GeneChip® Mapping 500K Assay Manual

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Chapter 1

Overview

Chapter 1

About This Manual

This manual is a guide for technical personnel conducting GeneChip® Mapping 500K experiments in the laboratory. It contains protocols for sample preparation, 96-well plate sample processing, and low throughput sample processing. It also includes instructions for washing, staining, and scanning arrays and generating genotype calls, as well as extensive troubleshooting information. 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 500K 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: 96-Well Plate Protocol: Detailed, step-by-step protocol for processing a full 96-well plate of human genomic DNA samples.

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: Analysis Workflow and Guidelines for Assessing Sample and Data Quality: Describes the analysis workflow in GeneChip® Operating Software (GCOS) and GeneChip® Genotyping Analysis Software (GTYPE), and provides information for assessing data quality.

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

Appendix A: Reagents, Equipment, and Supplies Required for 96-Well Plate Protocol: Includes vendor and part number information for the equipment and reagents required to run the 96-Well Plate Protocol.

Appendix B: Thermal Cycler Programs Required for 96-Well Plate Protocol: Includes a list of the thermal cycler programs required for the 96-Well Plate Protocol.

Appendix C: Low Throughput Protocol: Detailed, step-by-step protocol for low throughput human genomic DNA sample processing. Included is a description of quality control checkpoints at various stages of the protocol which enable array performance to be monitored.

Appendix D: Reagents, Instruments, and Supplies Required for Low Throughput Protocol: A complete list of the equipment and reagents required to run the Low Throughput Protocol.

About Whole Genome Sampling Analysis

Long before the completion of the sequence of the human genome, it was clear that sites of genetic variation could be used as markers to identify disease segregation patterns among families. This approach successfully led to the identification of a number of genes involved in rare, monogenic disorders [1]. Now that the genome sequence has been completed and is publicly available [2, 3], attention has turned to the challenge of identifying genes involved in common, polygenic diseases [4, 5]. The markers of choice that have emerged for whole-genome linkage scans and association studies are single nucleotide polymorphisms (SNPs). Although there are multiple sources of genetic variation that occur among individuals, SNPs are the most common type of sequence variation and are powerful markers due to their abundance, stability, and relative ease of scoring [6]. Current estimates of the total human genetic variation suggest that there are over 7 million SNPs with a minor allele frequency of at least 5% [7]. The ongoing international effort to build a haplotype map will identify a standard set of common-allele SNPs that are expected to provide the framework for new genome-wide studies designed to identify the underlying genetic basis of complex diseases, pathogen susceptibility, and differential drug responses [8, 9]. Genome-wide association studies, which are based on the underlying principle of linkage disequilibrium (LD) in which a disease predisposing allele co-segregates with a particular allele of a SNP, have been hampered by the lack of whole-genome genotyping methodologies [10]. As new genotyping technologies develop, coupled with ongoing studies into

LD patterns and haplotype block structure across the genome, improvements in the design and power of association studies will be feasible [11-18].

We have developed an assay termed whole-genome sampling analysis (WGSA) for highly multiplexed SNP genotyping of complex DNA [19, 20]. This method reproducibly amplifies a subset of the human genome through a single primer amplification reaction using restriction enzyme digested, adaptor-ligated human genomic DNA. In contrast, many alternative genotyping technologies depend on multiple (2 to 6) locus-specific oligonucleotides per SNP, which often precludes scaling due to cost and technical difficulty to the magnitude required for linkage and association studies. This assay was first developed for simultaneous genotyping of over 10,000 SNPs on a single array (GeneChip® Human Mapping 10K Array Xba 142 2.0) and has been used to date for both linkage studies [21-34] and association studies [35-39]. Recently, the WGSA assay has been extended to allow highly accurate SNP genotyping of over 100,000 SNPs using the two array GeneChip® Human Mapping 100K Set [40]. With an average inter-marker distance of 23.6 kb, the arrays provide increasingly dense coverage for whole-genome association studies [41]. Recently, landmark breakthroughs in age-related macular degeneration and multiple sclerosis provide additional examples that this approach has now been proven to work for whole-genome association studies [42, 43]. The same characteristics that make SNPs useful markers for genetic studies also make SNPs powerful markers for additional biological applications such as the analysis of DNA copy number changes which include but are not limited to loss of heterozygosity (LOH), deletions, and gene amplifications [44-55]. Furthermore, integration of DNA copy number changes with gene expression changes provides a powerful paradigm for elucidating gene function [56].With the recent identification of large-scale copy number polymorphisms in the human genome as well, it is increasingly clear that a detailed understanding of the role of genomic alterations and structure will be important in the context of both the normal and disease state [57-60], and the high-resolution of the genotyping arrays should prove valuable in this arena. Additionally, large-scale SNP genotyping provides the basis for complex studies on population and admixture structure [61, 62].

The third-generation product in the mapping portfolio, the GeneChip® Human Mapping 500K Array Set, also uses the WGSA assay that has been the hallmark characteristic of all previous mapping arrays. An outline of the assay steps is shown in Figure 1.1 and Figure 1.2. By changing the choice of the restriction enzymes used in WGSA, and therefore the complexity of the resulting PCR products, along with an increase in the information capacity of the high-density arrays themselves, genotyping of 500,000 SNPs has been enabled on two arrays. This increase in the number of SNPs allows for higher density, genome-wide mapping sets, which in turn will increase the amount of information that can be extracted for association studies, the identification of DNA copy number alterations, and population sub-structure. Estimates for the number of SNPs that need to be genotyped for whole-genome association studies using large population based samples range from 100,000 to 1,000,000 depending on the disease, population or number of samples being studied [63-66]. In order to leverage the power of SNPs in genetic studies, we have developed a DNA target preparation method that, when combined with allelic discrimination via hybridization and with an automated genotype calling algorithm [67], allows rapid and accurate genotypic information at a fraction of the cost of current approaches. The Mapping 500K Set thus provides a powerful, flexible, cost-effective approach for scoring SNP genotypes in large numbers of samples and will provide a new technological paradigm for the design of whole-genome association studies.

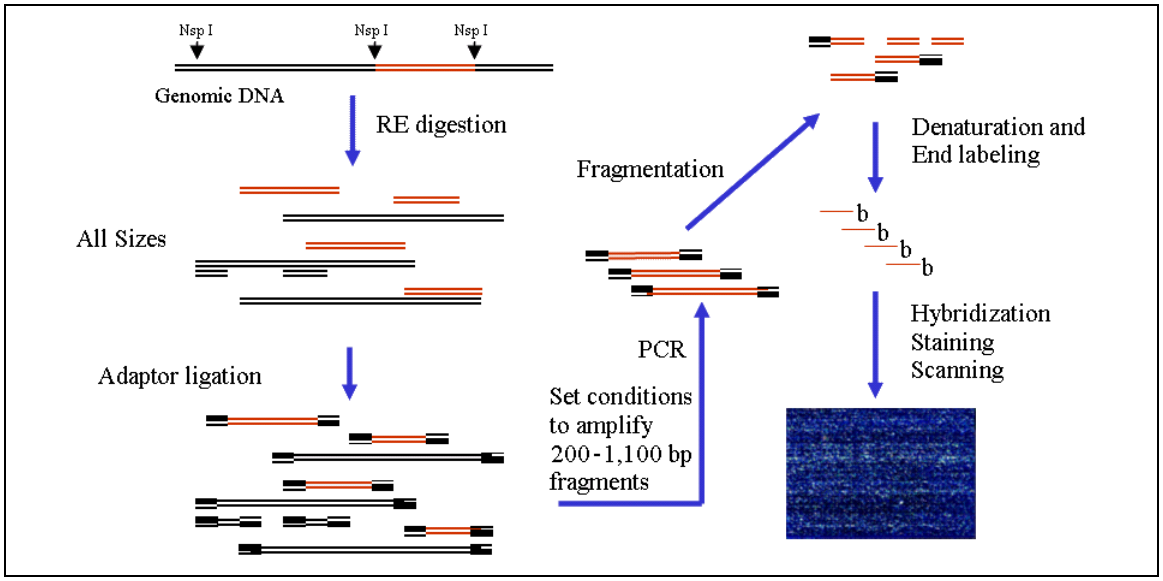

Figure 1.1
GeneChip® Mapping Assay - Nsp I

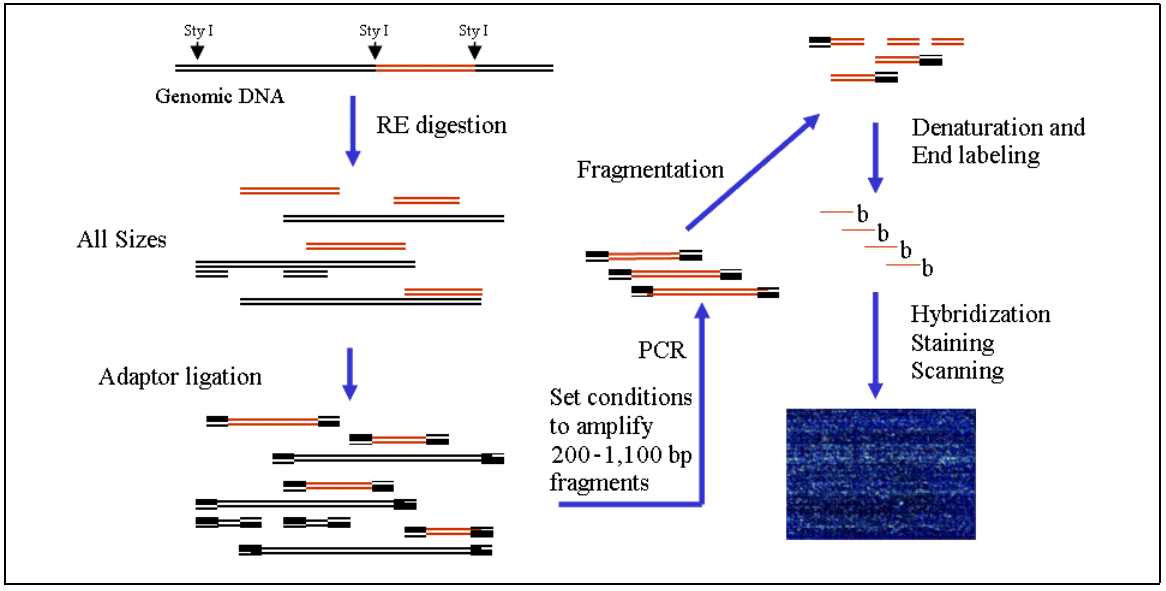

Figure 1.2
GeneChip® Mapping Assay - Sty I

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Chapter 2

Laboratory Setup

Chapter 2

Introduction to Laboratory Setup

IMPORTANT **!** **Please read this chapter before planning any experiments.**

The GeneChip® Human Mapping 500K Array Set has been optimized to reliably and accurately detect greater than 500,000 SNPs in each sample. The assay reduces the complexity of the genome by preferentially amplifying approximately 200-1100 base pair (bp) Nsp I fragments and approximately 200-1100 bp Sty I fragments using a single PCR primer from only 250 ng DNA per restriction enzyme. As with all genotyping applications, care should be taken to minimize possible sources of contamination that would reduce genotyping accuracy, call rate, and consequently, genetic power.

To reduce the possibility of cross-contamination, Affymetrix recommends maintaining a single direction workflow.

The GeneChip® Human Mapping 250K Nsp assay and the GeneChip® Human Mapping 250K Sty assay should be performed separately to avoid sample mix up.

IMPORTANT **!** **The most likely potential source of contamination for the GeneChip® Mapping 500K Assay is previously amplified PCR product.**

IMPORTANT **!** **Maintain an ambient laboratory environment throughout the procedure.**

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. A summary of the areas is shown in Figure 2.1.

IMPORTANT !

Once you enter the Main Lab, do not return to the Pre-PCR Room or the PCR Staging Room until you have showered and changed into freshly laundered clothing.

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 adaptor 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 to minimize the risk of trace levels of contaminants being brought into the Pre-PCR Clean Area. This room should contain dedicated pipets, tips, vortex, etc. Refer to Appendix A or Appendix D as appropriate for more details.

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, booties, and gloves is recommended to prevent PCR carryover.

IMPORTANT !

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

Main Lab

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.

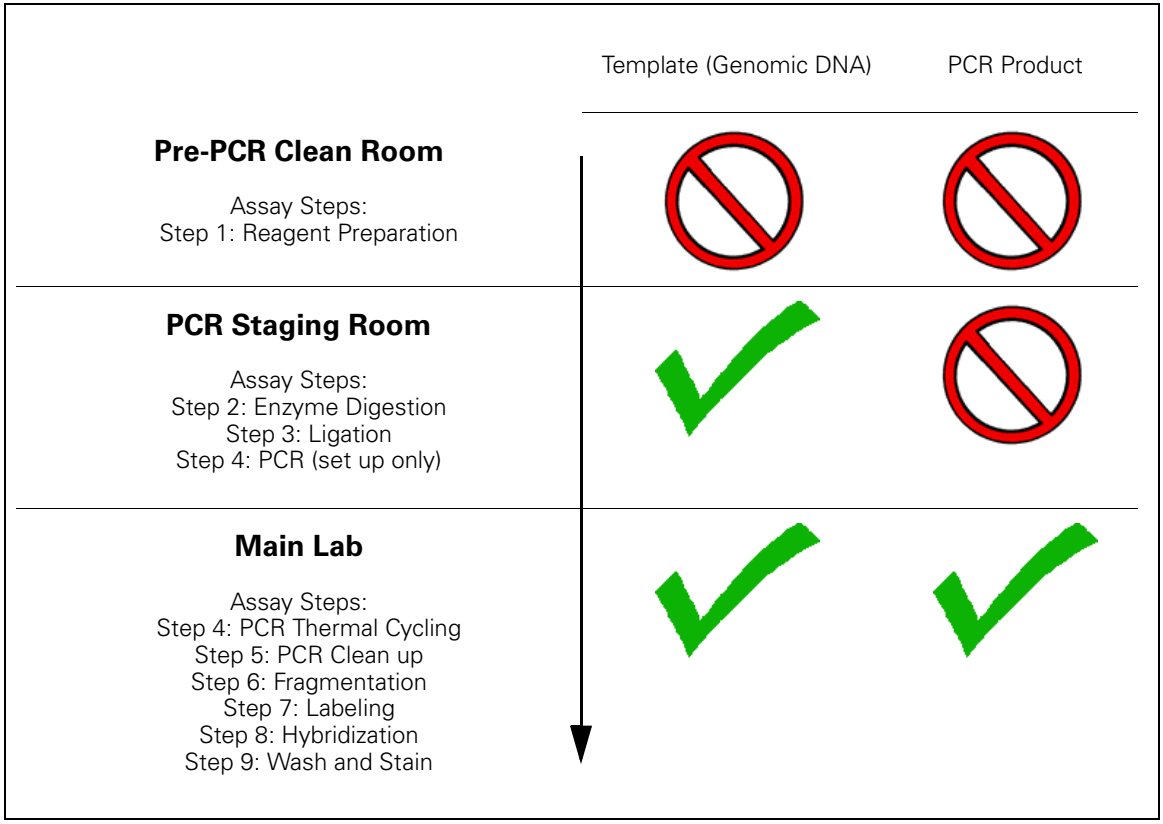

Figure 2.1
A summary of rooms required to perform the GeneChip® Mapping 500K Assay

Safety Precautions

The GeneChip® Mapping 250K Assay Kits as well as the GeneChip® 250K Nsp Array and the GeneChip® 250K Sty Array 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 toxins. 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 General Requirements

Chapter 3

Introduction

This chapter describes the general requirements for genomic DNA sources and extraction methods. The success of this assay requires the amplification of PCR fragments between 200 and 1100 bp in size throughout the genome. To achieve this, the genomic DNA must be of high quality, and must be free of contaminants that would affect the enzymatic reactions carried out.

A genomic DNA control (Reference Genomic DNA, 103) is provided in both of the GeneChip® Mapping 250K Assay Kits (Nsp and Sty). This control DNA meets the requirements outlined below. The size of the starting genomic DNA can be compared with Ref103 DNA to assess the quality. The control DNA should also 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

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, *96-Well Plate Protocol* or Appendix C, *Low Throughput Protocol* as appropriate.
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.

4. DNA must not be highly degraded. For any particular SNP, the genomic DNA fragment containing the SNP must have Nsp I (or Sty I) restriction sites intact 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% or 2% agarose gel using an appropriate size standard control. Reference Genomic DNA 103 can be run on the same gel for side-by-side comparison. 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. Pre-amplified genomic DNA has been tested with the Human Mapping 500K Assay and found to give results comparable to the standard DNA preparation methods. The Repli-G® Kit (a Φ29 whole genome amplification kit; QIAGEN) was used to amplify 10 ng genomic DNA, and the amplified products were immediately used in the subsequent protocol steps. This DNA gave call rates between 96 and 98% (Nsp) and 95 and 98% (Sty), and concordance greater than 99%. Other pre-amplification methods or pre-digestion with restriction enzymes other than Nsp I or Sty I have not been tested by Affymetrix. If these other methods are desired, it is recommended that the user conduct experiments to evaluate these other methods with the Mapping 500K Assay.

Sources of Human Genomic DNA

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 27.

- blood
- cell line

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 27.

Genomic DNA Extraction/Purification Methods

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 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

If a genomic DNA preparation is suspected to contain inhibitors, the following cleanup procedure can be used:

1. Add 0.5 volumes of 7.5 M NH_4OAc, 2.5 volumes of absolute ethanol (stored at –20°C), and 0.5 µL of glycogen (5 mg/mL) to 250 ng genomic DNA.
2. Vortex and incubate at –20°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 reduced EDTA TE buffer (10 mM Tris, pH 8.0, 0.1 mM EDTA, pH 8.0).

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Chapter 4

96-Well Plate Protocol

Chapter 4

96-Well Plate Protocol

ABOUT THIS PROTOCOL

The 96-well plate protocol described in this chapter is designed for processing one full 96-well reaction plate, one enzyme at a time. The protocol is presented in stages:

- *Stage 1: Genomic DNA Plate Preparation*
- *Stage 2: Restriction Enzyme Digestion*
- *Stage 3: Ligation*
- *Stage 4: PCR*
- *Stage 5: PCR Product Purification and Elution*
- *Stage 6: Quantitation and Normalization*
- *Stage 7: Fragmentation*
- *Stage 8: Labeling*
- *Stage 9: Target Hybridization*

The protocol and guidelines include key points regarding the various molecular biology steps that comprise whole-genome sampling analysis (WGSA).

Successful performance of the various molecular biology steps in this protocol requires accuracy and attention to detail. Many of these stages involve specific yet distinct enzymatic reactions. For example, in stage 2 the genomic DNA is initially digested with the restriction enzymes *Nsp* I or *Sty* I. In stage 3, it is ligated to a common adaptor with T4 DNA ligase. Following ligation, the template undergoes PCR (stage 4) using TITANIUM™ *Taq* DNA polymerase. Once the product has been purified and normalized (stage 5), it is then fragmented in stage 7 with Fragmentation Reagent (DNAse I), and end-labeled using terminal deoxynucleotidyl transferase (stage 8).

The stages involving enzymatic reactions are the most critical of the assay. Thus, it is important to carefully monitor and control any variables such as pH, salt concentrations, time, and temperature, all of which can adversely modulate enzyme activity.

Successful sample processing can be achieved by incorporating the following principles:

- Use only fresh reagents from the recommended vendors to help eliminate changes in pH or the salt concentration of buffers.
- Properly store all enzyme reagents. Storage methods can profoundly impact activity.
- When using reagents at the lab bench:
  - Closely follow the protocol and ensure that enzymes are kept at −20°C until needed.
  - Keep all master mixes and working solutions in chilled cooling chambers as indicated.
  - Properly chill essential equipment such as centrifuges, cooling chambers and reagent coolers before use.
  - Since enzyme activity is a function of temperature, ensure that all temperature transitions are rapid and/or well-controlled to help maintain consistency across samples.

Along with the enzymatic stages, lab instrumentation plays an important role in WGSA. To aid in maintaining consistency across samples and operators, all equipment should be well maintained and calibrated including:

- The thermal cyclers used (pre-PCR, PCR, and post-PCR)
- GeneChip® Hybridization Oven 640
- GeneChip® Fluidics Station 450
- GeneChip® Scanner 3000 7G
- The UV spectrophotometer plate reader
- All multi-channel pipettes

Since WGSA involves a series of ordered stages, it follows that the output of one stage directly impacts the performance of the subsequent stage. For example, the quantity and purity of the DNA after purification and normalization can affect the kinetics of the Fragmentation Reagent during the subsequent fragmentation stage. Variation in either the quantity or the relative purity of the DNA can result in over- or under-fragmentation, which can lead to variability in genotype call rates.

To efficiently process samples in a 96-well plate, it is essential to be proficient with the use of 12-channel pipettes. Attempting to use a

single channel pipette for plate-based samples requires too many pipetting steps, thus creating too high of a chance for error. To familiarize yourself with the use of multi-channel pipettes, we strongly recommend practicing several times before processing actual samples. You can use water to get a feel for the aspiration and dispensing of a solution to multiple wells simultaneously.

The post-PCR stages (5 through 8) are best performed by the more experienced operators in your laboratory. These operators should be proficient in:

- The use of multi-channel pipettes
- High throughput sample processing

Even if you feel comfortable with the protocol and with multichannel pipetting, do not attempt to transition to 96 samples immediately. We recommend a moderately paced ramp-up, wherein the operator first establishes proficiency with 24 samples. Your field application specialist (FAS) will provide additional guidance on best practices for a successful lab scale up.

When you are ready to process full 96-well plates, we recommend that the same operator should not perform too many stages in a given day. Small teams of individuals dedicated to different stages of the process has proven to be a highly effective method of managing this workflow.

