleotide Control Reagent: Affymetrix; P/N 900440, available in Box 3 of the GeneChip® Mapping 10K Xba Assay Kit, P/N 900441

### Reagent Preparation

#### 12 X MES Stock

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



### IMPORTANT

*Do not autoclave. Store between 2°C and 8°C, and shield from light. Discard solution if it turns yellow.*

#### For 1000 mL:

70.4 g MES hydrate

193.3 g MES Sodium Salt

800 mL 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.

## Hybridization Procedure

### IMPORTANT

*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.*

1. Prepare the following Hybridization Cocktail Master Mix (for multiple samples make 5% excess):

| Reagent   | 1X                           | Final Conc. in Sample |
|---|------------------------------|-----------------------|
| 12X MES Stock (1.22 M MES, 0.89 M [Na <sup>+</sup> ]) | 12 $\mu$ L                   | 0.056 M               |
| DMSO (100%)   | 13 $\mu$ L                   | 5.0%                  |
| Denhardt's Solution (50X)                             | 13 $\mu$ L                   | 2.5                   |
| EDTA (0.5 M)  | 3 $\mu$ L                    | 5.77 mM               |
| HSDNA (10 mg/mL)                                      | 3 $\mu$ L                    | 0.115 mg/mL           |
| Oligonucleotide Control Reagent                       | 2 $\mu$ L                    |                       |
| Human Cot-1 (1 mg/mL)                                 | 3 $\mu$ L                    | 11.5 mg/mL            |
| Tween-20 (3%)   | 1 $\mu$ L                    | 0.0115%               |
| TMACL (5M)  | 140 $\mu$ L                  | 2.69 M                |
| <b>Total</b>  | <b>190 <math>\mu</math>L</b> |                       |

2. Aliquot 190  $\mu$ L of the Hybridization Cocktail Master Mix into 70  $\mu$ L of labeled DNA samples as described below:

| Reagent           | Volume/Sample                |
|-------------------|------------------------------|
| Labeled DNA       | 70 $\mu$ L                   |
| Hybridization Mix | 190 $\mu$ L                  |
| <b>Total</b>      | <b>260 <math>\mu</math>L</b> |

### ✓ Note

*This Hybridization Cocktail Master Mix can be stored at -20°C before proceeding to the next step.*

3. Heat the 260  $\mu\text{L}$  of hybridization mix and labeled DNA at 95°C in a heat block for 10 minutes to denature.
4. Cool on crushed ice for 10 seconds.

**✓ Note**

*Do not leave on ice for longer than 10 seconds.*

5. Spin briefly at 2,000 rpm for 1 minute in microfuge to collect any condensate.
6. Place the tubes at 48°C for 2 minutes.
7. Inject 80  $\mu\text{L}$  of the denatured hybridization sample into the probe array.
8. Hybridize at 48°C for 16 to 18 hours at 60 rpm.

**✓ Note**

*The remaining hybridization mix can be stored at -20°C for future use.*

**✓ Note**

*The hybridization temperature is 48°C. This is different from the GeneChip expression assay.*