For example, the full process can be sub-divided into four teams in which each team is responsible for the following stages:

- Team 1: pre-PCR (digestion, ligation, PCR set-up)
- Team 2: post PCR (purification, quantitation)
- Team 3: post-PCR (normalization, fragmentation, labeling)
- Team 4: array processing (hybridization, fluidics, scanning)

Again, your FAS can provide additional guidance on how best to configure the lab personnel for the 96-well plate protocol.

IMPORTANT ! **Arrays from different enzyme fractions should not be processed by the same technician on the same day.**

Before You Begin

MASTER MIX PREPARATION

Carefully follow each master mix recipe. Use pipettes that have been calibrated to ± 5%. When molecular biology-grade water is specified, be sure to use the AccuGENE® water listed in Appendix A, *Reagents, Equipment, and Consumables Required for 96-Well Plate Protocol*. Using in-house ddH_2O or other water can negatively affect your results. The enzymatic reaction in *Stage 7: Fragmentation* is particularly sensitive to pH and metal ion contamination.

If you run out of master mix during any of these procedures, a volume error has been made or the pipettes are not accurate. We recommend that you stop and repeat the experiment.

REAGENT HANDLING AND STORAGE

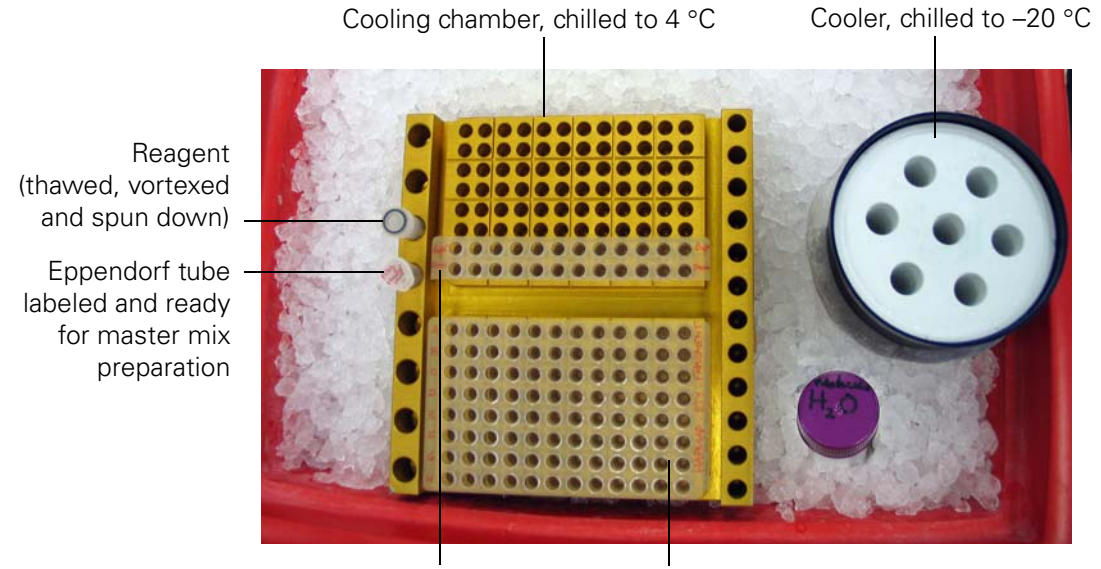
Follow these guidelines for reagent handling and storage.

- When working on the bench top, keep all reagents (except enzymes) on ice in a cooling chamber that has been chilled to 4 °C.
- Always leave enzymes at –20 °C until immediately prior to adding them to master mixes. When removed from the freezer, immediately place in a cooler that has been chilled to –20 °C and placed on ice.
- Store the reagents used for the restriction digestion, ligation and PCR steps in the Pre-PCR Clean Area.
- Do not re-enter the Pre-PCR Clean Area after entering the PCR-Staging Room or the Main Lab. Aliquot each of the reagents in the Pre-PCR Clean Area before starting the rest of the experiment.
- Dedicate one cooler for the Pre-PCR Clean Area and one for the Main Lab (post-PCR).
- When performing the steps for Stages 2 through 8:
  - Leave all of the tubes on ice or in a cooling chamber on ice.
  - Keep all plates in a cooling chamber on ice.
- Consult the appropriate MSDS for reagent storage and handling requirements.

PREPARING THE WORK AREA FOR EACH STAGE

Many of the stages in the Mapping 500K 96-well plate protocol must be performed rapidly and on ice to carefully control enzyme activity and temperature transitions. Therefore, we recommend that you gather and set up all of the equipment, consumables and reagents (except for the enzymes) prior to beginning each stage.

Below is an illustration of the setup for *Stage 7: Fragmentation*. Pipettes and tips are not shown.



Have pipettes and pipette tips ready as well. Begin each stage with full racks of pipette tips.

Figure 4.1
Example of Work Area Preparation

THERMAL CYCLERS, PLATES AND PLATE SEALS

The Mapping 500K 96-well plate protocol has been optimized using the following thermal cyclers, reaction plates and adhesive film.

IMPORTANT !

Use only the PCR plate, adhesive film and thermal cyclers listed in Table 4.1. Using other PCR plates and film that are incompatible with these thermal cyclers can result in crushed tubes, loss of sample, or poor results.

Table 4.1
Thermal Cyclers, PCR Plate and Adhesive Film Optimized for Use With the 500K 96-well Plate Protocol

Area	Thermal Cyclers Validated for Use	Plate	Film
Pre-PCR	2720 Thermal Cycler or GeneAmp® PCR System 9700 by Applied Biosystems MJ Tetrad PTC-225 by Bio-Rad	Multiplate 96-Well Unskirted PCR Plates Bio-Rad, P/N MLP-9601	MicroAmp® Clear Adhesive Films Applied Biosystems, P/N 4306311
PCR and Post-PCR	GeneAmp® PCR System 9700 by Applied Biosystems (silver block or gold-plated silver block) MJ Tetrad PTC-225 by Bio-Rad	Multiplate 96-Well Unskirted PCR Plates Bio-Rad, P/N MLP-9601	MicroAmp® Clear Adhesive Films Applied Biosystems, P/N 4306311

PROGRAM YOUR THERMAL CYCLERS

The Mapping 500K 96-well plate protocol includes the thermal cycler programs listed below. Before you begin processing samples, enter and store these programs on the thermal cyclers that will be used for this protocol.

Thermal cycler program details are listed in Appendix B, *Thermal Cycler Programs*.

Table 4.2
Thermal Cycler Programs Required for the 96-well Plate Protocol

Program Name	# of Thermal Cyclers Required (based on 96 sample throughput)	Laboratory
500K Digest	1	Pre-PCR Clean Area
500K Ligate	1	Pre-PCR Clean Area
500K PCR	3	Main Lab
500K Fragment	1	Main Lab
500K Label	1	Main Lab
500K Hyb	1	Main Lab

CUTTING STRIP TUBES FROM PLATES

Strip tubes are required for many of the steps in the 96-well plate protocol.

The Mapping 500K 96-well plate protocol has been optimized using strips of tubes that have been cut from the Bio-Rad plates recommended in Table 4.1 on page 40 and in Appendix A, *Reagents, Equipment, and Consumables Required for 96-Well Plate Protocol*.

Stage 1: Genomic DNA Plate Preparation

ABOUT THIS STAGE

The human genomic DNA you will process using the Mapping 500K 96-well plate protocol should meet the general requirements listed in Chapter 3, *Genomic DNA General Requirements*. During this stage, you will prepare the genomic DNA by:

1. Determining the concentration of each sample.
2. Diluting each sample to 50 ng/µL using reduced EDTA TE buffer.
3. Aliquoting 5 µL of each sample to a 96-well plate (one sample per well).

LOCATION AND DURATION

- Pre-PCR Clean Area
- Hands-on time: time will vary; can be up to 4 hours

INPUT REQUIRED

This protocol is written for processing 96 samples of genomic DNA, one enzyme at a time.

Quantity	Item
96	Genomic DNA samples that meet the general requirements listed in Chapter 3, *Genomic DNA General Requirements*.

EQUIPMENT AND CONSUMABLES REQUIRED

The following equipment and consumables are required for this stage. Refer to Appendix A, *Reagents, Equipment, and Consumables Required for 96-Well Plate Protocol* for vendor and part number information.

Table 4.3
Equipment and Consumables Required for Stage 1: Genomic DNA Plate Preparation

Quantity	Item
1	Cooling chamber, double, chilled to 4 °C (do not freeze)
1	Ice bucket, filled with ice
1	Plate centrifuge
1	Pipette, single channel P20
1	Pipette, 12-channel P200
1	Pipette, single channel P200
As needed	Pipette tips
As needed	Reaction plates, 96-well**
As needed	Plate seals**
1	Spectrophotometer plate reader
1	Vortexer

IMPORTANT !

**** Use only the PCR plate, adhesive film and thermal cyclers listed in Table 4.1 on page 40.**

REAGENTS REQUIRED

The following reagents are required for this stage. Refer to Appendix A, *Reagents, Equipment, and Consumables Required for 96-Well Plate Protocol* for vendor and part number information.

Table 4.4
Reagents Required for Stage 1: Genomic DNA Plate Preparation

Quantity	Item
As needed	Reduced EDTA TE Buffer (10 mM Tris HCL, 0.1 mM EDTA, pH 8.0)

PREPARING THE GENOMIC DNA PLATE

This protocol has been optimized using UV absorbance to determine genomic DNA concentrations. Other quantitation methods such as PicoGreen will give different readings. Therefore, you should correlate readings from other methods to the equivalent UV absorbance reading.

To prepare the genomic DNA plate:

1. Thoroughly mix the genomic DNA by vortexing at high speed for 3 sec.
2. Determine the concentration of each genomic DNA sample.
3. Based on OD measurements, dilute each sample to 50 ng/µL using reduced EDTA TE buffer.

   Apply the convention that 1 absorbance unit at 260 nm equals 50 µg/mL for double-stranded DNA. This convention assumes a path length of 1 cm. Consult your spectrophotometer handbook for more information. If using a quantitation method other than UV absorbance, correlate the reading to the equivalent UV absorbance reading.
4. Thoroughly mix the diluted DNA by vortexing at high speed for 3 sec.

IMPORTANT ! **An elevated EDTA level may interfere with subsequent reactions.**

ALIQUOTING PREPARED GENOMIC DNA

To aliquot the prepared genomic DNA:

1. Vortex the plate of genomic DNA at high speed for 10 sec, then spin down at 2000 rpm for 30 sec.
2. Aliquot 5 µL of each DNA to the corresponding wells of a 96-well reaction plate.

   5 µL of the 50 ng/µL working stock is equivalent to 250 ng genomic DNA per well.

   For this protocol, one plate is required to process Nsp samples; a second plate is required to process Sty samples. Do not process Nsp and Sty samples on the same day.
3. If continuing immediately to the next stage, place the plate with prepared genomic DNA in a double cooling chamber on ice.

   Otherwise, seal each plate with adhesive film.

WHAT YOU CAN DO NEXT

Do one of the following:

- Proceed to the next stage, processing one plate of samples, one enzyme at a time.
- Store the sealed plates of diluted genomic DNA at –20 °C.

Stage 2: Restriction Enzyme Digestion

ABOUT THIS STAGE

During this stage, the genomic DNA is digested by one of two restriction enzymes: Nsp I or Sty I. You will prepare the Digestion Master Mix, then add it to the samples. The samples are then placed onto a thermal cycler and the *500K Digest* program is run.

LOCATION AND DURATION

- Pre-PCR Clean Area
- Hands-on time: 30 minutes
- *500K Digest* thermal cycler program time: 2.3 hours

INPUT REQUIRED FROM PREVIOUS STAGE

The input required from *Stage 1: Genomic DNA Plate Preparation* is:

Quantity	Item
1 Plate, 96-well	Genomic DNA prepared as instructed in the previous stage (5 µL at 50 ng/µL in each well). Keep in a cooling chamber on ice.

EQUIPMENT AND CONSUMABLES REQUIRED

The following equipment and consumables are required for this stage. Refer to Appendix A, *Reagents, Equipment, and Consumables Required for 96-Well Plate Protocol* for vendor and part number information.

IMPORTANT !

**** Use only the PCR plate, adhesive film and thermal cyclers listed in Table 4.1 on page 40.**

Table 4.5
Equipment and Consumables Required for Stage 2: Restriction Enzyme Digestion

Quantity	Item
1	Cooler, chilled to –20 °C
1	Cooling chamber, double, chilled to 4 °C (do not freeze)
1	Ice bucket, filled with ice
1	Marker, fine point, permanent
1	Microcentrifuge
1	Pipette, single channel P100
1	Pipette, single channel P200
1	Pipette, single channel P1000
1	Pipette, 12-channel P20
As needed	Pipette tips for pipettes listed above; full racks
1	Plate centrifuge
1	Plate seal**
1	Thermal cycler**
1 strip	Tubes, strip, 12 per strip
1	Tube, Eppendorf 2.0 mL
1	Vortexer

REAGENTS REQUIRED

The following reagents are required for this stage. Refer to Appendix A, *Reagents, Equipment, and Consumables Required for 96-Well Plate Protocol* for vendor and part number information. The amounts listed are sufficient to process one full 96-well reaction plate.

Table 4.6
Reagents Required for Stage 2: Restriction Enzyme Digestion

Quantity	Reagent
1 vial	BSA (100X; 10 mg/mL)
1 vial	NE Buffer 2 or 3 (10X) • If Nsp, use NE Buffer 2 • If Sty, use NE Buffer 3
1 vial	Sty I or Nsp I (10 U/µL; NEB)
2.0 mL	AccuGENE® Water, molecular biology-grade

IMPORTANT INFORMATION ABOUT THIS STAGE

To help ensure the best results, carefully read the information below before you begin this stage of the protocol.

IMPORTANT !

- The same team or individual operator should not perform Nsp 1 and Sty 1 digestion reactions on the same day.

About Using Controls

Positive Controls

Reference Genomic DNA 103 can be used as a positive control. It is supplied in both the Sty and Nsp GeneChip® Mapping 250K Assay Kits.

- Nsp 100 Rxn Kit P/N 900753
- Sty 100 Rxn Kit P/N 900754

Negative Controls

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.

PREPARE THE REAGENTS, EQUIPMENT AND CONSUMABLES

Thaw Reagents and Genomic DNA Plate

1. Allow the following reagents to thaw on ice:
   - NE Buffer
   - BSA
2. If the plate of genomic DNA from stage 1 was frozen, allow it to thaw in a cooling chamber on ice.

IMPORTANT !

Leave the NSP I or STY I enzyme at –20°C until ready to use.

Prepare Your Work Area

To prepare the work area:

1. Place a double cooling chamber and a cooler on ice (Figure 4.1 on page 39).
2. Label the following tubes, then place in the cooling chamber:
   - One strip of 12 tubes labeled *Dig*
   - A 2.0 mL Eppendorf tube labeled *Dig MM*
3. Place the AccuGENE® water on ice.
4. Prepare the plate of genomic DNA from Stage 1 as follows:
   A. Vortex the center of the plate at high speed for 3 sec.
   B. Spin down the plate at 2000 rpm for 30 sec.
   C. Place back in the cooling chamber on ice.
5. To prepare the reagents (except for the enzyme):
   A. Vortex 3 times, 1 sec each time.

B. Pulse spin for 3 sec.

C. Place in the cooling chamber.

Preheat the Thermal Cycler Lid

Power on the thermal cycler to preheat the lid. Leave the block at room temperature.

PREPARE THE DIGESTION MASTER MIX

IMPORTANT !

The same team or individual operator should not process samples with both Nsp and Sty enzymes on the same day. Best practice is to process samples for either Nsp or Sty on a given day.

Keeping all reagents and tubes on ice, prepare the Digestion Master Mix as follows:

1. To the 2.0 mL Eppendorf tube, add the appropriate volumes of the following reagents based on Table 4.7 (Nsp) or Table 4.8 (Sty):
   - AccuGENE® Water
   - NE Buffer
   - BSA
2. Remove the appropriate enzyme (Nsp I or Sty I) from the freezer and immediately place in a cooler.
3. Pulse spin the enzyme for 3 sec.
4. Immediately add the enzyme to the master mix, then place remaining enzyme back in the cooler.
5. Vortex the master mix at high speed 3 times, 1 sec each time.
6. Pulse spin for 3 sec.
7. Place in the cooling chamber.
8. Return any remaining enzyme to the freezer.
9. Proceed immediately to *Add Digestion Master Mix to Samples* on page 51.

Table 4.7
Nsp I Digestion Master Mix

Reagent	1 Sample	96 Samples (15% extra)
AccuGENE® Water	11.55 µL	1275.1 µL
NE Buffer 2 (10X)	2 µL	220.8 µL
BSA (100X; 10 mg/mL)	0.2 µL	22.1 µL
Nsp I (10 U/µL)	1 µL	110.4 µL
Total	**14.75 µL**	**1628.4 µL**

Table 4.8
Sty I Digestion Master Mix

Reagent	1 Sample	96 Samples (15% extra)
AccuGENE® Water	11.55 µL	1275.1 µL
NE Buffer 3 (10X)	2 µL	220.8 µL
BSA (100X; 10 mg/mL)	0.2 µL	22.1 µL
Sty I (10 U/µL)	1 µL	110.4 µL
Total	**14.75 µL**	**1628.4 µL**

ADD DIGESTION MASTER MIX TO SAMPLES

To add Digestion Master Mix to samples:

1. Using a single channel P200 pipette, aliquot 135 µL of Digestion Master Mix to each tube of the strip tubes labeled *Dig*.
2. Using a 12-channel P20 pipette, add 14.75 µL of Digestion Master Mix to each DNA sample in the cooling chamber on ice.

   The total volume in each well is now 19.75 µL.

Reagent	Volume/Sample
Genomic DNA (50 ng/µL)	5 µL
Digestion Master Mix	14.75 µL
Total Volume	19.75 µL

3. Seal the plate tightly with adhesive film.
4. Vortex the center of the plate at high speed for 3 sec.
5. Spin down the plate at 2000 rpm for 30 sec.
6. Ensure that the lid of thermal cycler is preheated.
7. Load the plate onto the thermal cycler and run the *500K Digest* program.

500K Digest Program	
Temperature	**Time**
37°C	120 minutes
65°C	20 minutes
4°C	Hold

8. When the program is finished, remove the plate and spin it down at 2000 rpm for 30 sec.
9. Do one of the following:
   - If proceeding directly to the next step, place the plate in a cooling chamber on ice.
   - If not proceeding directly to the next step, store the samples at –20 °C.

Stage 3: Ligation

ABOUT THIS STAGE

During this stage, the digested samples are ligated using either the Nsp or Sty Adaptor. You will prepare the Ligation Master Mix, then add it to the samples. The samples are then placed onto a thermal cycler and the *500K Ligate* program is run.

When the program is finished, you will dilute the ligated samples with AccuGENE® water.

LOCATION AND DURATION

- Pre-PCR Clean Area
- Hands-on time: 30 minutes
- *500K Ligate* thermal cycler program time: 3.3 hours

INPUT REQUIRED FROM PREVIOUS STAGE

The input required from *Stage 2: Restriction Enzyme Digestion* is:

Quantity	Item
1	Plate of digested samples in a cooling chamber on ice.

EQUIPMENT AND CONSUMABLES REQUIRED

The following equipment and consumables are required for this stage. Refer to Appendix A, *Reagents, Equipment, and Consumables Required for 96-Well Plate Protocol* for vendor and part number information.

Table 4.9
Equipment and Consumables Required for Stage 3: Ligation

Quantity	Item
1	Cooler, chilled to –20 °C
1	Cooling chamber, double, chilled to 4 °C (do not freeze)
1	Ice bucket, filled with ice
1	Marker, fine point, permanent
1	Microcentrifuge
1	Pipette, single channel P100
1	Pipette, single channel P1000
1	Pipette, 12-channel P20
1	Pipette, 12-channel P200
As needed	Pipette tips for pipettes listed above; full racks
1	Plate centrifuge
2	Plate seal**
1	Solution basin, 55 mL
1	Thermal cycler**
1 strip	Tubes, strip, 12 per strip
1	Tube, Eppendorf 2.0 mL
1	Vortexer

IMPORTANT !

**** Use only the PCR plate, adhesive film and thermal cyclers listed in Table 4.1 on page 40.**

REAGENTS REQUIRED

The following reagents are required for this stage. Refer to Appendix A, *Reagents, Equipment, and Consumables Required for 96-Well Plate Protocol* for vendor and part number information. The amounts listed are sufficient to process one full 96-well reaction plate.

Table 4.10
Reagents Required for Stage 3: Ligation

Quantity	Reagent
1 vial	T4 DNA Ligase (400 U/µL; NEB)
1 vial	T4 DNA Ligase Buffer (10X)
1 vial	Adaptor, Nsp or Sty as appropriate (50 µM)
10 mL	AccuGENE® water, molecular biology-grade

IMPORTANT INFORMATION ABOUT THIS PROCEDURE

To help ensure the best results, carefully read the information below before you begin this stage of the protocol.

IMPORTANT !

- Aliquot the T4 DNA Ligase Buffer (10X) after thawing for the first time to avoid multiple freeze-thaw cycles. See vendor instructions.
- Be sure to use the correct adaptor (Nsp or Sty).

PREPARE THE REAGENTS, CONSUMABLES AND OTHER COMPONENTS

Thaw the Reagents and Digestion Stage Plate

To thaw the reagents and Digestion Stage Plate:

1. Allow the following reagents to thaw on ice:
   - Adaptor Nsp I or Sty I as appropriate
   - T4 DNA Ligase Buffer (10X)

   Takes approximately 20 minutes to thaw.

2. If the Digestion Stage plate was frozen, allow to thaw in a cooling chamber on ice.

IMPORTANT **Leave the T4 DNA Ligase at –20 °C until ready to use.**

Prepare Your Work Area

To prepare the work area:

1. Place a double cooling chamber and a cooler on ice (Figure 4.1 on page 39).
2. Label the following tubes, then place in the cooling chamber:
   - One strip of 12 tubes labeled *Lig*
   - A 2.0 mL Eppendorf tube labeled *Lig MM*
   - Solution basin
3. Prepare the Digestion Stage plate as follows:
   A. Vortex the center of the plate at high speed for 3 sec.
   B. Spin down the plate at 2000 rpm for 30 sec.
   C. Place back in the cooling chamber on ice.
4. To prepare the reagents:
   A. Vortex at high speed 3 times, 1 sec each time (except for the enzyme).
   B. Pulse spin for 3 sec.
   C. Place in the cooling chamber.

IMPORTANT **T4 DNA Ligase Buffer (10X) contains ATP and should be thawed on ice. Vortex the buffer as long as necessary before use to ensure precipitate is re-suspended and that the buffer is clear. Avoid multiple freeze-thaw cycles per vendor instructions.**

Preheat the Thermal Cycler Lid

Power on the thermal cycler to preheat the lid. Leave the block at room temperature.

The lid must be preheated before samples are loaded.

PREPARE THE LIGATION MASTER MIX

Keeping all reagents and tubes on ice, prepare the Ligation Master Mix as follows:

1. To the 2.0 mL Eppendorf tube, add the following reagents based on the volumes shown in Table 4.11 (for Nsp) or Table 4.12 (for Sty):
   - Adaptor (Nsp or Sty)
   - T4 DNA Ligase Buffer (10X)
2. Remove the T4 DNA Ligase from the freezer and immediately place in the cooler on ice.
3. Pulse spin the T4 DNA Ligase for 3 sec.
4. Immediately add the T4 DNA Ligase to the master mix; then place back in the cooler.
5. Vortex the master mix at high speed 3 times, 1 sec each time.
6. Pulse spin for 3 sec.
7. Place the master mix on ice.
8. Proceed immediately to *Add Ligation Master Mix to Reactions*.

Table 4.11
Nsp I Ligation Master Mix

Reagent	1 Sample	96 Samples (15% extra)
Adaptor Nsp I (50 µM)	0.75 µL	82.8 µL
T4 DNA Ligase Buffer (10X)	2.5 µL	276 µL
T4 DNA Ligase (400 U/µL)	2 µL	220.8 µL
Total	**5.25 µL**	**579.6 µL**

Table 4.12
Sty I Ligation Master Mix

Reagent	1 Sample	96 Samples (15% extra)
Adaptor Sty I (50 µM)	0.75 µL	82.8 µL
T4 Ligase Buffer (10X)	2.5 µL	276 µL
T4 DNA Ligase (400U/µL)	2 µL	220.8 µL
Total	**5.25 µL**	**579.6 µL**

ADD LIGATION MASTER MIX TO REACTIONS

To add Ligation Master Mix to samples:

1. Using a single channel P100 pipette, aliquot 48 µL of Ligation Master Mix to each tube of the strip tubes on ice.
2. Using a 12-channel P20 pipette, aliquot 5.25 µL of Ligation Master Mix to each reaction on the Digestion Stage Plate.

Reagent	Volume/Sample
Digested DNA	19.75 µL
Ligation Master Mix*	5.25 µL
Total	**25 µL**

* Contains ATP and DTT. Keep on ice.

3. Seal the plate tightly with adhesive film.
4. Vortex the center of the plate at high speed for 3 sec.
5. Spin down the plate at 2000 rpm for 30 sec.
6. Ensure that the thermal cycler lid is preheated.
7. Load the plate onto the thermal cycler and run the *500K Ligate* program.

500K Ligate Program	
Temperature	**Time**
16°C	180 minutes
70°C	20 minutes
4°C	Hold

DILUTE THE SAMPLES

To dilute the samples:

8. Place the AccuGENE® Water on ice 20 minutes prior to use.

1. When the 500K Ligate program is finished, remove the plate and spin it down at 2000 rpm for 30 sec.
2. Place the plate in a cooling chamber on ice.
3. Dilute each reaction as follows:

   A. Pour 10 mL AccuGENE® water into the solution basin.

   B. Using a 12-channel P200 pipette, add 75 µL of the water to each reaction.

   The total volume in each well is 100 µL.

Reagent	Volume/Sample
Ligated DNA	25 µL
AccuGENE® water	75 µL
Total	**100 µL**

4. Seal the plate tightly with adhesive film.
5. Vortex the center of the plate at high speed for 3 sec.
6. Spin down the plate at 2000 rpm for 30 sec.

IMPORTANT !

It is crucial to dilute the ligated DNA with AccuGENE® water prior to PCR.

WHAT YOU CAN DO NEXT

Do one of the following:

- If proceeding to the next step, store the plate in a cooling chamber on ice for up to 60 minutes.
- If not proceeding directly to the next step, store the plate at –20 °C.

Stage 4: PCR

ABOUT THIS STAGE

During this stage, you will transfer equal amounts of each ligated sample into three new 96-well plates (Figure 4.2 on page 67). You will then prepare the PCR Master Mix, and add it to each sample.

Each plate is placed onto a thermal cycler and the *500K PCR* program is run. When the program is finished, you will check the results of this stage by running 3 µL of each PCR product on a 2% TBE gel.

LOCATION AND DURATION

- Pre-PCR Clean Area: PCR Master Mix preparation
- PCR Staging Area: PCR set up
- Main Lab: PCR Plates placed on thermal cyclers
- Hands-on time: 1 hour
- *500K PCR* thermal cycler program time: 1.5 hours; samples can be held overnight

INPUT REQUIRED FROM PREVIOUS STAGE

The input required from *Stage 3: Ligation* is:

Quantity	Item
1	Plate of diluted ligated samples Keep in a cooling chamber on ice.

EQUIPMENT AND MATERIALS REQUIRED

The following equipment and materials are required to perform this stage. Refer to Appendix A, *Reagents, Equipment, and Consumables Required for 96-Well Plate Protocol* for vendor and part number information.

Table 4.13
Equipment and Consumables Required for Stage 4: PCR

Quantity	Item
1	Cooler, chilled to –20 °C
2 double or 4 single	Cooling chambers, chilled to 4 °C (do not freeze)
1	Ice bucket, filled with ice
1	Marker, fine point, permanent
1	Microcentrifuge
1	Pipette, single channel P20
1	Pipette, single channel P100
1	Pipette, single channel P200
1	Pipette, single channel P1000
1	Pipette, 12-channel P20
1	Pipette, 12-channel P200
As needed	Pipette tips for pipettes listed above; full racks
6	Plates, 96-well reaction**
1	Plate centrifuge
7	Plate seal**
1	Solution basin, 55 mL
3	Thermal cycler**
1	Tube, Falcon 50 mL
1	Vortexer

IMPORTANT !

**** Use only the PCR plate, adhesive film and thermal cyclers listed in Table 4.1 on page 40.**

REAGENTS REQUIRED

The following reagents are required for this stage. Refer to Appendix A, *Reagents, Equipment, and Consumables Required for 96-Well Plate Protocol* for vendor and part number information. The amounts listed are sufficient to process one full 96-well reaction plate.

Table 4.14
Reagents Required for Stage 4: PCR

Quantity	Reagent
• 15 mL	AccuGENE® water, molecular biology-grade
875 µL (2 vials)	PCR Primer 002 (100 µM)
The following reagents from the Clontech TITANIUM™ DNA Amplification Kit	
• 1.28 mL	dNTPs (2.5 mM each)
• 1 mL	GC-Melt (5M)
• 100 µL	TITANIUM™ *Taq* DNA Polymerase (50X)
• 600 µL	TITANIUM™ *Taq* PCR Buffer (10X)

GELS AND RELATED MATERIALS REQUIRED

The following gels and related materials are required for this stage. Refer to Appendix A, *Reagents, Equipment, and Consumables Required for 96-Well Plate Protocol* for vendor and part number information. The amounts listed are sufficient to process one full 96-well reaction plate.

Table 4.15
Gels and Related Materials Required for Stage 4: PCR

Quantity	Reagent
50 µL	DNA Marker
13	Gels, 2% TBE
As needed	Gel loading solution
3	Plates, 96-well reaction

IMPORTANT INFORMATION ABOUT THIS STAGE

To help ensure the best results, carefully read the information below before you begin this stage of the protocol.

IMPORTANT !

- Make sure the ligated DNA was diluted to 100 µL with AccuGENE® water.
- Prepare PCR Master Mix immediately prior to use, and prepare in Pre-PCR Clean room. To help ensure the correct distribution of fragments, be sure to add the correct amount of primer to the master mix. Mix the master mix well to ensure the even distribution of primers.
- Set up the PCRs in PCR Staging Area.
- To ensure consistent results, take 3 µL aliquots from each PCR to run on gels *before* adding EDTA.

About Controls

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.

PREPARE THE REAGENTS, CONSUMABLES AND OTHER COMPONENTS

Thaw Reagents and Ligation Stage Plate

To thaw the reagents and Ligation Stage Plate:

1. Allow the following reagents to thaw on ice.
   - TITANIUM *Taq* PCR Buffer
   - dNTPs
   - PCR Primer 002

IMPORTANT !

Leave the TITANIUM *Taq* DNA Polymerase at –20 °C until ready to use.

2. If the Ligation Stage plate was frozen, allow to thaw in a cooling chamber on ice.

Prepare Your Work Area (Pre-PCR Clean Area)

To prepare the work area:

1. Place two double or four single cooling chambers and one cooler on ice (Figure 4.1 on page 39).
2. Label the following, then place in a cooling chamber:
   - Three 96-well reaction plates labeled *P1, P2, P3*; see Figure 4.2 on page 67
   - A 50 mL Falcon tube labeled *PCR MM*
3. Place on ice:
   - AccuGENE® water
   - GC-Melt
   - Solution basin

4. Prepare the Ligation Stage plate as follows:
   - **A.** Vortex the center of the plate at high speed for 3 sec.
   - **B.** Spin down the plate at 2000 rpm for 30 sec.
   - **C.** Label the plate *Lig*.
   - **D.** Place back in the cooling chamber on ice.
5. To prepare the reagents:
   - **A.** Vortex at high speed 3 times, 1 sec each time (except for the enzyme).
   - **B.** Pulse spin for 3 sec.
   - **C.** Place in a cooling chamber.

Preheat the Thermal Cycler Lids (Main Lab)

Have someone in the Main Lab power on the thermal cyclers to be used for the PCR to preheat the lids. The lids must be preheated before loading samples; leave the blocks at room temperature.

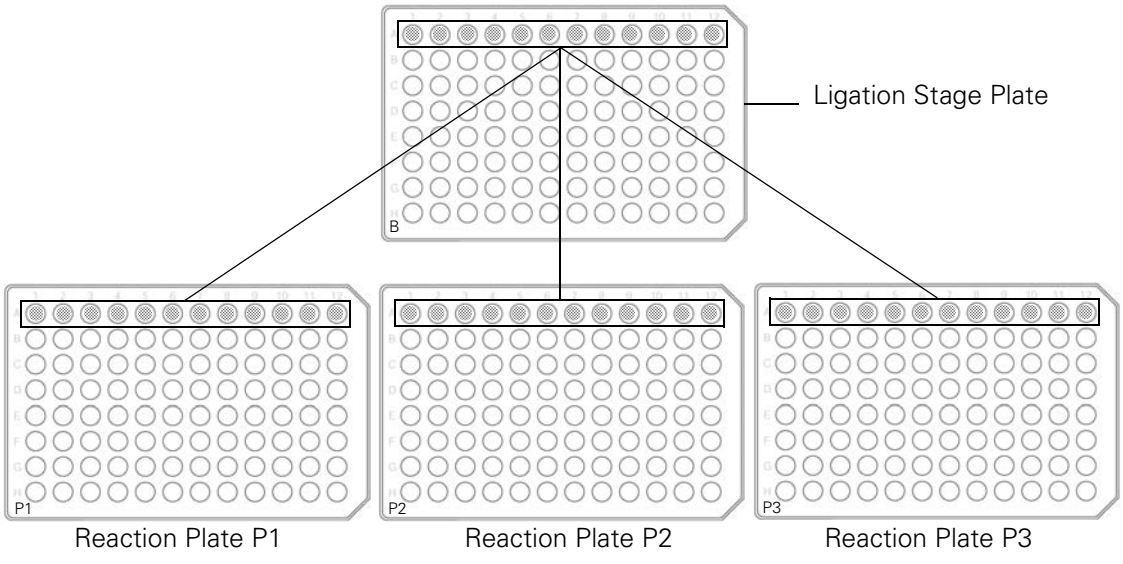
If you are preparing the plates for PCR, it is best not to go from the Pre-PCR Room or Staging Area to the Main Lab and then back again.

ADD DNA TO THE REACTION PLATES

To add DNA to the reaction plates:

1. Working one row at a time and using a 12-channel P20 pipette, transfer 10 µL of sample from each well of the Ligation Plate to the corresponding well of each reaction plate.

   Example (Figure 4.2 on page 67): Transfer 10 µL of sample from each well of row A on the Ligation Plate to the corresponding wells of row A on reaction plates P1, P2 and P3.
2. Seal each plate with adhesive film, and leave in cooling chambers on ice.



An equal aliquot of each sample from the Ligation Stage Plate is transferred to the corresponding well of each PCR Plate. For example, an equal aliquot of each sample from row A on the Ligation Stage Plate is transferred to the corresponding wells of row A on PCR Plates P1, P2 and P3.

Figure 4.2
Transferring Equal Aliquots of Diluted, Ligated Samples to Three Reaction Plates

PREPARE THE PCR MASTER MIX

Prepare enough PCR Master Mix to run three PCR reactions per sample.

Location

Pre-PCR Clean Room

Prepare the PCR Master Mix

To prepare the PCR Master Mix:

IMPORTANT !

The PCR reaction is sensitive to the concentration of primer used. It is critical that the correct amount of primer be added to the PCR Master Mix to achieve the correct distribution of fragments (200 to 1100 bp) in the products.

Check the PCR reactions on a gel to ensure that the distribution is correct (see Figure 4.3). 90 µg of PCR product is needed for fragmentation.

1. Keeping the 50 mL Falcon tube in the cooling chamber, add the reagents in Table 4.16 in the order shown.
2. Remove the TITANIUM *Taq* DNA Polymerase from the freezer and immediately place in a cooler.
3. Pulse spin the *Taq* DNA polymerase for 3 sec.
4. Immediately add the *Taq* DNA polymerase to the master mix; then return the tube to the cooler on ice.
5. Vortex the master mix at high speed 3 times, 1 sec each time.
6. Pour the mix into the solution basin, keeping the basin on ice.

Table 4.16
PCR Master Mix

Reagent	For 1 Reaction	For 3 PCR Plates (15% extra)
AccuGENE® water	39.5 µL	13.082 mL
TITANIUM *Taq* PCR Buffer (10X)	10 µL	3.312 mL
GC-Melt (5M)	20 µL	6.624 mL
dNTP (2.5 mM each)	14 µL	4.637 mL
PCR Primer 002 (100 µM)	4.5 µL	1.490 mL
TITANIUM *Taq* DNA Polymerase (50X)	2 µL	0.663 mL
Total	**90 µL**	**29.808 mL**

ADD PCR MASTER MIX TO SAMPLES

Location

PCR Staging Area

Procedure

To add PCR Master Mix to samples:

1. Using a 12-channel P200 pipette, add 90 µL PCR Master Mix to each sample.

   The total volume in each well is 100 µL.
2. Seal each reaction plate tightly with adhesive film.
3. Vortex the center of each reaction plate at high speed for 3 sec.
4. Spin down the plates at 2000 rpm for 30 sec.
5. Keep the reaction plates in cooling chambers on ice until loaded onto the thermal cyclers.

LOAD PCR PLATES ONTO THERMAL CYCLERS

IMPORTANT !

PCR protocols for the MJ Tetrad PTC-225 and Applied Biosystems thermal cyclers are different. See Table 4.17 and Table 4.18 below.

Location

Main Lab

Procedure

To load the plates and run the *500K PCR* program:

1. Transfer the reaction plates to the Main Lab.
2. Ensure that the thermal cycler lids are preheated.

   The block should be at room temperature.
3. Load each reaction plate onto a thermal cycler.
4. Run the *500K PCR* program.

   The program varies depending upon the thermal cyclers you are using. See Table 4.17 for Applied Biosystems thermal cyclers and Table 4.18 for Bio-Rad thermal cyclers.

IMPORTANT !

If using GeneAmp® PCR System 9700 thermal cyclers, be sure the blocks are silver or gold-plated silver. Do NOT use thermal cyclers with aluminum blocks. It is not easy to visually distinguish between silver and aluminum blocks.

Table 4.17
500K PCR Thermal Cycler Program for the GeneAmp® PCR System 9700 (silver or gold-plated silver blocks)

500K PCR Program for GeneAmp® PCR System 9700		
Temperature	**Time**	**Cycles**
94°C	3 minutes	1X
94°C 60°C 68°C	30 sec 45 sec 15 sec	} 30X
68°C	7 minutes	1X
4°C	HOLD (Can be held overnight)	
Volume: 100 µL		
Specify *Maximum* mode.		

Table 4.18
500K PCR Thermal Cycler Program for the MJ Tetrad PTC-225

500K PCR Program for MJ Tetrad PTC-225		
Temperature	**Time**	**Cycles**
94°C	3 minutes	1X
94°C 60°C 68°C	30 sec 30 sec 15 sec	} 30X
68°C	7 minutes	1X
4°C	HOLD (Can be held overnight)	
Volume: 100 µL		
Use *Heated Lid* and *Calculated Temperature*		

RUNNING GELS

Before Running Gels

To ensure consistent results, take 3 µL aliquot from each PCR before adding EDTA.

WARNING ▲ **Wear the appropriate personal protective equipment when handling ethidium bromide.**

Run the Gels

When the *500K PCR* program is finished:

1. Remove each plate from the thermal cycler.
2. Spin down plates at 2000 rpm for 30 sec.
3. Place plates in cooling chambers on ice or keep at 4 °C.
4. Label three fresh 96-well reaction plates *P1Gel*, *P2Gel* and *P3Gel*.
5. Aliquot 3 µL of 2X Gel Loading Dye to each well of the three plates.
6. Using a 12-channel P20 pipette, transfer 3 µL of each PCR product from plates *P1*, *P2* and *P3* to the corresponding plate, row and wells of plates *P1Gel*, *P2Gel* and *P3Gel*.

   Example: 3 µL of each PCR product from each well of row A on plate P1 is transferred to the corresponding wells of row A on plate P1Gel.
7. Seal plates *P1Gel*, *P2Gel* and *P3Gel.*
8. Vortex the center of plates *P1Gel*, *P2Gel* and *P3Gel,* then spin down at 2000 rpm for 30 sec.
9. Load all 6 µL from each well of plates *P1Gel*, *P2Gel* and *P3Gel* onto 2% TBE gels.
10. Run the gels at 120V for 40 minutes to 1 hour.
11. Verify that the PCR product distribution is between ~250 bp to 1100 bp (see Figure 4.3).

    90 µg of PCR product is needed for fragmentation.

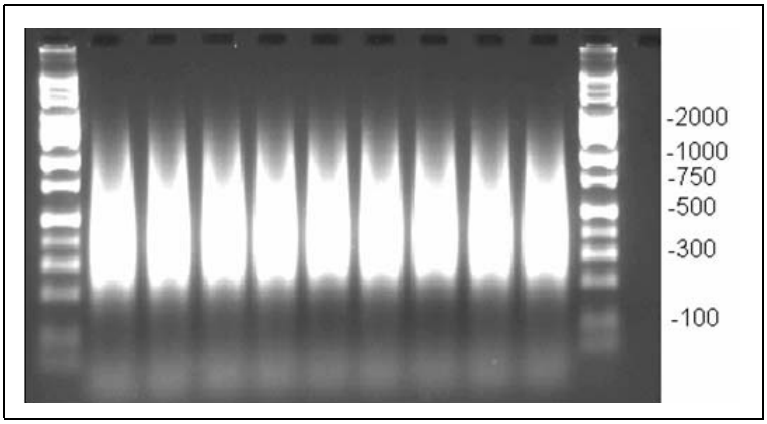


Figure 4.3
Example of PCR products run on 2% TBE agarose gel at 120V for 1 hour. Average product distribution is between ~250 to 1100 bp.

WHAT YOU CAN DO NEXT

Do one of the following:

- Proceed to the next stage within 60 minutes.
- If not proceeding directly to the next stage, seal the plates with PCR product and store at –20 °C.

Stage 5: PCR Product Purification and Elution

ABOUT THIS STAGE

During this stage, you will:

- Add diluted EDTA to each PCR product
- Pool each corresponding PCR product back to a single reaction on a Clontech Clean-Up Plate (Figure 4.4 on page 80).
- Place the Clean-Up Plate onto a manifold and concentrate the PCR products.
- Wash the PCR products three times while still on the manifold using AccuGENE® water.
- Elute the PCR products using RB Buffer and transfer them to a new 96-well plate.

LOCATION AND DURATION

- Main Lab
- Hands-on time: 1 hour
- Initial dry down: 1.5 to 2 hours
- First two water washes: 15 to 20 minutes each
- Third water wash: 45 to 75 minutes

INPUT REQUIRED FROM PREVIOUS STAGE

The input required from *Stage 4: PCR* is:

Quantity	Item
3	Plates of PCR product in cooling chambers on ice.

EQUIPMENT AND CONSUMABLES REQUIRED

The following equipment and consumables are required for this stage. Refer to Appendix A, *Reagents, Equipment, and Consumables Required for 96-Well Plate Protocol* for vendor and part number information.

Table 4.19
Equipment and Consumables Required for Stage 5: PCR Product Purification and Elution

Quantity	Item
1	Jitterbug
As needed	Kimwipes
1	Manifold, QIAvac Multiwell
1	Marker, fine point, permanent
1	Pipette, single channel P200
1	Pipette, single channel P1000
1	Pipette, 12-channel P20
1	Pipette, 12-channel P200
As needed	Pipette tips for pipettes listed above; full racks
1	Plate, 96-well PCR
1	Plate centrifuge
1	Plate, Clontech Clean-Up
4	Plate holders
5	Plate seal**
4	Plate supports
1	Regulator (QIAGEN)
1	Solution basin, 55 mL
1	Vortexer

IMPORTANT !

**** Use only the PCR plate, adhesive film and thermal cyclers listed in Table 4.1 on page 40.**

REAGENTS REQUIRED

The following reagents are required for this stage. Refer to Appendix A, *Reagents, Equipment, and Consumables Required for 96-Well Plate Protocol* for vendor and part number information. The amounts listed are sufficient to process one full 96-well reaction plate.

Table 4.20
Reagents Required for Stage 5: PCR Product Purification and Elution

Quantity	Reagent
1	Clean-Up Plate (Clontech)
3 mL	EDTA, diluted to 0.1M (working stock is 0.5 M, pH 8.0)
5 mL	RB Buffer
75 mL	AccuGENE® water, molecular biology-grade

IMPORTANT INFORMATION ABOUT THIS STAGE

To help ensure the best results, carefully read the information below before you begin this stage of the protocol.

IMPORTANT !

- The working stock of EDTA must be diluted to 0.1 M before use.
- The AccuGENE® water listed in Appendix A must be used for this stage. Using in-house ddH_2O is not acceptable and can negatively impact downstream stages, particularly *Stage 7: Fragmentation*. The fragmentation reaction is very sensitive to pH and metal ion contamination.
- To avoid cross-contamination and the introduction of air bubbles, pipette very careful when pooling the three PCR reactions for each sample onto the Clontech Clean-Up Plate.
- Maintain the vacuum at 600 mbar.

- The PCR reactions contain significant contaminants including EDTA. These contaminants can affect subsequent steps unless removed by washing. Therefore, be sure to perform three water washes.
- After the third wash, the wells must be *completely* dry before eluting the samples with RB Buffer. Any extra water carried with the RB Buffer to the next stage can result in over-fragmentation.
- Immediately upon removal from the manifold, blot the bottom of the plate and wipe the bottom of each well. Any remaining liquid will quickly seep back into the wells.

PREPARE THE REAGENTS, CONSUMABLES AND OTHER COMPONENTS

Prepare the PCR Product Plates

To prepare the PCR Product Plates from the previous stage:

1. Place the three PCR product plates on the bench top in plate holders.

   If frozen, allow them to thaw to room temperature.
2. Once at room temperature, vortex the center of each plate at high speed for 3 sec.
3. Spin down each plate at 2000 rpm for 30 sec.

Dilute the Working Solution of EDTA

Dilute the working stock of EDTA to a concentration of 0.1 M. A higher concentration may interfere with downstream steps.

Setup the Manifold

To set up the manifold:

1. Connect the manifold and regulator to a suitable vacuum source able to maintain 600 mbar.
2. Place the waste tray inside the base of the manifold.

   Do not turn on the vacuum at this time.

ADD DILUTED EDTA TO THE PCR PRODUCTS

To add diluted EDTA to the PCR products:

1. Add 3 mL of diluted EDTA (0.1M) to a solution basin.
2. Using a 12-channel P20 pipette, aliquot 8 µL of diluted EDTA to each well with PCR product on each PCR product plate.
3. Tightly seal each plate.
4. Vortex the center of each plate at high speed for 3 sec.
5. Spin down each plate at 2000 rpm for 30 sec.
6. Place each plate back in a plate holder.

PREPARE THE CLEAN-UP PLATE

Follow the steps as described below. Consult the Clontech Clean-Up Plate Handbook for the general procedure.

To prepare the Clean-Up Plate:

1. Label the plate to indicate its orientation *CUP BL (Clean-Up Plate bottom left*).
2. If not processing a full plate of samples, cover the wells that will not be used with adhesive film as follows:
   - **A.** Apply pressure around the edges of the plate to make the film stick.
   - **B.** Cut the film between the used and unused wells.
   - **C.** Remove the portion that covers the wells you want to use.

POOL THE PCR PRODUCTS

CAUTION

To avoid piercing the Clean-Up Plate membrane, do not pipette up and down in the plate, and do not touch the bottom of the plate.

Be very careful when pooling the third set of PCR products, as the wells are very full. Avoid cross-contaminating neighboring wells with small droplets.

Also, pipette very carefully to avoid the formation of air bubbles. Air bubbles will slow drying.

Working one row at a time, pool the PCR products as follows:

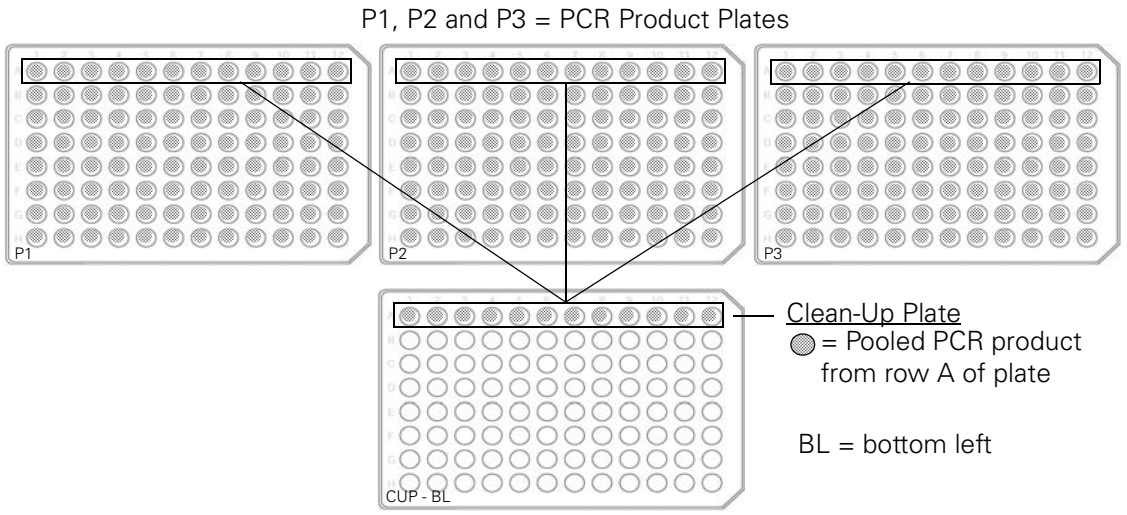
1. Cut the adhesive film from the first row of each reaction plate.
2. Using a 12-channel P200 pipette, transfer and pool the samples from the same row and well of each PCR product plate to the corresponding row and well of the Clean-Up Plate.

   Example (Figure 4.4 on page 80): transfer each sample from row A of plates P1, P2 and P3 to the corresponding wells of row A on the Clean-Up Plate.

   To avoid piercing the membrane, do not pipette up and down in the Clean-Up Plate.
3. Change your pipette tips.

   Be sure to change pipette tips after each of the three corresponding rows of sample are pooled onto the Clean-Up Plate.
4. Repeat these steps until all of the PCR products are pooled.
5. Examine the three PCR product plates to be sure that the full volume of each well was transferred and that the plates are empty.

   The final volume in each well on the Clontech Clean-Up Plate should be approximately 320 µL.



Transfer and pool each PCR product from plates P1, P2 and P3 to the corresponding well of the Clean-Up Plate. For example, transfer and pool the PCR product from well A1 of plates P1, P2 and P3 to the corresponding row and well on the Clean-Up Plate.

Figure 4.4
Pooling PCR Products Onto the Clean-Up Plate

PURIFY THE PCR PRODUCTS

IMPORTANT !

Three water washes must be performed to properly purify the PCR products. Be sure to completely dry the membrane after the third wash.

To purify the PCR products:

1. Load the Clontech Clean-Up Plate with samples onto the manifold.
2. Cover the plate to protect the samples from environmental contaminants.

   For example, you can use the lid from a pipette tip box. Do not put a plate seal on the wells containing sample.

3. Turn on the vacuum and slowly bring it up to 600 mbar.
4. Check the vacuum by gently trying to lift the middle section of the manifold off the base.

   Be very careful not to lose any sample. You should not be able to lift the middle section off the base.
5. Maintain the vacuum at 600 mbar until all of the wells are dry (approximately 1.5 to 2 hours).

   The vacuum regulator may sound like it is leaking. This sound is the pressure release working to limit the vacuum to 600 mbar.
6. Wash the PCR products three times as follows, keeping the vacuum on the entire time:

   A. Add 75 mL AccuGENE® Water to a solution basin.

   B. Using a 12-channel P200 pipette, add 50 µL water to each well.

   C. Dry the wells for 15 to 20 minutes.

   The top and bottom rows may take longer to filter and dry.

   D. Repeat steps B and C two additional times for a total of 3 water washes.
7. After the third wash, tap the manifold firmly on the bench to force any drops on the sides of the wells to move to the bottom and be pulled through the plate.
8. Allow the samples to dry completely.

   Drying after the third wash may take 45 to 75 minutes.
9. Tilt and inspect the plate to confirm that the top and bottom rows are completely dry.

   Do not allow the plate to sit on the manifold or the bench top for more than 90 minutes after the wells are completely dried.

IMPORTANT !

To prevent the dilution of DNA with water, ensure that every well is completely dry before adding RB Buffer.

ELUTE THE PCR PRODUCTS

To elute the PCR products:

1. When the wells are completely dry after the third wash, turn off the vacuum.
2. Carefully remove the plate from the manifold and immediately:
   - **A.** Blot the bottom of the plate on a thick stack of clean absorbent paper to remove any remaining liquid.
   - **B.** Dry the bottom of each well with an absorbent wipe.

IMPORTANT ! **Immediately blot the bottom of the plate and dry the bottom of each well. Any remaining liquid will quickly seep back into the wells.**

3. Aliquot 5 mL RB Buffer to a solution basin.
4. Using a 12-channel P200 pipette, add 45 µL RB buffer to each well of the plate.
5. Tightly seal the plate.
6. Load the plate onto a Jitterbug plate shaker.
7. Set the Jitterbug to setting 5 and moderately shake the plate for 10 minutes at room temperature.

   This setting (approximately 1000 rpm) allows as much movement as possible without losing liquid to the sides of the wells and film.
8. Transfer 45 µL of each eluted sample from the Clontech Clean-Up Plate to the corresponding well of a fresh 96-well plate following these guidelines:
   - Use a 12-channel P200 pipette set to 60 µL.
   - Tilt the Clontech Clean-Up Plate at a 30 to 45 degree angle to move the liquid to one side of the well.

     Optional: use a plate support to keep the plate tilted at an angle (Well Plate Stand: Diversified Biotech, P/N WPST-1000).
   - Pipette up and down 3 to 4 times before removing and transferring the eluate to a fresh 96-well reaction plate.

- Go back into the well a second time and remove any remaining liquid. It is OK to touch the bottom of the filter.

WHAT YOU CAN DO NEXT

Do one of the following:

- Proceed immediately to the next step.
- If not proceeding immediately to the next step:
  - **A.** Seal the plate with the eluted samples.
  - **B.** Store the plate at –20 °C.

Stage 6: Quantitation and Normalization

ABOUT THIS STAGE

During this stage, you will prepare three independent dilutions of each PCR product in optical plates. You will then quantitate the diluted PCR products and average the OD measurements from each plate.

Once the concentration of each reaction is determined, you will normalize each reaction to 2 µg/µL in RB Buffer.

LOCATION AND DURATION

- Main Lab
- Hands-on time
  - Quantitation: 1 hour
  - Normalization: 1 hour

INPUT REQUIRED FROM PREVIOUS STAGE

Input required from *Stage 5: PCR Product Purification and Elution* is:

Quantity	Item
1	Plate of purified PCR product

EQUIPMENT AND CONSUMABLES REQUIRED

The following equipment and consumables are required for this stage. Refer to Appendix A, *Reagents, Equipment, and Consumables Required for 96-Well Plate Protocol* for vendor and part number information.

IMPORTANT !

**** Use only the PCR plate, adhesive film and thermal cyclers listed in Table 4.1 on page 40.**

Table 4.21
Equipment and Consumables Required for Stage 6: Quantitation and Normalization

Quantity	Item
1	Cooling chamber, double, chilled to 4 °C (do not freeze)
1	Ice bucket, filled with ice
1	Marker, fine point, permanent
1	Pipette, single channel P20
1	Pipette, single channel P100
1	Pipette, single channel P1000
1	Pipette, 12-channel P20 (accurate to within ± 5%)
1	Pipette, 12-channel P200
As needed	Pipette tips for pipettes listed above; full racks
4	Plate, optical For example, the UV Star Transparent, 96-well. Use the optical plate recommended for use with your plate reader.
1	Plate, 96-well reaction
1	Plate centrifuge
5	Plate seal**
1	Spectrophotometer plate reader
1	Solution basin, 100 mL
1	Vortexer

REAGENTS REQUIRED

The following reagents are required for this stage. Refer to Appendix A, *Reagents, Equipment, and Consumables Required for 96-Well Plate Protocol* for vendor and part number information. The amounts listed are sufficient to process one full 96-well reaction plate.

Table 4.22
Reagents Required for Stage 6: Quantitation and Normalization

Quantity	Reagent
As needed	RB Buffer (from Clontech DNA Amplification Clean-Up Kit)
75 mL	AccuGENE® water, molecular biology-grade

IMPORTANT INFORMATION ABOUT THIS STAGE

To help ensure the best results, carefully read the information below before you begin this stage of the protocol.

IMPORTANT !

- Prepare three independent dilutions of each sample for accurate concentration measurement. Average the results for each individual sample before normalizing.
- The sample in each well must be normalized to 2 µg/µL in RB Buffer (90 µg in 45 µL RB Buffer). Do NOT determine an average concentration to use for every well.
- The amount of DNA added to the arrays has been optimized for the best performance. Since not all wells will contain the same amount of DNA after purification, the eluted PCR products must be carefully normalized to 2 µg/µL before continuing to *Stage 7: Fragmentation*.
- Normalize samples using RB Buffer (not water) to maintain the correct pH for subsequent steps.
- The accuracy of the OD measurement is critical. Carefully follow the steps below and be sure the OD measurement is within the quantitative linear range of the instrument (0.2 to 0.8 OD).
- The spectrophotometer plate reader should be calibrated regularly to ensure correct readings.
- This protocol has been optimized using a UV spectrophotometer plate reader for quantitation.

  NOTE: The NanoDrop® will give different quantitation results. This protocol has not been optimized for use with this instrument. In

addition, the NanoDrop quantifies a single sample at a time and is not amenable to 96-well plate processing.

PREPARE THE REAGENTS, EQUIPMENT AND CONSUMABLES

Turn on the Spectrophotometer Plate Reader

Turn on the spectrophotometer now and allow it to warm for 10 minutes before use.

Prepare Your Work Area

To prepare the work area:

1. Place a double cooling chamber on ice.
2. Label the 96-well reaction plate *Fragment* as this plate will also be used for the next stage), and place on the cooling chamber.
3. Place the following on the bench top:
   - Optical plates
   - Solution basin
   - AccuGENE® water
4. Label each optical plate as follows: *OP1, OP2, OP3, OP4*.
5. Vortex the RB Buffer and place on the bench top.
6. Prepare the purified, eluted PCR product plate as follows:
   A. If the plate was frozen, allow it to thaw in a cooling chamber on ice.
   B. Vortex the center of the plate at high speed for 3 sec.
   C. Spin down the plate at 2000 rpm for 30 sec.
   D. Place the plate on the bench top.

PREPARE DILUTED ALIQUOTS OF PURIFIED SAMPLE

IMPORTANT !

Two of the wells on each optical plate must be set up as blanks containing AccuGENE® water only.

The 12-channel P20 pipette must be accurate to within ± 5%.

To prepare three diluted aliquots of the purified samples:

1. Pour 75 mL of room temperature AccuGENE® water into the solution basin.
2. Using a 12-channel P200 pipette, aliquot 198 µL of water to:
   - **A.** Each well of optical plates 1, 2 and 3.
   - **B.** The first four rows of optical plate 4.
3. Using a 12-channel P20 pipette:
   - **A.** Transfer 2 µL of each purified PCR product from rows A through G of the purified sample plate to the corresponding rows and wells of optical plates 1, 2 and 3 (see Figure 4.5 on page 89).
   - **B.** Pipette up and down 2 times after each transfer to ensure that all of the product is dispensed.
   - **C.** Examine the pipette tips and aliquots before and after each dispense to ensure that exactly 2 µL has been transferred.
   - **D.** Transfer 2 µL of each purified PCR product from row H of the purified sample plate to the corresponding rows and wells of optical plate 4.
   - **E.** Again, pipette up and down 2 times after each transfer, and examine the pipette tips and aliquots before and after each dispense.

     The result is a 100-fold dilution.

     Two of the wells containing water only will serve as blanks.
4. Set a 12-channel P200 pipette to 180 µL.
5. Mix each sample by pipetting up and down 5 to 10 times.

   Be careful not to scratch the bottom of the plate, or to introduce air bubbles.

6. Repeat this procedure and prepare three plates of diluted PCR product to test.

   Be sure to keep two wells as blanks (water only) on each plate.

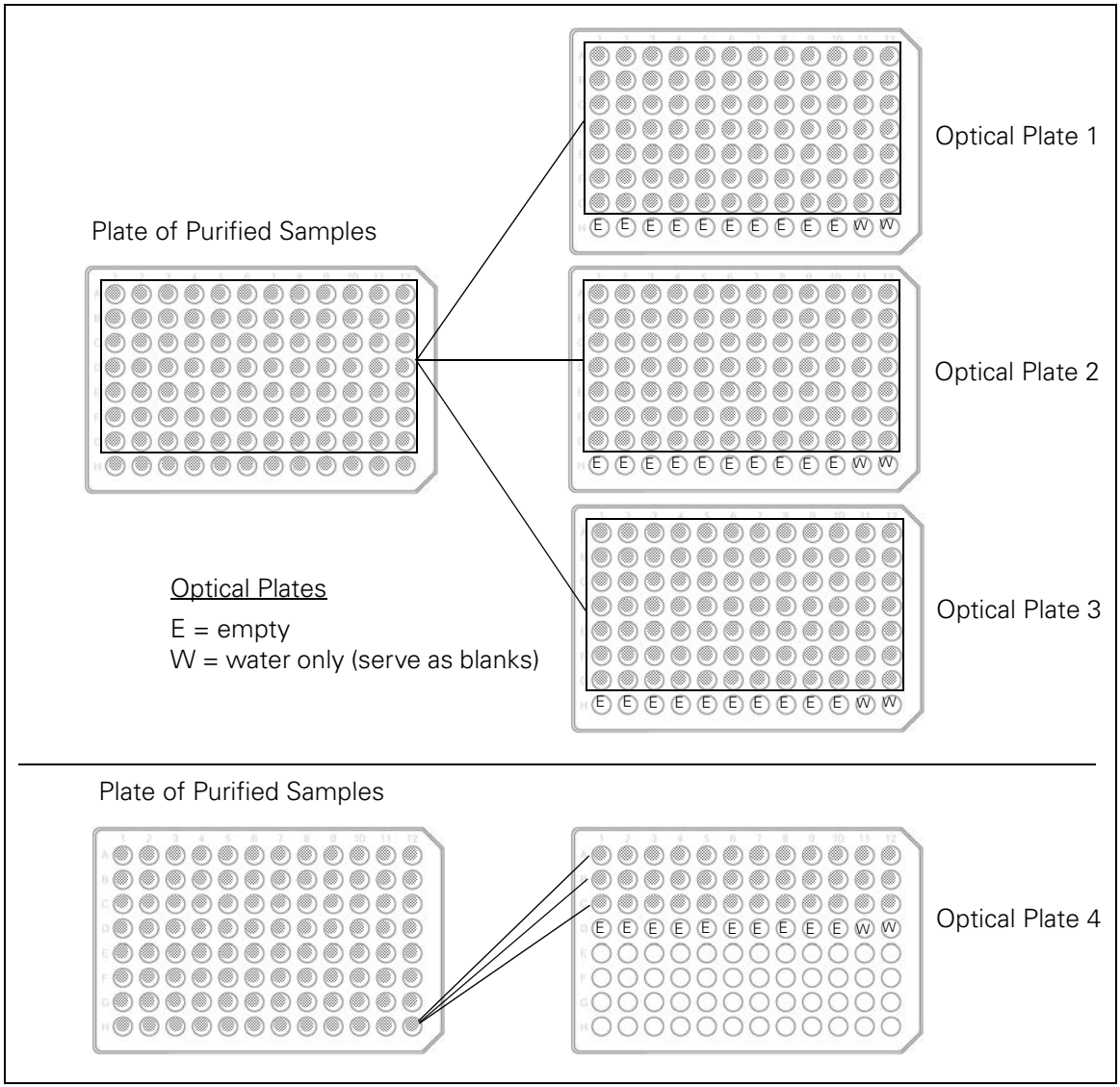


Figure 4.5
Loading Optical Plates with Purified Sample

QUANTITATE THE DILUTED PCR PRODUCT

To quantitate the diluted PCR product:

1. Measure the OD of each PCR product at 260, 280 and 320 nm.

   OD280 and OD320 are used as process controls. Their use is described under *Process Control Metrics* below.

2. Determine the OD260 measurement for the water blank.

3. Determine the concentration of each PCR product as follows:

   A. Take 3 OD readings for every sample (1 from each optical plate; P1, P2, P3 and P4 in Figure 4.5 on page 89).

   OD1 = (sample OD) – (water blank OD)
   OD2 = (sample OD) – (water blank OD)
   OD3 = (sample OD) – (water blank OD)

   B. Average the 3 readings for each sample to obtain an *Average Sample OD*:

   Average Sample OD = (OD1 + OD2 + OD3) ÷ 3

   C. Calculate the undiluted sample concentration for each sample using the Average Sample OD:

   Sample concentration in µg/µL =
   Average Sample OD × (0.05 µg/µL) × 100

   Apply the convention that 1 absorbance unit at 260 nm equals 50 µg/mL (equivalent to 0.05 µg/µL) for double-stranded PCR products. This convention assumes a path length of 1 cm. Consult your spectrophotometer handbook for further information.

ASSESS THE OD READINGS

Follow the guidelines below for assessing and troubleshooting OD readings.

Average Sample OD

A typical average sample OD is 0.5 to 0.7. This OD range is equivalent to a final PCR product concentration of 2.5 to 3.5 µg/µL. It is based on the use of a conventional UV spectrophotometer plate reader and assumes a path length of 1 cm.

Process Control Metrics

Evaluate the process control metrics as follows:

- The OD260/OD280 ratio should be between 1.8 and 2.0.
  Do not proceed if this metric falls outside of this range.
- The OD320 measurement should be very close to zero (0 ± 0.005).

OD Troubleshooting Guidelines

Refer to the tables below when troubleshooting OD readings.

Table 4.23
PROBLEM: Average Sample OD is greater than 0.7 (3.5 µg/µL)

If the average sample OD of three independent measurements is greater than 0.7 (calculated concentration greater than 3.5 µg/µL), a problem exists with either the elution of PCR products or the OD reading. The limit on PCR yield is approximately 3.5 µg/µL, as observed in practice and as predicted by the mass of dNTPs in the reaction.

Possible causes include:

- The purified PCR product was eluted in a volume less than 45 µL.
- The purified PCR product was not mixed adequately before making the 1:100 dilution.
- The diluted PCR product was not mixed adequately before taking the OD reading.
- The water blank reading was not subtracted from each sample OD reading.
- The spectrophotometer plate reader may require calibration.
- Pipettes may require calibration.
- There may be air bubbles or dust in the OD plate.
- There may be defects in the plastic of the plate.
- The settings on the spectrophotometer plate reader or the software may be incorrect.
- OD calculations may be incorrect and should be checked.

Reliance on any single OD reading may give an outlier result. You should make three independent dilutions and take three independent OD readings per dilution.

Table 4.24
PROBLEM: Average Sample OD is Less Than 0.5 (2.5 µg/µL)

If the average sample OD of three independent measurements is less than 0.5 (calculated concentration less than 2.5 µg/µL), a problem exists with either the genomic DNA, the PCR reaction, the elution of purified PCR products, or the OD readings.

Possible problems with input genomic DNA that would lead to reduced yield include:

- The presence of inhibitors (heme, EDTA, etc.).
- Severely degraded genomic DNA.
- Inaccurate concentration of genomic DNA.

  NOTE: Check the OD reading for the PCR products derived from RefDNA 103 as a control for these issues.

To prevent problems with the PCR reaction that would lead to reduced yield:

- Use the recommended reagents and vendors (including AccuGENE® water) for all PCR mix components.
- Thoroughly mix all components before making the PCR Master Mix.
- Pipette all reagents carefully, particularly the PCR Primer, when making the master mix.
- Check all volume calculations for making the master mix.
- Store all components and mixes on ice when working at the bench. Do not allow reagents to sit at room temperature for extended periods of time.
- Be sure to use the recommended PCR plates. Plates from other vendors may not fit correctly in the thermal cycler block. Differences in plastic thickness and fit with the thermal cycler may lead to variance in temperatures and ramp times.
- Be sure to use the correct cycling mode when programming the thermal cycler (*maximum mode* on the GeneAmp® PCR System 9700; *calculated mode* on the MJ Tetrad PTC-225).
- Be sure to use silver or gold-plated silver blocks on the GeneAmp® PCR System 9700 (other blocks are not capable of maximum mode, which will affect ramp times).
- Use the recommended plate seal. Make sure the seal is tight and that no significant evaporation occurs during the PCR.

NOTE: The Mapping 500K PCR reaction amplifies a size range of fragments that represents 15-20% of the genome. The Mapping 500K arrays are designed to detect the SNPs that are amplified in this complex fragment population. Subtle changes in the PCR conditions may not affect the PCR yield, but may shift the amplified size range up or down very slightly. This can lead to reduced amplification of SNPs that are assayed on the array set, subsequently leading to lower call rates.

Table 4.24
PROBLEM: Average Sample OD is Less Than 0.5 (2.5 µg/µL)

Troubleshooting Possible Problems with the Elution or OD Readings – possible causes include:

- The purified PCR product was eluted in a volume greater than 45 µL.
- The purified PCR product was not mixed adequately before making the 1:100 dilution.
- The diluted PCR product was not mixed adequately before taking the OD reading.
- The water blank reading was not subtracted from each sample OD reading.
- The spectrophotometer plate reader may require calibration.
- Pipettes may require calibration.
- There may be air bubbles or dust in the OD plate.
- There may be defects in the plastic of the plate.
- The settings on the spectrophotometer plate reader or the software may be incorrect.
- OD calculations may be incorrect and should be checked.

Reliance on any single OD reading may give an outlier result. You should make three independent dilutions and take three independent OD readings per dilution.

Table 4.25
PROBLEM: OD260/OD280 ratio is not between 1.8 and 2.0

Possible causes include:

- The PCR product may be not be sufficiently purified. Be sure to perform three water washes and check to be sure the vacuum manifold is working properly.
- An error may have been made while taking the OD readings.

Table 4.26
PROBLEM: The OD320 measurement is significantly larger than zero (0 ± 0.005)

Possible causes include:
• Precipitate may be present in the eluted samples. Be sure to add diluted EDTA to PCR products before purification. • There may be defects in the OD plate. • Air bubbles in the OD plate or in solutions.

NORMALIZE THE SAMPLES

To normalize the samples:

1. Calculate the volume of RB Buffer required to normalize each sample.

   Formula X µL RB Buffer = 45 µL – (Y µL purified PCR product)

   Where:

   Y = The volume of purified PCR product that contains 90 µg

   The value of Y is calculated as:
   Y µL purified PCR product = (90 µg) ÷ (Z µg/µL)

   Z = the concentration of purified PCR product in µg/µL

2. Using a single-channel P20 pipette, add the calculated volume of RB Buffer to each well of a new 96-well reaction plate (the value of X).
3. Using a single-channel P100 pipette, add the calculated volume of purified PCR product (the value of Y) to the corresponding well with RB Buffer.

   The total volume of each well is now 45 µL.

   After normalization, each well should contain 90 µg of purified PCR product in a volume of 45 µL (or 2 µg/µL).
4. Seal the plate with adhesive film.
5. Vortex the center of the plate at high speed for 3 sec.

6. Spin down the plate at 2000 rpm for 30 sec and place back in the cooling chamber.

IMPORTANT !

Because the DNA concentration in each sample is different, the volume transferred to each well will differ. For optimal performance, it is critical that the contents of each well be normalized to 2 µg of DNA/µL before proceeding to the next step.

WHAT YOU CAN DO NEXT

Do one of the following:

- Proceed immediately to the next step.
- If not proceeding immediately to the next step:
  - **A.** Seal the plate with the eluted samples.
  - **B.** Store the plate at –20 °C.

Stage 7: Fragmentation

ABOUT THIS STAGE

During this stage the purified, normalized PCR products will be fragmented using Fragmentation Reagent. You will first dilute the Fragmentation Reagent by adding the appropriate amount of Fragmentation Buffer and AccuGENE® water.

You will then quickly add the diluted reagent to each reaction, place the plate onto a thermal cycler, and run the 500K Fragment program.

Once the program is finished, you will check the results of this stage by running 4 µL of each reaction on a 4% TBE gel.

LOCATION AND DURATION

- Main Lab
- Hands-on time: 30 minutes
- *500K Fragment* thermal cycler program time: 1 hour

INPUT REQUIRED FROM PREVIOUS STAGE

The input required from *Stage 6: Quantitation and Normalization* is:

Quantity	Item
1	Plate of quantitated, normalized PCR product in a cooling chamber on ice.

EQUIPMENT AND CONSUMABLES REQUIRED

The following equipment and consumables are required for this stage. Refer to Appendix A, *Reagents, Equipment, and Consumables Required for 96-Well Plate Protocol* for vendor and part number information.

IMPORTANT !

**** Use only the PCR plate, adhesive film and thermal cyclers listed in Table 4.1 on page 40.**

Table 4.27
Equipment and Consumables Required for Stage 7: Fragmentation

Quantity	Item
1	Cooler, chilled to –20 °C
1	Cooling chamber, double, chilled to 4 °C (do not freeze)
1	Ice bucket, filled with ice
1	Marker, fine point, permanent
1	Microcentrifuge
1	Pipette, single channel P20
1	Pipette, single channel P100
1	Pipette, single channel P1000
1	Pipette, 12-channel P20 (accurate to within ± 5%)
As needed	Pipette tips for pipettes listed above; full racks
1	Plate centrifuge
1	Plate seal**
1	Thermal cycler**
2	Tube, Eppendorf 1.5 mL
2	Tubes, strips of 12 cut from the Bio-Rad 96-well unskirted PCR plate, P/N MLP-9601 IMPORTANT For this stage, the strip tubes must be cut from this particular plate.
1	Vortexer

REAGENTS REQUIRED

The following reagents are required for this stage. Refer to Appendix A, *Reagents, Equipment, and Consumables Required for 96-Well Plate Protocol* for vendor and part number information. The amounts listed are sufficient to process one full 96-well reaction plate.

Table 4.28
Reagents Required for Stage 7: Fragmentation

Quantity	Reagent
1 vial	Fragmentation Buffer (10X)
1 vial	Fragmentation Reagent (DNase I)
2 mL	AccuGENE® water, molecular biology-grade

GELS AND RELATED MATERIALS REQUIRED

The following gels and related materials are required for this stage. Refer to Appendix A, *Reagents, Equipment, and Consumables Required for 96-Well Plate Protocol* for vendor and part number information. The amounts listed are sufficient to process one full 96-well reaction plate.

Table 4.29

Quantity	Reagent
5	4% TBE Gel
10	DNA Markers, 5 µL each
As needed	Gel loading solution

IMPORTANT INFORMATION ABOUT THIS STAGE

To help ensure the best results, carefully read the information below before you begin this stage of the protocol.

IMPORTANT !

- Purified PCR product must be normalized to 90 µg DNA in 45 µL RB Buffer.
- The degree of fragmentation is critical. Perform this stage carefully to ensure uniform, reproducible fragmentation.
- The Fragmentation Reagent is extremely temperature sensitive. It rapidly loses activity at higher temperatures. To avoid loss of activity:
  - Dilute the Fragmentation Reagent immediately prior to use.
  - Keep at –20 °C until ready to use. Transport and hold in a –20 °C cooler. Return to the cooler immediately after use.
  - Perform these steps rapidly and without interruption.
- The Fragmentation Reagent (DNase I) may adhere to the walls of some microfuge tubes and 96-well plates. To ensure the accurate amount of DNase I in the fragmentation reaction (*Stage 7: Fragmentation*), the strip tubes used for this stage must be cut from Bio-Rad 96-well unskirted PCR plates, P/N MLP-9601. See *Cutting Strip Tubes From Plates* on page 41 and Table 4.1 on page 40 for more information on these plates.
- The Fragmentation Reagent is viscous and requires extra care when pipetting. Follow these guidelines:
  - When aspirating, allow enough time for the correct volume of solution to enter the pipette tip.
  - Avoid excess solution on the outside of the pipette tip.
- Be sure to use the AccuGENE® water listed in Appendix A. Using in-house ddH_2O or other water can negatively affect your results. The reaction in *Stage 7: Fragmentation* is particularly sensitive to pH and metal ion contamination.
- All additions, dilutions and mixing must be performed on ice.

PREPARE THE REAGENTS, CONSUMABLES AND OTHER COMPONENTS

Thaw Reagents

Thaw the Fragmentation Buffer (10X) on ice.

IMPORTANT **Leave the Fragmentation Reagent at –20 °C until ready to use.**

Prepare Your Work Area

To prepare the work area (Figure 4.6 on page 101):

1. Place a double cooling chamber and a cooler on ice.
2. Place the AccuGENE® Water on ice.
3. Prepare the Fragmentation Buffer as follows:
   - **A.** Vortex 3 times, 1 sec each time.
   - **B.** Pulse spin for 3 sec.
   - **C.** Place the buffer in the cooling chamber on ice.
4. Cut two strips of 12 tubes from a Bio-Rad 96-well unskirted PCR plate (P/N MLP-9601).

   Strip tubes must be cut from this particular plate. See *Important Information About This Stage* on page 99 for more information.
5. Label and place the following in the cooling chamber on ice:
   - Two strips of 12 tubes labeled *Buffer* and *FR*.
   - One 1.5 mL Eppendorf tube labeled *Frag MM*.
   - Plate of purified, normalized PCR product from the previous stage.

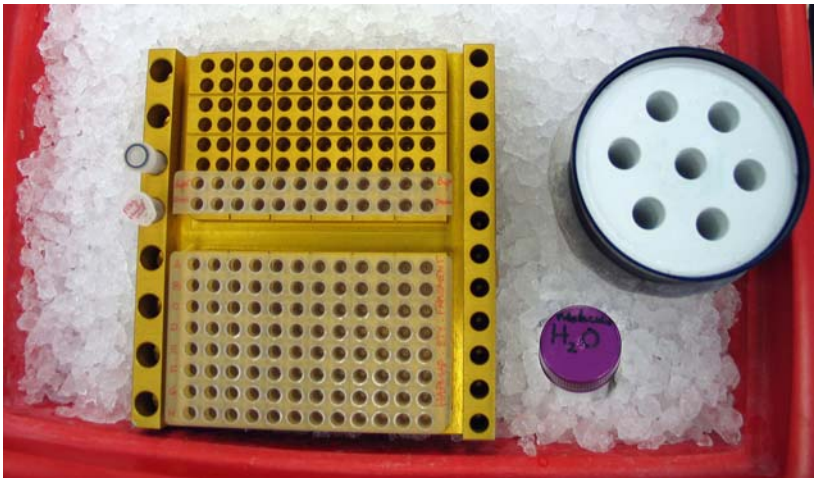


Figure 4.6
Work Area Set Up for Stage 7: Fragmentation

Preheat the Thermal Cycler Block

The block must be heated to 37 °C before samples are loaded.

To preheat the thermal cycler:

1. Power on the thermal cycler and preheat the block to 37 °C.
2. Allow it to heat for 10 minutes before loading samples.

PREPARE THE SAMPLES FOR FRAGMENTATION

Add Fragmentation Buffer to Samples

IMPORTANT !

All of the additions in this procedure must be performed on ice.

To prepare the samples for Fragmentation:

1. Aliquot 50 µL of 10X Fragmentation Buffer to each tube of the 12-tube strip labeled Buffer.
2. Using a 12-channel P20 pipette, add 5 µL of Fragmentation Buffer to each sample in the 96-well reaction plate.

   Check your pipette tips each time to ensure that all of the buffer has been dispensed.

   The total volume in each well is now 50 µL.

Dilute the Fragmentation Reagent

IMPORTANT !

The concentration of stock Fragmentation Reagent (U/µL) may vary from lot-to-lot. Therefore, read the label on the tube and record the stock concentration before diluting this reagent. Use the formula provided to accurately calculate the dilution required.

To dilute the Fragmentation Reagent:

1. Read the Fragmentation Reagent tube label and record the concentration.

Table 4.30
Dilution Recipes for Fragmentation Reagent Concentrations of 2 and 3 U/µL

Reagent	Fragmentation Reagent Concentration	
	2 U/µL	3 U/µL
AccuGENE® water	525 µL	530 µL
Fragmentation Buffer	60 µL	60 µL
Fragmentation Reagent	15 µL	10 µL
Total (enough for 96 samples)	**600 µL**	**600 µL**

2. If the concentration is 2 or 3 U/µL, dilute the Fragmentation Reagent using the volumes show in Table 4.30 above.
3. If the concentration is not 2 or 3 U/µL, use the formula below to calculate the dilution required to bring the reagent to a final concentration of 0.05 U/µL.

Formula $Y = \frac{0.05\ U/\mu L * 600\ \mu L}{X\ U/\mu L}$

Where:

- Y = number of µL of stock Fragmentation Reagent
- X = number of U of stock Fragmentation Reagent per µL (per label on tube)
- 0.05 U/µL = final concentration of Fragmentation Reagent
- 600 µL = final volume of diluted Fragmentation Reagent (enough for 96 reactions)

4. Dilute the Fragmentation Reagent to 0.05 U/µL as follows using the recipes in Table 4.30 or the dilution formula calculation:

   A. To the 1.5 mL Eppendorf tube on ice:

   1) Add the AccuGENE® water and Fragmentation Buffer.

2) Allow to cool on ice.

B. Remove the Fragmentation Reagent from the freezer and:

1) Immediately pulse spin for 3 sec.

Spinning is required because the Fragmentation Reagent tends to cling to the top of the tube, making it warm quicker.

2) Immediately place in a cooler.

C. Add the Fragmentation Reagent to the 1.5 mL Eppendorf tube.

D. Vortex the diluted Fragmentation Reagent at high speed 3 times, 1 sec each time.

E. Pulse spin for 3 sec and immediately place on ice.

5. Proceed immediately to the next set of steps, *Add Diluted Fragmentation Reagent to the Samples*.

Add Diluted Fragmentation Reagent to the Samples

To add diluted Fragmentation Reagent to the samples:

1. Quickly and on ice, aliquot 50 µL of diluted Fragmentation Reagent to each tube of the 12 tube strip labeled *FR*.

2. Using a 12-channel P20 pipette, add 5 µL of diluted Fragmentation Reagent to each sample.

Do not pipette up and down.

Reagent	Volume/Sample
Sample with Fragmentation Buffer	50 µL
Diluted Fragmentation Reagent (0.05 U/µL)	5 µL
Total	**55 µL**

3. Seal the plate and inspect the edges to ensure that it is tightly sealed.

IMPORTANT !

To minimize solution loss due to evaporation, make sure that the plate is tightly sealed prior to loading onto the thermal cycler. The MJ thermal cyclers are more prone to evaporation.

4. Vortex the center of the plate at high speed for 3 sec.
5. Place the plate in a chilled plastic plate holder and spin it down at 4 °C at 2000 rpm for 30 sec.
6. Immediately load the plate onto the pre-heated block of the thermal cycler (37 °C) and run the *500K Fragment* program.

500K Fragment Program	
Temperature	**Time**
37°C	35 minutes
95°C	15 minutes
4°C	Hold

7. Discard any remaining diluted Fragmentation Reagent. Diluted Fragmentation Reagent should never be reused.

WHAT YOU CAN DO NEXT

Proceed directly to the next stage. Concurrently, check the fragmentation reaction by running gels as described below.

CHECK THE FRAGMENTATION REACTION

To ensure that fragmentation was successful:

1. When the 500K Fragment program is finished:
   - **A.** Remove the plate from the thermal cycler.
   - **B.** Spin down the plate at 2000 rpm for 30 sec, and place in a cooling chamber on ice.
2. Dilute 4 µL of each fragmented PCR product with 4 µL gel loading dye.
3. Run on 4% TBE gel with the BioNexus All Purpose Hi-Lo ladder at 120V for 30 minutes to 1 hour.
4. Inspect the gel and compare it against the example shown in Figure 4.7 below.

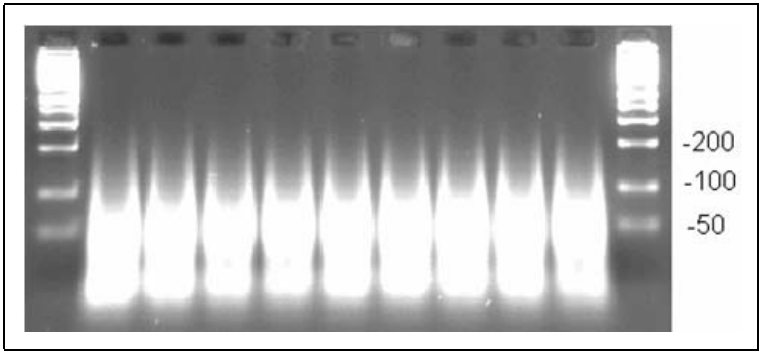


Figure 4.7
Typical example of fragmented PCR products run on 4% TBE agarose gel at 120V for 30 minutes to 1 hour. Average fragment size is < 180 bp.

Stage 8: Labeling

ABOUT THIS STAGE

During this stage, the fragmented samples will be labeled using the GeneChip® DNA Labeling Reagent.

You will prepare the Labeling Master Mix, add the mix to each sample, place the samples onto a thermal cycler and run the *500K Label* program.

LOCATION AND DURATION

- Main Lab
- Hands-on time: 30 minutes
- *500K Label* thermal cycler program time: 4.25 hours

INPUT REQUIRED FROM PREVIOUS STAGE

The input required from *Stage 7: Fragmentation* is:

Quantity	Item
1	Plate of fragmented DNA

EQUIPMENT AND CONSUMABLES REQUIRED

The following equipment and consumables are required for this stage. Refer to Appendix A, *Reagents, Equipment, and Consumables Required for 96-Well Plate Protocol* for vendor and part number information.

IMPORTANT !

**** Use only the PCR plate, adhesive film and thermal cyclers listed in Table 4.1 on page 40.**

Table 4.31
Equipment and Consumables Required for Stage 8: Labeling

Quantity	Item
1	Cooler, chilled to –20 °C
1	Cooling chamber, double, chilled to 4 °C (do not freeze)
1	Ice bucket, filled with ice
1	Marker, fine point, permanent
1	Microcentrifuge
1	Pipette, single channel P200
1	Pipette, single channel P1000
1	Pipette, 12-channel P20 (accurate to within ± 5%)
As needed	Pipette tips for pipettes listed above; full racks
1	Plate centrifuge
1	Plate seal**
1	Thermal cycler**
1	Tube, centrifuge 15 mL
1	Tubes, strip of 12
1	Vortexer

REAGENTS REQUIRED

The following reagents are required for this stage. Refer to Appendix A, *Reagents, Equipment, and Consumables Required for 96-Well Plate Protocol* for vendor and part number information. The amounts listed are sufficient to process one full 96-well reaction plate.

Table 4.32
Reagents Required for Stage 8: Labeling

Quantity	Reagent
1 vial	GeneChip® DNA Labeling Reagent (30 mM)
1 vial	Terminal Deoxynucleotidyl Transferase (TdT; 30 U/µL)
2 vials	Terminal Deoxynucleotidyl Transferase Buffer (TdT Buffer; 5X)

IMPORTANT INFORMATION ABOUT THIS STAGE

To help ensure the best results, carefully read the information below before you begin this stage of the protocol.

IMPORTANT !

- To minimize sample loss due to evaporation, be sure that the plate is tightly sealed before running the *500K Label* thermal cycler program.

PREPARE THE REAGENTS, CONSUMABLES AND OTHER COMPONENTS

Thaw Reagents

Thaw the following reagents on ice:

- 5X TdT Buffer
- GeneChip® DNA Labeling Reagent

IMPORTANT !

Leave the TdT enzyme at –20 °C until ready to use.

Prepare Your Work Area

To prepare the work area:

1. Place a double cooling chamber and a cooler on ice.
2. Prepare the reagents as follows:
   A. Vortex each reagent at high speed 3 times, 1 sec each time.
   B. Pulse spin for 3 sec.
   C. Place in the cooling chamber.
3. Label and place the following in the cooling chamber:
   - One strip of 12 tubes labeled *MM*
   - One 15 mL centrifuge tube labeled *MM*
   - Plate of fragmented reactions from the previous stage

Preheat the Thermal Cycler Block

The block must be heated to 37 °C before samples are loaded.

To preheat the thermal cycler block:

1. Turn on the thermal cycler and preheat the block to 37 °C.
2. Allow it to heat for 10 minutes before loading samples.

PREPARE THE LABELING MASTER MIX

Preparation

Keep all reagents and tubes on ice while preparing the Labeling Master Mix.

To prepare the Labeling Master Mix:

1. Add the following to the 15 mL centrifuge tube on ice using the volumes shown in Table 4.33:
   - 5X TdT Buffer
   - GeneChip® DNA Labeling Reagent
2. Remove the TdT enzyme from the freezer and immediately place in the cooler.

3. Pulse spin the enzyme for 3 sec; then immediately place back in the cooler.
4. Add the TdT enzyme to the master mix.
5. Vortex the master mix at high speed 3 times, 1 sec each time.
6. Pulse spin for 3 sec.
7. Immediately proceed to the next set of steps, *Add the Labeling Master Mix to the Samples*.

Table 4.33
Labeling Master Mix

Reagent	1 Sample	96 Samples (15% extra)
TdT Buffer (5X)	14 µL	1545.6 µL
GeneChip® DNA Labeling Reagent (30 mM)	2 µL	220.8 µL
TdT enzyme (30 U/µL)	3.5 µL	386.4 µL
Total	19.5 µL	2152.8 µL

ADD THE LABELING MASTER MIX TO THE SAMPLES

To add the Labeling Master Mix to the samples:

Keep samples in the cooling chamber and all tubes on ice when making additions.

1. Aliquot 178 µL of Labeling Master Mix to each tube of the strip tubes.
2. Add the Labeling Master Mix as follows:
   - **A.** Using a 12-channel P20 pipette, aliquot 19.5 µL of Labeling Master Mix to each sample.
   - **B.** Pipette up and down one time to ensure that all of the mix is added to the samples.

     The total volume in each well is now 70 µL.

Reagent	Volume/Rx
Fragmented DNA (less the 4 µL used for gel analysis)	50.5 µL
Labeling Mix	19.5 µL
Total	**70 µL**

3. Seal the plate tightly with adhesive film.

IMPORTANT !

Check to ensure that the plate is tightly sealed, particularly around the wells on the edge of the plate. The plate must be tightly sealed to minimize evaporation while on the thermal cycler.

4. Vortex the center of the plate at high speed for 3 sec.
5. Spin down the plate at 2000 rpm for 30 sec.
6. Place the plate on the pre-heated thermal cycler block, and run the *500K Label* program.

   Samples can remain at 4 °C overnight.

500K Label Program	
Temperature	**Time**
37°C	4 hours
95°C	15 minutes
4°C	Hold
Samples can remain at 4 °C overnight.	

7. When the 500K Labeling program is finished:

   A. Remove the plate from the thermal cycler.

   B. Spin down the plate at 2000 rpm for 30 sec.

WHAT YOU CAN DO NEXT

Do one of the following:

- Proceed to the next stage.
- If not proceeding directly to the next stage, freeze the samples at –20 °C.

Stage 9: Target Hybridization

ABOUT THIS STAGE

During this stage, each sample is loaded onto either a GeneChip® Human Mapping 250K Sty Array or a 250K Nsp Array. Three methods for performing this stage are presented.

- Method 1 — Using a GeneAmp® PCR System 9700

  Requires the use of a GeneAmp® PCR System 9700 located adjacent to the hybridization ovens. Samples are on a 96-well reaction plate. See *Method 1 — Using a GeneAmp® PCR System 9700* on page 124.

- Method 2 — Using an Applied Biosystems 2720 Thermal Cycler or an MJ Tetrad PTC-225 Thermal Cycler.

  Requires the use of an Applied Biosystems 2720 Thermal Cycler or an MJ Tetrad PTC-225 Thermal Cycler located adjacent to the hybridization ovens. Samples are on a 96-well reaction plate. See *Method 2 — Using an Applied Biosystems 2720 Thermal Cycler or an MJ Tetrad PTC-225 Thermal Cycler* on page 127.

- Method 3 — Using Heat Blocks

  Requires the use of two heat blocks and Eppendorf tubes, one per sample. See *Hybridizing Samples Using Heat Blocks* on page 130.

First, you will prepare a Hybridization Master Mix and add the mix to each sample. Then, based on the method you are using, you will denature the samples on a thermal cycler (methods 1 and 2) or on a heat block (method 3).

After denaturation, you will load each sample onto the appropriate GeneChip Human Mapping 250K Array (Nsp or Sty) – one sample per array. The arrays are then placed into a hybridization oven that has been preheated to 49 °C. Samples are left to hybridize for 16 to 18 hours.

Two operators are required for all of the methods.

LOCATION AND DURATION

- Main Lab
- Hands-on time: approximately 2 hours
- Hybridization time: 16 to 18 hours

INPUT REQUIRED FROM PREVIOUS STAGE

The input required from *Stage 8: Labeling* is:

Quantity	Item
1	Plate of labeled DNA

EQUIPMENT AND CONSUMABLES REQUIRED

The following equipment and consumables are required for this stage. Refer to Appendix A, *Reagents, Equipment, and Consumables Required for 96-Well Plate Protocol* for vendor and part number information.

IMPORTANT !

Increased variability in Mapping 500K performance has been observed in GeneChip® Hybridization Oven 640 models (P/N 800138 or 800189) manufactured prior to 2001. Check the serial number of your hybridization oven(s). If the serial numbers are 11214 or lower, contact Affymetrix for an upgrade.

Hybridizing Samples Using a Thermal Cycler

The following table lists the equipment and consumables required if hybridizing samples using a thermal cycler.

Table 4.34
Equipment and Consumables Required for Stage 9: Target Hybridization Using a Thermal Cycler

Quantity	Item
1	Cooling chamber, chilled to 4 °C (do not freeze)
96	GeneChip® 250K Arrays (one array per sample)
1	GeneChip® Hybridization Oven 640
1	Ice bucket, filled with ice
1	Pipette, single channel P200
1	Pipette, single channel P1000
As needed	Pipette tips for pipettes listed above; full racks
1	Plate, Bio-Rad 96-well, P/N MLP-9601**
1	Plate centrifuge
2	Plate holders, centrifuge
1	Plate seal**
1	Solution basin, 55 mL
1	Thermal cycler** See About this Stage on page 114.
2 per array	Tough-Spots®
1	Tube, centrifuge 50 mL
1	Vortexer

IMPORTANT !

**** Use only the PCR plate, adhesive film and thermal cyclers listed in Table 4.1 on page 40.**

Hybridizing Samples Using Heat Blocks

The following table lists the equipment and consumables required if hybridizing samples using heat blocks.

Table 4.35
Equipment and Consumables Required for Stage 9: Target Hybridization Using Heat Blocks

Quantity	Item
1	Cooling chamber, chilled to 4 °C (do not freeze)
96	• GeneChip® Human Mapping 250K Sty Array or • GeneChip® Human Mapping 250K Nsp Array One array per sample is required.
1	GeneChip® Hybridization Oven 640
2	Heat block
1	Ice bucket, filled with ice
1	Pipette, single channel P200
1	Pipette, single channel P1000
As needed	Pipette tips for pipettes listed above; full racks
1	Plate centrifuge
1	Plate seal**
1	Solution basin, 55 mL
4	Timers
1	Tube, centrifuge 50 mL
96	Tube, Eppendorf® Safe-Lock, 1.5 mL (one tube per sample)
2 per array	Tough-Spots®
1	Vortexer

REAGENTS REQUIRED

The following reagents are required for this stage. Refer to Appendix A, *Reagents, Equipment, and Consumables Required for 96-Well Plate Protocol* for vendor and part number information. The amounts listed are sufficient to process one full 96-well reaction plate.

Table 4.36
Reagents Required for Stage 9: Target Hybridization

Quantity	Reagent
5 mL (1 tube)	Denhart's Solution
1.5 mL (1 tube)	DMSO
0.5 mL (1 vial)	EDTA
1 mL (1 vial)	Herring Sperm DNA (HSDNA)
500 µL (1 vial)	Human Cot-1 DNA®
80 g	MES Hydrate SigmaUltra
200 g	MES Sodium Salt
16 mL (1 tube)	Tetramethyl Ammonium Chloride (TMACL; 5M)
10 mL (1 vial)	Tween-20, 10%
250 µL (1 vial)	Oligo Control Reagent (OCR), 0100

IMPORTANT INFORMATION ABOUT THIS STAGE

To help ensure the best results, carefully read the information below before you begin this stage of the protocol.

IMPORTANT !

- This procedure requires two operators working simultaneously when loading samples onto arrays and placing arrays in the hybridization ovens.
- If using a thermal cycler, it is critical that the samples remain at 49 °C after denaturation and while being loaded onto arrays. If you have a GeneAmp PCR System 9700 located adjacent to the hybridization ovens, we recommend using method 1. Otherwise,

you must use method 2 or method 3 (see *About this Stage* on page 114).

- About DMSO:
  - When adding to the Hybridization Master Mix, pipette DMSO into the middle of the tube. Do not touch the sides of the tube as the DMSO can leach particles out of the plastic which, in turn, may cause high background.
  - DMSO is light sensitive and must be stored in a dark glass bottle. Do not store in a plastic container.
- Be sure to allow the arrays to equilibrate to room temperature; otherwise, the rubber septa may crack and the array may leak.
- An accurate hybridization temperature is critical for this assay. Therefore, we recommend that your hybridization ovens be serviced at least once per year to ensure that they are operating within manufacture specifications.
- Gloves, safety glasses, and lab coats must be worn when preparing the Hybridization Master Mix.
- Consult the appropriate MSDS for reagent storage and handling requirements.

PREPARE THE REAGENTS, CONSUMABLES AND OTHER COMPONENTS

Prepare a 12X MES Stock Solution

The 12X MES stock solution can be prepared in bulk and kept for at least one month if properly stored. Proper storage:

- Protect from light using aluminum foil
- Keep at 4 °C

IMPORTANT !

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

To prepare 1000 mL of 12X MES Stock Solution:
(1.25 M MES, 0.89 M [Na^+])

1. Combine:
   - 70.4 g MES hydrate
   - 193.3 g MES sodium salt
   - 800 mL AccuGENE® water
2. Mix and adjust volume to 1,000 mL.
3. Test the pH.
   The pH should be between 6.5 and 6.7.
4. Filter the solution through a 0.2 µm filter.
5. Protect from light using aluminum foil and store at 4 °C.

Preheat the Hybridization Ovens

To preheat the hybridization ovens:

1. Turn each oven on and set the temperature to 49 °C.
2. Set the rpm to 60.
3. Turn the rotation on and allow to preheat for 1 hour before loading arrays.

IMPORTANT !

An accurate hybridization temperature is critical for this assay. Therefore, we recommend that your hybridization ovens be serviced at least once per year to ensure that they are operating within manufacture specifications.

Thaw Reagents

If the labeled samples from the previous stage were frozen:

1. Thaw the plate on the bench top.
2. Vortex the center of the plate at high speed for 3 sec.
3. Spin down the plate at 2000 rpm for 30 sec.
4. Place in a cooling chamber on ice.
5. If hybridizing samples using Method 1 or 2, the labeled samples must be placed in a Bio-Rad unskirted 96-well plate (P/N MLP-9601). For Method 2, the plate will be cut into 4 strips of 24 wells each.

Preheat the Thermal Cycler Lid

A thermal cycler is required only if you are hybridizing samples using Method 1 or 2. See *Hybridizing Samples Using a Thermal Cycler* on page 124.

Power on the thermal cycler to preheat the lid. Leave the block at room temperature.

PREHEAT THE HEAT BLOCKS

Heat blocks are required only if you are hybridizing samples using Method 3. See *Hybridizing Samples Using Heat Blocks* on page 130.

To prepare the heat blocks:

1. Turn on both heat blocks.
2. Preheat one to 99 °C.
3. Preheat the other to 49 °C.

PREPARE THE ARRAYS

To prepare the arrays:

1. Unwrap the arrays and place on the bench top, septa-side up.
2. Mark each array with a meaningful designation (e.g., a number) to ensure that you know which sample is loaded onto each array.
3. Allow the arrays to warm to room temperature by leaving on the bench top 10 to 15 minutes.
4. Insert a 200 µL pipette tip into the upper right septum of each array.

WARNING ▲ **To ensure that the data collected during scanning is associated with the correct sample, number the arrays in a meaningful way. It is critical that you know which sample is loaded onto each array.**

PREPARE THE HYBRIDIZATION MASTER MIX

As an option, you can prepare a larger volume of Hybridization Master Mix than required. The extra mix can be aliquoted and stored at –20 °C for up to one week.

Preparing Fresh Hybridization Master Mix

To prepare the Hybridization Master Mix:

1. To the 50 mL centrifuge tube, add the reagents in the order shown in Table 4.37.

   DMSO addition: pipette directly into the solution of other reagents. Avoid pipetting along the side of the tube.
2. Mix well.
3. If making a larger volume, aliquot out 20.9 mL, and store the remainder at –20 °C for up to one week.

Table 4.37
Hybridization Master Mix

Reagent	1 Array	96 Arrays (15% extra)
MES (12X; 1.25 M)	12 µL	1320 µL
Denhardt's Solution (50X)	13 µL	1430 µL
EDTA (0.5 M)	3 µL	330 µL
HSDNA (10 mg/mL)	3 µL	330 µL
OCR, 0100	2 µL	220 µL
Human Cot-1 DNA® (1 mg/mL)	3 µL	330 µL
Tween-20 (3%)	1 µL	110 µL
DMSO (100%)	13 µL	1430 µL
TMACL (5 M)	140 µL	1540 mL
Total	**190 µL**	**20.9 mL**

Using Premixed Hybridization Master Mix

Hybridization Master Mix can be made ahead of time, aliquoted and stored for 1 week.

To prepare stored Hybridization Master Mix:

1. Place the stored Hybridization Master Mix on the bench top, and allow to warm to room temperature.
2. Vortex at high speed until the mixture is homogeneous and without precipitates (up to 5 minutes).
3. Pulse spin for 3 sec.

HYBRIDIZING SAMPLES USING A THERMAL CYCLER

Method 1 — Using a GeneAmp® PCR System 9700

The thermal cycler used for this method must be a GeneAmp PCR System 9700 located adjacent to the hybridization ovens. This particular thermal cycler is required because of the way the lid operates. You can slide it back one row at a time as samples are loaded onto arrays. Keeping the remaining rows covered prevents condensation in the wells.

Add Hybridization Master Mix and Denature the Samples

To add Hybridization Master Mix and denature the samples:

1. Pour 20.9 mL Hybridization Master Mix into a solution basin.
2. Using a 12-channel P200 pipette, add 190 µL of Hybridization Master Mix to each sample on the Label Plate.

   Total volume in each well is 260 µL.
3. IMPORTANT Seal the plate tightly with adhesive film.
4. Vortex the center of the plate for 3 minutes.
5. Spin down the plate at 2000 rpm for 30 sec.
6. Cut the adhesive film between each row of samples.

   Do not remove the film.
7. Place the plate onto the thermal cycler and close the lid.
8. Run the *500K Hyb* program.

500K Hyb Program	
Temperature	**Time**
95°C	10 minutes
49°C	Hold

Load the Samples onto Arrays

This procedure requires 2 operators working simultaneously. Operator 1 loads the samples onto the arrays; Operator 2 covers the septa with Tough-Spots and loads the arrays into the hybridization ovens.

To load the samples onto arrays:

Operator 1 Tasks

1. When the plate reaches 49 °C, slide back the lid on the thermal cycler enough to expose the first row of samples only.
2. Remove the film from the first row.
3. Using a single-channel P200 pipette, remove 200 µL of denatured sample from the first well.
4. Immediately inject the sample into an array.
5. Pass the array to Operator 2.

   NOTE: The tasks for Operator 2 are listed below.
6. Remove 200 µL of sample from the next well and immediately inject it into an array.
7. Pass the array to Operator 2.
8. Repeat this process one sample at a time until the entire row is loaded.
9. Place a fresh strip of adhesive film over the completed row.
10. Slide the thermal cycler lid back to expose the next row of samples.
11. Repeat steps 3 through 10 until all of the samples have been loaded onto arrays.

Operator 2 Tasks

1. Cover the septa on each array with a Tough-Spot (Figure 4.8).
2. For every 4 arrays:
   - **A.** Load the arrays into an oven tray evenly spaced.
   - **B.** Immediately place the tray into the hybridization oven.

     Do not allow loaded arrays to sit at room temperature for more than approximately 1.5 minute. Ensure that the oven is balanced as the trays are loaded, and ensure that the trays are rotating at 60 rpm at all times.

     Because you are loading 4 arrays per tray, each hybridization oven will have a total of 32 arrays.

Operators 1 and 2

- Load no more than 32 arrays in one hybridization oven at a time.
- All 96 samples should be loaded within 1 hour.
- Store the remaining samples and any samples not yet hybridized in a tightly sealed plate at -20 °C.
- Allow the arrays to rotate at 49 °C, 60 rpm for 16 to 18 hours.

IMPORTANT !

Allow the arrays to rotate in the hybridization ovens for 16 to 18 hours at 49°C and 60 rpm. This temperature is optimized for this product, and should be stringently followed.

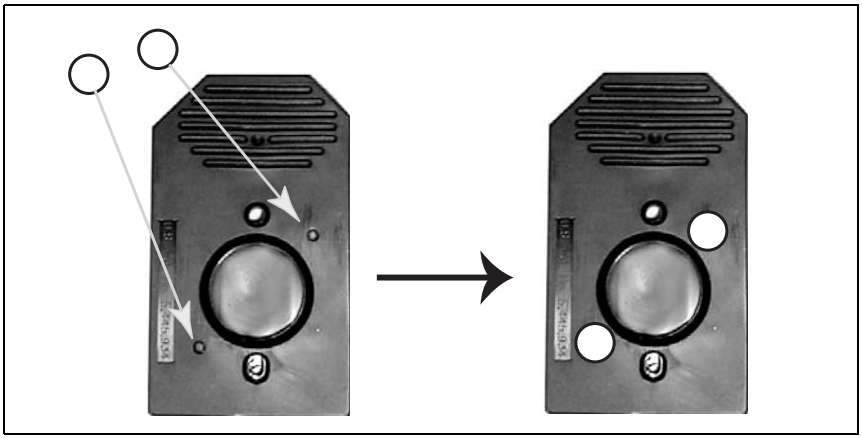


Figure 4.8
Applying Tough-Spots® to the array cartridge

Method 2 — Using an Applied Biosystems 2720 Thermal Cycler or an MJ Tetrad PTC-225 Thermal Cycler

For this method, you can use an Applied Biosystems 2720 Thermal Cycler or an MJ Tetrad PTC-225 Thermal Cycler. The thermal cycler must be located adjacent to the hybridization ovens. Because the lids on these thermal cyclers do not slide back, you will process 24 samples at a time.

Add Hybridization Master Mix and Denature

To add Hybridization Master Mix and denature the samples:

1. Pour 20.9 mL Hybridization Master Mix into a solution basin.
2. Using a 12-channel P200 pipette, add 190 µL of Hybridization Master Mix to each sample on the Label Plate.

   Total volume in each well is 260 µL.
3. IMPORTANT Seal the plate tightly with adhesive film.
4. Vortex the center of the plate for 3 minutes.
5. Cut the plate into 4 strips of two rows each.
6. Put each strip of 24 samples into a plate holder, 2 strips per holder.
7. Spin down the strips at 2000 rpm for 30 sec.

8. Cut the adhesive film between each row of samples.
   Do not remove the film.
9. Place one set of 24 wells onto the thermal cycler and close the lid.
10. Keep the remaining sets of wells in a cooling chamber on ice.
11. Run the *500K Hyb* program.

500K Hyb Program	
Temperature	**Time**
95°C	10 minutes
49°C	Hold

Load the Samples onto Arrays

This procedure requires 2 operators working simultaneously. Operator 1 loads the samples onto the arrays; Operator 2 covers the septa with Tough-Spots and loads the arrays into the hybridization ovens.

To load the samples onto arrays:

Operator 1 Tasks

1. When the plate reaches 49 °C, open the lid on the thermal cycler.
2. Remove the film from the first row.
3. Using a single-channel P200 pipette, remove 200 µL of denatured sample from the first well.
4. Immediately inject the sample into an array.
5. Pass the array to Operator 2.
   NOTE: The tasks for Operator 2 are listed below.
6. Remove 200 µL of denatured sample and immediately inject it into an array.
7. Pass the array to Operator 2.
8. Repeat this process one sample at a time until all 24 samples are loaded onto arrays.

9. Cover the wells with a fresh strip of adhesive film and place in the cooling chamber on ice.
10. Remove the next strip of 24 wells and place it on the thermal cycler.
11. Run the *500K Hyb* program.
12. Repeat steps 1 through 11 until all of the samples have been loaded onto arrays.

Operator 2 Tasks

1. Cover the septa on each array with a Tough-Spot (Figure 4.8).
2. When 4 arrays are loaded and the septa are covered:
   - **A.** Load the arrays into an oven tray evenly spaced.
   - **B.** Immediately place the tray into the hybridization oven.

     Do not allow loaded arrays to sit at room temperature for more than approximately 1 minute. Ensure that the oven is balanced as the trays are loaded, and ensure that the trays are rotating at 60 rpm at all times.

     Because you are loading 4 arrays per tray, each hybridization oven will have a total of 32 arrays.

Operators 1 and 2

- Load no more than 32 arrays in one hybridization oven at a time.
- All 96 samples should be loaded within 1 hour.
- Store the remaining samples and any samples not yet hybridized in a tightly sealed plate at -20 °C.
- Allow the arrays to rotate at 49 °C, 60 rpm for 16 to 18 hours.

HYBRIDIZING SAMPLES USING HEAT BLOCKS

About this Procedure

The following instructions require 2 operators working simultaneously, each processing two samples at a time. Batches of sixteen samples at a time are denatured and loaded onto arrays.

Two heat blocks are required: one set to 99 °C; the other set to 49 °C.

Load Samples Onto a Heat Block

1. If the heat blocks are not turned on, preheat them now (set one to 99 °C; the other to 49 °C).
2. Add 190 µL of Hybridization Master Mix to each 1.5 mL Eppendorf Safe-Lock tube.
3. Transfer the labeled sample from the reaction plate to an Eppendorf tube containing Hybridization Master Mix (one sample per tube).

   The total volume is now 260 µL.

Reagent	Volume/Sample
Hybridization Master Mix	190 µL
Labeled DNA	70 µL
Total	260 µL

4. Vortex at high speed 3 times, 1 sec each time.
5. Pulse spin for 3 sec.
6. Do one of the following:
   - If denaturing and loading samples onto arrays now, place the tubes on ice.
   - If not proceeding to denature and hybridization at this time, store the samples at –20 °C (the mix will not freeze).
7. Place the tubes in batches of 16 at a time onto a heat block as follows:

A. Place four tubes onto a heat block at 99 °C and set a timer for 10 minutes.

B. Wait 3 to 4 minutes, then place another 4 tubes onto the heat block and set another timer for 10 minutes.

C. Repeat this procedure until there are 16 samples loaded onto the heat block.

Remove Samples from Heat Block and Load Onto Arrays

Two operators will perform this procedure at the same time, two samples per person.

To load samples onto arrays, 16 samples at a time:

1. When the first timer indicates 10 minutes has transpired:
   A. Immediately remove the first samples (two per operator).
   B. Cool on crushed ice for 10 sec, then remove immediately.

IMPORTANT !

Cool for 10 sec only. If left on ice longer, aggregates may form. These aggregates will not break apart at 49 °C and will reduce your call rate.

Cooling on ice is required for this method only due to the loose fit of the tubes on the heat blocks. This step helps to ensure that the samples cool quickly to 49 °C.

2. Pulse spin for 3 sec.
3. Place the tubes back on the heat block at 49 °C for 1 minute.
4. Remove tubes from the heat block, and check for precipitate.
5. Using a single-channel P200 pipette, remove 200 µL of denatured sample from one tube.
6. Immediately inject the sample into an array.
7. Cover each septa with a Tough-Spot (Figure 4.8 on page 127).
8. Repeat steps 5 through 7 for the next sample.
9. Immediately load the arrays into a hybridization oven tray, 4 arrays per tray evenly spaced.

10. Place the trays in the oven, 32 arrays per oven.

    Do not allow arrays to sit on the bench top for more than 1 minute after the sample has been loaded. Ensure that the oven is balanced as the trays are loaded, and ensure that the trays are rotating at 60 rpm at all times.

    Because you are loading 4 arrays per tray, each hybridization oven will have a total of 32 arrays.

11. Repeat this procedure until all 16 samples are loaded onto arrays and placed in the oven.

Process the Remaining Samples

To process the remaining samples:

1. Repeat the procedures listed under:
   - Load Samples Onto a Heat Block, and
   - Remove Samples from Heat Block and Load Onto Arrays
2. Allow the arrays to rotate at 49 °C, 60 rpm for 16 to 18 hours.
3. Store the remaining sample and any samples not yet hybridized in a tightly sealed plate at -20 °C.

IMPORTANT !

Allow the arrays to rotate for 16 to 18 hours at 49°C. This temperature has been optimized for this product, and should be stringently followed.

Chapter 5

Washing, Staining, and Scanning Arrays

Chapter 5

Introduction

This chapter contains instructions for using the Fluidics Station 450 to automate the washing and staining of GeneChip® Mapping 250K Arrays, and instructions for scanning probe arrays using the GeneChip® Scanner 3000 7G.

After completing the procedures described in this chapter, the scanned probe array image (.dat file) is ready for analysis.

Reagents and Materials Required

The following reagents and materials are recommendations and have been tested and evaluated by Affymetrix scientists. Information and part numbers listed are based on U.S. catalog information.

- Water, Molecular Biology Grade: BioWhittaker Molecular Applications/Cambrex, P/N 51200
- Distilled water: Invitrogen Life Technologies, P/N 15230147
- 20X SSPE (3 M NaCl, 0.2 M NaH_2PO_4, 0.02 M EDTA): BioWhittaker Molecular Applications/Cambrex, P/N 51214
- Anti-streptavidin antibody (goat), biotinylated: Vector Laboratories, P/N BA-0500; reconstitute according to product instructions
- R-Phycoerythrin Streptavidin: Molecular Probes, P/N S-866
- 10% Surfact-Amps® 20 (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
- 5 M 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 9185-0000

Reagent Preparation

Wash A: Non-Stringent Wash Buffer

(6X SSPE, 0.01% Tween 20)

For 1000 mL:
300 mL of 20X SSPE
1.0 mL of 10% Tween-20
699 mL of water
Filter through a 0.2 µm 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 µm filter.
Store at room temperature.
The pH should be 8.

IMPORTANT !

Prepare Wash B in smaller quantities to avoid long term storage. The container must be sealed tightly to avoid changes in salt concentration due to evaporation.

0.5 mg/mL Anti-Streptavidin Antibody

Resuspend 0.5 mg in 1 mL of water.
Store at 4°C.

12X MES Stock Buffer

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

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^+], 0.01% Tween-20)

For 100 mL:
8.3 mL of 12X MES Stock Buffer
18.5 mL of 5 M 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) 1.4 client (1.3 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.

   - The top half of the display pane refers to the sample and the bottom half refers to the experiment.

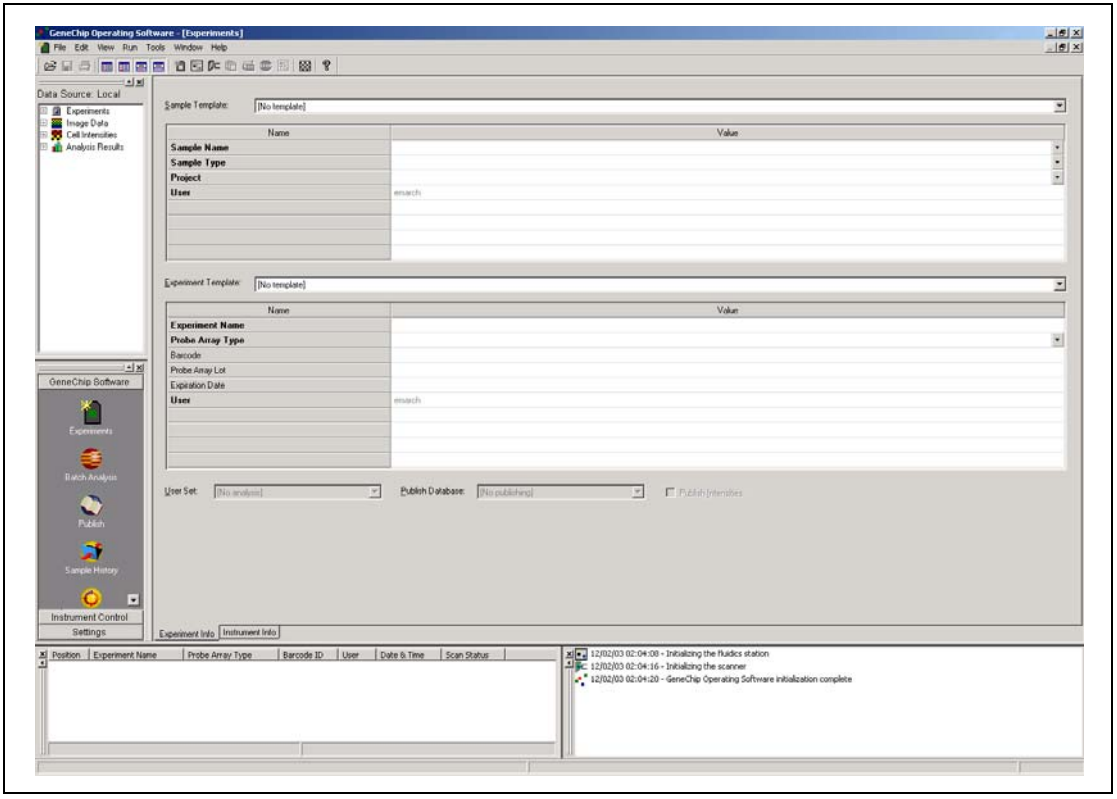


Figure 5.1
GCOS Sample Entry Pane

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.
- Appropriate 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 *GeneChip® Operating Software User's Guide* for further information.

STEP 2: PREPARING THE FLUIDICS STATION

The Fluidics Station 450 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 *GeneChip® 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 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_450 for the respective modules in the Protocol drop-down list.
3. Change the intake buffer reservoir A to Non-Stringent Wash Buffer, and intake buffer reservoir B to Stringent Wash Buffer.
4. Click Run for each module to begin priming.
5. Follow LCD instructions.

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 Phycoerythin (SAPE) stain, followed by an antibody amplification step and final stain with Streptavidin Phycoerythin (SAPE). Following staining, the array is filled with Array Holding Buffer prior to scanning as outlined in Table 5.5.

1. After 16 to 18 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 270 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. Mix well.

Table 5.1
Stain Buffer

Components	1X	Final Concentration
H_2O	800.04 µL	
SSPE (20X)	360 µL	6X
Tween-20 (3%)	3.96 µL	0.01%
Denhardt's (50X)	24 µL	1X
Subtotal	1188 µL	
Subtotal/2	594 µL	

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	594 µL	1X
1 mg/mL Streptavidin Phycoerythrin (SAPE)	6.0 µL	10 µg/mL
Total	600 µL	

Mix well.

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

Antibody Stain Solution

Table 5.3
Antibody Solution Mix

Components	Volume	Final Concentration
Stain Buffer	594 µL	1X
0.5 mg/mL biotinylated antibody	6 µL	5 µg/mL
Total	600 µL	

Mix well.

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

Array Holding Buffer

Table 5.4
Array Holding Buffer

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

Add 820 µL of Array Holding Buffer to each microcentrifuge tube. One tube is needed per module used.

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

Table 5.5
FS-450 Fluidics Protocol - Antibody Amplification for Mapping Targets

	49 Format (Standard) Mapping 500Kv1_450
Post Hyb Wash #1	6 cycles of 5 mixes/cycle with Wash Buffer A at 25°C
Post Hyb Wash #2	24 cycles of 5 mixes/cycle with Wash Buffer B at 45°C
Stain	Stain the probe array for 10 minutes in SAPE solution at 25°C
Post Stain Wash	6 cycles of 5 mixes/cycle with Wash Buffer A at 25°C
2nd Stain	Stain the probe array for 10 minutes in Antibody Stain Solution at 25°C
3rd Stain	Stain the probe array for 10 minutes in SAPE solution at 25°C
Final Wash	10 cycles of 6 mixes/cycle with Wash Buffer A at 30°C. The final holding temperature is 25°C
Filling Array	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

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

Using the Fluidics Station 450

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 Mapping500Kv1_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 fluidics station).
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 vials into the sample holders 1, 2 and 3 on the fluidics station.
   - Place one vial containing 600 µL Streptavidin Phycoerythrin (SAPE) stain solution mix in sample holder 1.
   - Place one vial containing 600 µL anti-streptavidin biotinylated antibody stain solution in sample holder 2.
   - Place one vial containing 820 µL 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 7G. Pull up on the cartridge lever to engage wash block and proceed to *Probe Array Scan* on page 148.

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 the arrays cannot be scanned promptly, keep the probe arrays at 4°C and in the dark until ready for scanning. Scan must be preformed 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 152.

Probe Array Scan

The scanner is also controlled by GCOS Software 1.4. The probe array is scanned after the wash protocols are complete. Make sure the laser is warmed up prior to scanning by turning the GeneChip Scanner 3000 7G 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 *GeneChip® Operating Software User's Guide* (P/N 701439), online help, and tutorials 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.

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 scanning the probe array, follow 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®, label spots can be applied to each of the two septa on the probe array cartridge to prevent 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 spot to each of the two septa. Press to ensure the spots remain flat. If the spots do not apply smoothly (that is, if bumps, bubbles, tears or curled edges are observed) do not attempt to smooth out the spot. Remove the spot and apply a new spot. See Figure 5.2.

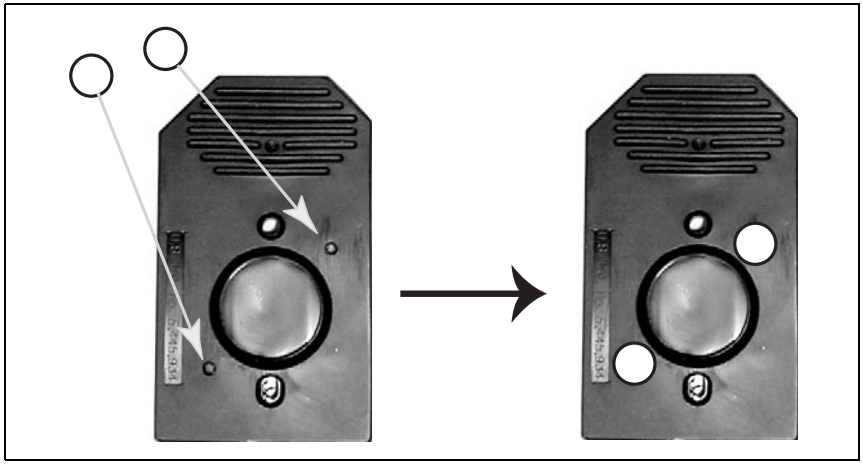


Figure 5.2
Applying Tough-Spots® to the probe array cartridge

3. Insert the cartridge into the scanner and test the autofocus to ensure the spots do not interfere with the focus. If a focus error message is observed, remove the spot and apply a new spot. Ensure that the spots lie flat.

SCANNING THE PROBE ARRAY

NOTE **Customers using the Autoloader should refer to the Autoloader User's Guide.**

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 being scanned.

   A previously run experiment can also be selected by using the Include Scanned Experiments option box. After selecting this option, previously scanned experiments appear in the drop-down list.

NOTE **If the experiment name is not seen in the scanner dialog box, open the workflow monitor, right-click your experiment, and select "Advance to Scan." Refer to the *GeneChip® Operating Software User's Guide* 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 7G.

WARNING **Please do not attempt to manually open or close the GeneChip® Scanner 3000 7G scanner door as this may damage the scanner.**

4. Once the experiment has been selected, click the Start button.

   A dialog box prompts to load the array into the scanner.

5. Pixel resolution and wavelength for the GeneChip Scanner 3000 7G are preset and cannot be changed.
6. Open the sample door of the scanner and insert the probe array into the holder. The door of the GeneChip Scanner 3000 7G 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. For the FS-450, gently lift up the cartridge lever to engage, or close, the washblock.

   The fluidics station automatically performs a Cleanout procedure. The LCD window indicates the progress of the Cleanout procedure.
3. When the fluidics station LCD window indicates REMOVE VIALS, the Cleanout procedure is complete.
4. Remove the sample microcentrifuge vial(s) from the sample holder(s).
5. If no other processing is to be performed, place wash lines into a bottle filled with deionized water.
6. Choose Shutdown_450 for all modules from the drop-down Protocol list in the Fluidics Station dialog box. Click the Run button for all modules.

   The Shutdown protocol is critical to instrument reliability. Refer to the appropriate Fluidics Station User's Guide for more information.
7. After Shutdown protocol is complete, flip the ON/OFF switch of the fluidics station to the OFF position.
8. Place buffer lines in a different bottle of deionized water than the one used for the shutdown protocol.

IMPORTANT !

To maintain the cleanliness of the fluidics station and obtain the highest quality image and data possible, a weekly bleach protocol is highly recommended.

Chapter 6

Fluidics Station Care and Maintenance

Chapter 6

Introduction

This chapter provides instructions on caring for and maintaining the instrument, and on troubleshooting if problems arise.

INSTRUMENT CARE

- Use a surge protector on the power line to the fluidics station.
- Always run a Shutdown protocol when the instrument will be off or unused overnight or longer. This will prevent salt crystals from forming within the fluidics system.
- When not using the instrument, leave the sample needles in the lowered position. Each needle should extend into an empty vial. This will protect them from accidental damage.
- Always use deionized water to prevent contamination of the lines. Change buffers with freshly prepared buffer at each system startup.
- The fluidics station should be positioned on a sturdy, level bench away from extremes in temperature and away from moving air.

IMPORTANT **!** **Before performing maintenance, turn off power to the station to avoid injury in case of a pump or electrical malfunction.**

INSTRUMENT MAINTENANCE

To ensure proper functioning of the fluidics station, perform periodic maintenance.

Fluidics Station Bleach Protocol

Affymetrix recommends a weekly cleaning protocol for the fluidics station. This protocol uses commonly purchased sodium hypochlorite bleach.

This protocol is designed to eliminate any residual SAPE-antibody complex that may be present in the fluidics station tubing and needles. The protocol runs a bleach solution through the system followed by a rinse cycle with deionized (DI) water. This protocol takes approximately one hour and forty minutes to complete. Affymetrix recommends running this protocol weekly, regardless of the frequency of use. The current version of the protocol can be found at: www.affymetrix.com/support/technical/fluidics_scripts.affx.

THE BLEACH CYCLE

To avoid carryover, or cross contamination, from the bleach protocol, Affymetrix recommends the use of dedicated bottles for bleach and DI water. Additional bottles can be obtained from Affymetrix.

Table 6.1
Affymetrix Recommended Bottles

Part Number	Description
400118	Media Bottle, SQ, 500 mL
400119	Media Bottle, SQ, 1000 mL

1. Disengage the washblock for each module by pressing down on the cartridge lever. Remove any probe array cartridge (Figure 6.1).

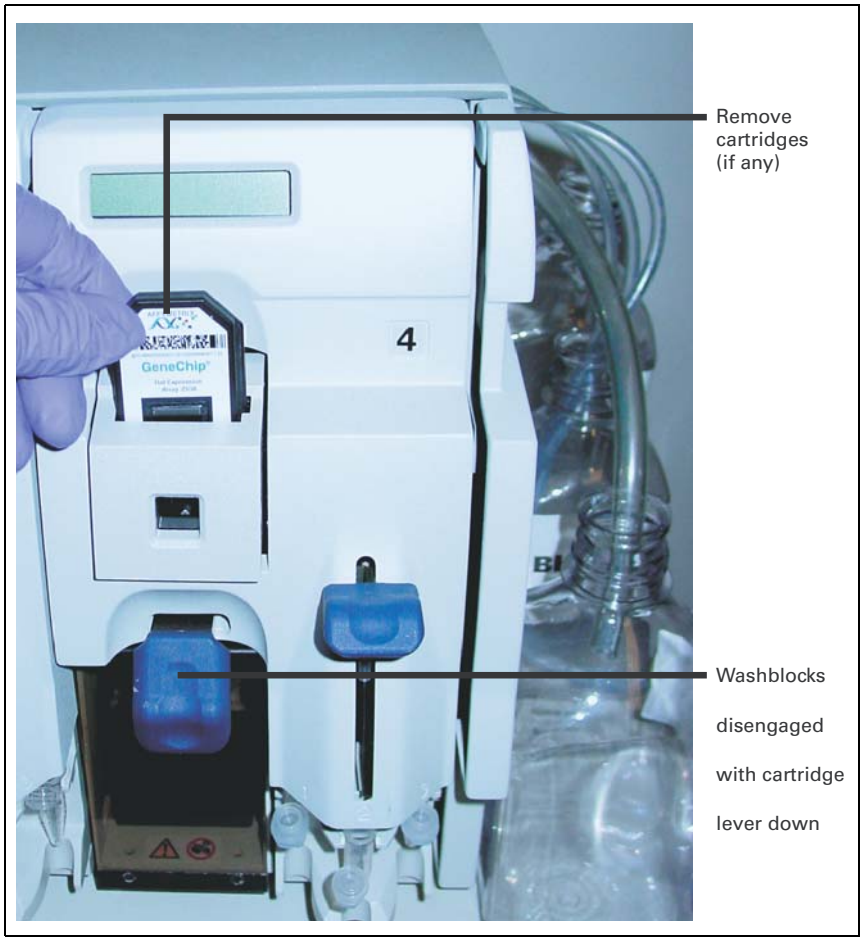


Figure 6.1
Disengaged washblocks showing cartridge levers in the down position. Remove any cartridges

2. Prepare 500 mL of 0.525% sodium hypochlorite solution using deionized water. For example, follow these directions to make 500 mL of bleach:

In a 1 liter plastic or glass graduated cylinder combine 43.75 mL of commercial bleach (such as Clorox® bleach, which is 6% sodium hypochlorite) with 456.25 mL of DI H_2O, mix well. Pour the solution into a 500 mL plastic bottle, and place

the plastic bottle on fluidics station.

IMPORTANT **The shelf life of this solution is 24 hours. After this period, you must prepare a fresh solution.**

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

3. Place on the fluidics station an empty one liter waste bottle, a 500 mL bottle of bleach and a one liter bottle of DI water as shown in Figure 6.2. Insert the waste line into the waste bottle (Figure 6.2).
4. Immerse all three wash and water lines of the fluidics station into the 500 mL of bleach solution (Figure 6.2). DO NOT IMMERSE THE WASTE LINE INTO THE BLEACH.

NOTE **The BLEACH protocol requires approximately one liter of DI water.**

Figure 6.2
The bleach cycle. Immerse the tubes into the 0.525% sodium hypochlorite solution. The waste line remains in the waste bottle.

5. Open GeneChip® Operating Software (GCOS), Microarray Suite, or the current version of the Affymetrix control software. Click Run → Fluidics... from the menu. Alternatively, click the down arrow on the Protocol list on the toolbar. The protocol window appears (Figure 6.3).

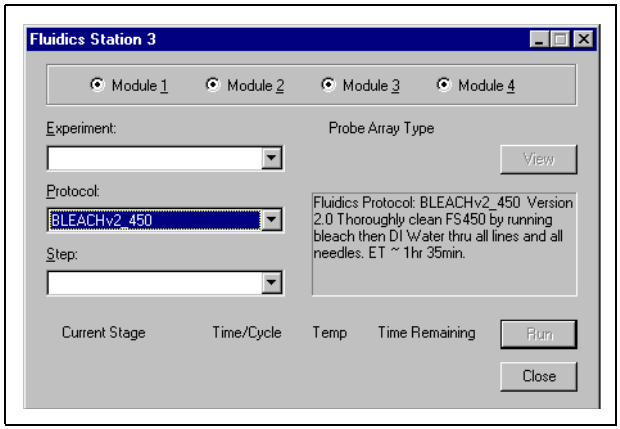


Figure 6.3
The Fluidics Station protocol window: select all modules.

6. Choose the current bleach protocol (as of the writing of this manual, it is BLEACHv2_450) for each of the respective modules in the Protocol drop-down list. Select all four modules, 1 to 4, and click **Run**. The fluidics station will not start the bleach protocol until the needle lever is pressed down (Figure 6.4).

NOTE **Temperature will ramp up to 50°C.**

7. Follow the prompts on each LCD. Load empty 1.5 mL vials onto each module if not already done so.
8. Press down on each of the needle levers to start the bleach protocol (Figure 6.4).

Figure 6.4
Press down on the needle levers to start the bleach protocol.

9. The fluidics station will begin the protocol, emptying the lines and performing the cleaning cycles using bleach solution.
10. After approximately 30 minutes, the LCD will prompt you when the bleach cycle is over and the rinse cycle is about to begin.

THE RINSE CYCLE

Once the bleach cycle has finished, the second part of the protocol is a rinse step. This step is essential to remove all traces of bleach from the system. Failure to complete this step can result in damaged arrays.

1. Follow the prompts on the LCD for each module. Lift up on the needle levers and remove the bleach vials. Load clean, empty vials onto each module.
2. Remove the three wash and water lines from the bleach bottle and transfer them to the DI water bottle (Figure 6.5). At this step, there is no need to be concerned about the bleach remaining in the lines.

Figure 6.5
Immerse the three wash and water lines in the DI water bottle.

3. Press down on the needle levers to begin the rinse cycle. The fluidics station will empty the lines and rinse the needles.
4. When the rinse is completed after approximately one hour, the fluidics station will bring the temperature back to 25°C and drain the lines with air. The LCD display will read CLEANING DONE.
5. Discard the vials employed for the bleach protocol.
6. After completing the bleach protocol, follow suggestions in Table 6.2 for storage of the Fluidics Station 450.

Table 6.2
Quick Reference Guide to Using the FS-450

If:	Then do this:
Planning to use the system immediately	After running the bleach protocol, remove the DI water supply used in the rinse phase and install the appropriate reagents for use in the next staining and washing protocol (including fresh DI water). • Perform a prime protocol without loading your probe arrays. **Failure to run a prime protocol will result in irreparable damage to the loaded hybridized probe arrays.**
Not planning to use the system immediately	Since the system is already well purged with water, there is no need to run an additional shutdown protocol. Remove the old DI water bottle and replace it with a fresh bottle.
Not planning to use the system for an extended period of time (longer than one week)	Remove the DI water and perform a "dry" protocol shutdown. This will remove most of the water from the system and prevent unwanted microbial growth in the supply lines. Also, remove the pump tubing from the peristaltic pump rollers.

NOTE **After completing the bleach protocol, discard the vials.**

Peristaltic Tubing Replacement

Periodically the peristaltic tubing requires replacement because of wear, contamination, or in order to avoid salt buildup. Inspect the tubing, and if there is evidence of these conditions, follow the procedure outlined below.

IMPORTANT !

For systems in routine use, Affymetrix recommends monthly replacement of the tubing. To ensure proper performance, use only tubing available from Affymetrix. This tubing is manufactured to the required specifications to ensure proper fluid delivery and array performance. Additional tubing can be obtained by ordering from Affymetrix:

Part Number	Description	Quantity
400110	Tubing, Silicone Peristaltic, 8.5	1

Wear gloves when changing tubing. Do not allow fluid from old tubing to spill onto surfaces.

1. Open the module door (Figure 6.6).

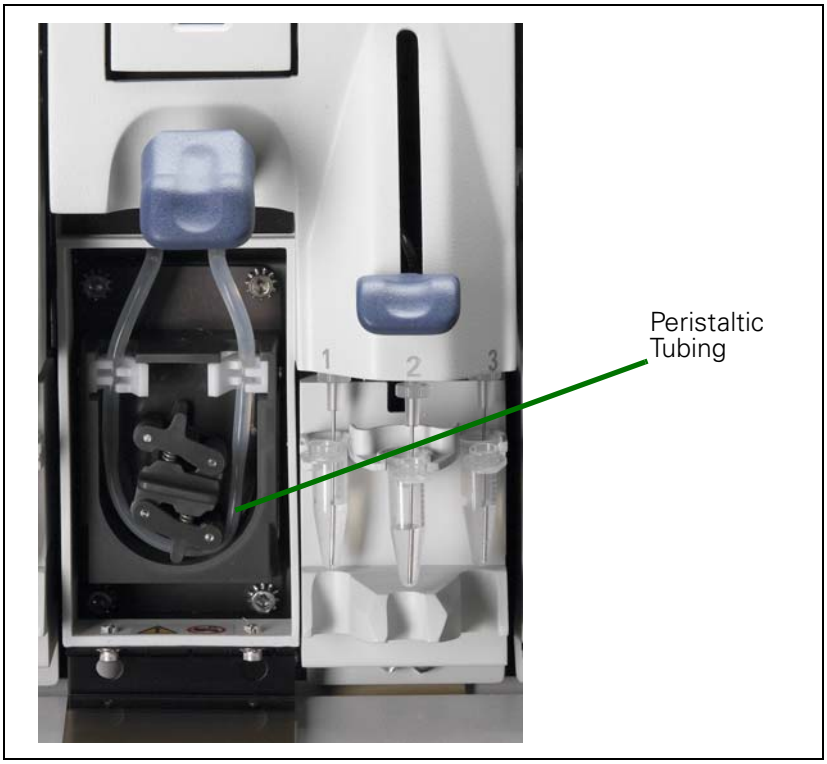


Figure 6.6
Module door open showing peristaltic tubing

2. Open the white clamps to release tubing on both sides. See Figure 6.7.

WARNING ▲

Do not attempt to replace the tubing on a module where the module has been removed from the case of the fluidics station. In this case, rotating the pump may damage the motor driver circuitry.

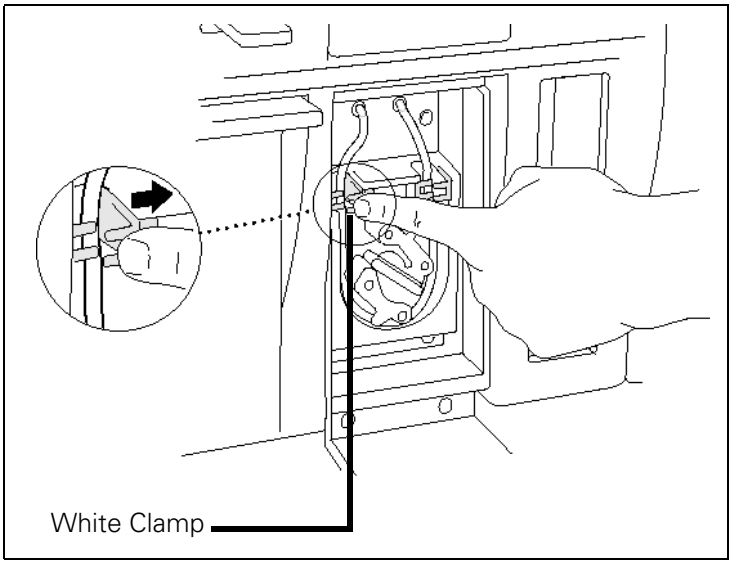


Figure 6.7
Releasing peristaltic tubing

3. Pull tubing off while gently turning the peristaltic pump head. Discard old tubing.
4. Replace tubing with new peristaltic tubing supplied with the accessory kit as described below:
   - **A.** Attach one end of the new tubing to the fitting on the right at the top of the pump enclosure.
   - **B.** Insert the tubing into the clamp under the fitting without stretching the portion of the tubing between the fitting and the clamp. There should be a small amount of slack in that portion of the tubing.
   - **C.** Work the tubing into the pump head while slowly turning the pump.
   - **D.** Insert the free end of the tubing into the other clamp, and attach it to the other fitting.
   - **E.** Close the drop-down module door.
5. Order more replacement tubing (P/N 400110).

Troubleshooting and Assistance

If problems arise with the fluidics station, use the following tables to locate the description that matches the problem. If a solution is not found, call Affymetrix Technical Support for assistance.

TROUBLESHOOTING DECISION TREE

The following simple flow charts (Figure 6.8 and Figure 6.9) show you how to begin troubleshooting the FS450/250 for a Missing Fluid Error (MFE).

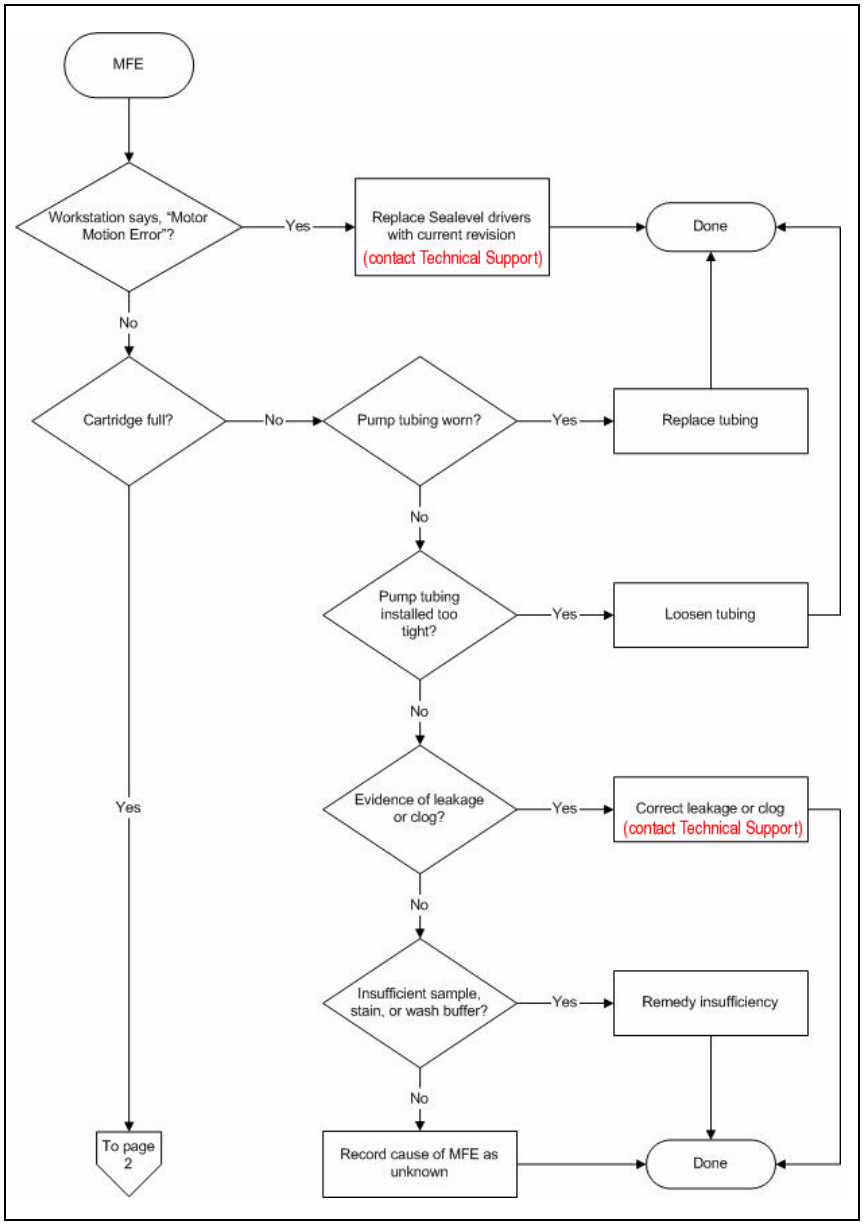


Figure 6.8
Troubleshooting decision tree, page 1

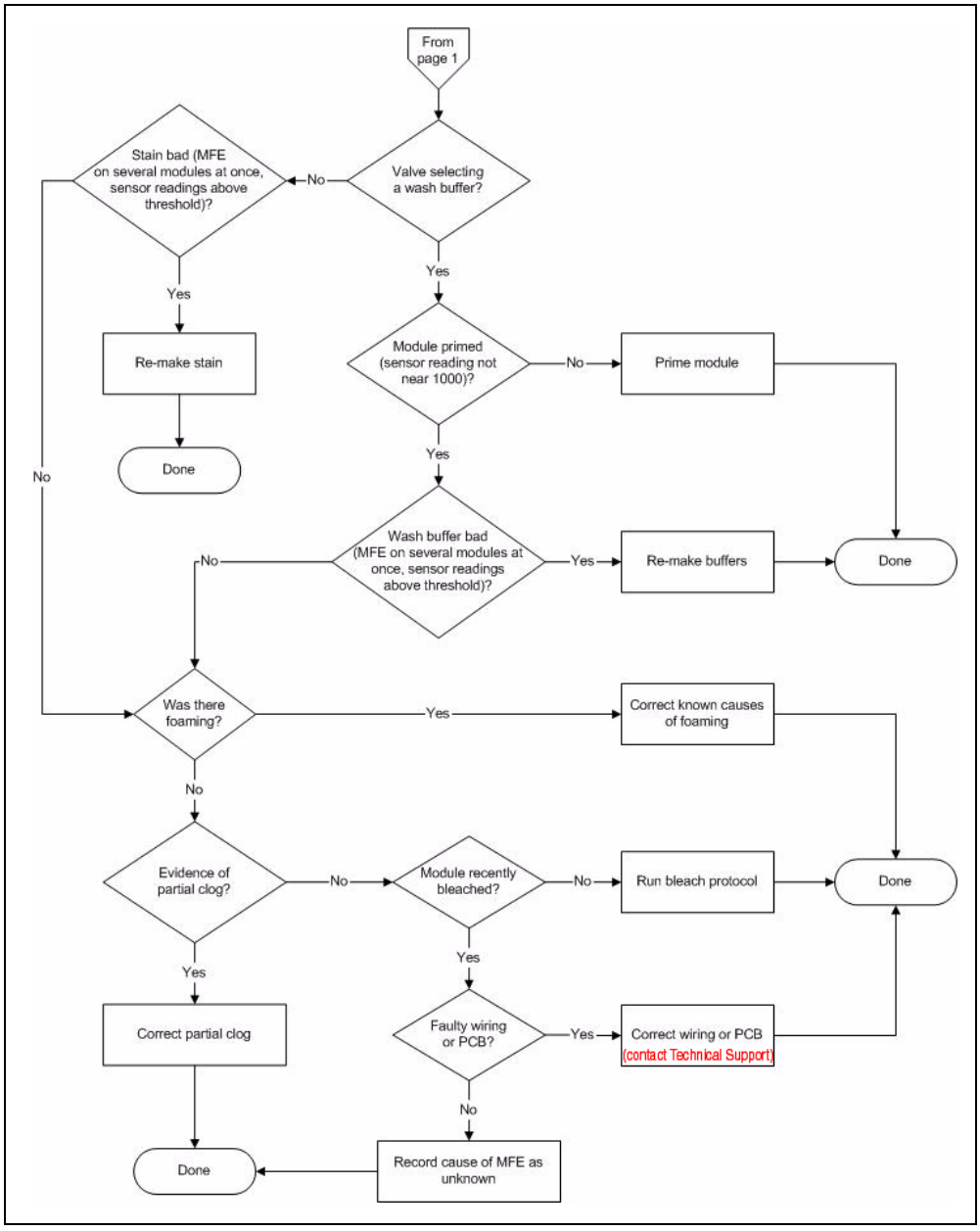


Figure 6.9
Troubleshooting decision tree, page 2

PROBLEMS AND SOLUTIONS

Table 6.3
Common error messages, their meanings, probable causes and solutions

Error Message	Problem	Possible Cause	Solution
Missing Fluid Error	Cartridge not filling completely with sample solution or buffer during initial stages of hybridization wash or staining protocol.	Possible holes in the septa of the cartridge.	Run Recover script, and then use another cartridge.
		Sample or staining solution not in place properly.	Run Recover script. Make sure sample or stain vial is in the sample holder.
		Insufficient volume of sample or staining solution (500 µL).	Run Recover script. Add more sample solution to the sample vial.
		Blocked sampling tube or line of the fluidics station.	Run Recover script. Run the Clean or Prime script with fresh deionized (DI) water to flush out salt blockage.
		Failure of one of the fluidics sensors.	Call Affymetrix Technical Support for service.
		Pump tubing stretched too tightly around the pump.	Loosen the tubing clamps, allow tubing to relax, close the clamps.
	Cartridge not filling completely with buffer during wash script	Buffer bottle empty.	Fill buffer bottles.
		Module not primed.	Prime module.
	System detects improper conditions while filling. Note where in protocol error occurred.	Missing or insufficient stain or antibody in vial.	Identify if chip is filled • If important to recover fluid in chip, then run **Recovery** script, followed by **Resume** function • If not important to recover fluid in chip, run **Resume** function
		Wash empty.	
		Air bubbles in line.	
		Leaks.	
	Recovered less sample than initial input during Recover script.	Loose tubing attachments inside the fluidics station.	Call Affymetrix Technical Support for service.

Table 6.3 (Continued)
Common error messages, their meanings, probable causes and solutions

Error Message	Problem	Possible Cause	Solution
Fluidics Station X Does Not Respond		Power not switched on at the fluidics station.	Turn fluidics station power on, and then try to connect again.
		Incorrect fluidics station designated for communication.	Designate correct fluidics station on workstation.
		Loose cables.	Firmly connect cables to fluidics station.
Sensor Timeout	"Sensor Timeout" error message on workstation.	No user response to "Remove Vial" prompt or other prompt.	Start the selected script again.
Error While Draining **Error While Filling**	Cartridge is not filling or draining properly.	Defective septa in cartridge.	Use a new cartridge.
		Insufficient sample or stain volume.	Add more sample solution to sample vial.
		Excessive bubbling in cartridge.	Change the buffer: reduce detergent.
		Buffer conductivity too low.	Change the buffer: increase salt.
		Failure of one of the fluid sensors.	Call Affymetrix Technical Support for service.
Error While Filling	System detects improper conditions while filling. Note where in protocol error occurred.	Missing or insufficient stain or antibody in vial.	Identify if chip is filled: • If important to recover fluid in chip, then run **Recovery** script, followed by **Resume** function • If not important to recover fluid in chip, run **Resume** function
		Wash or DI water empty.	
		Air bubbles in line. Leaks.	

Table 6.3 (Continued)
Common error messages, their meanings, probable causes and solutions

Error Message	Problem	Possible Cause	Solution
Invalid Command	Communications error detected. Note where in protocol error occurred.		Identify if chip is filled. • If important to recover fluid in chip, then run **Recovery** script. • Attempt to rerun script if sample loss can be tolerated. If problem persists, contact Affymetrix for service • If sample loss cannot be tolerated, do not attempt to rerun script. Contact Affymetrix for service
Temperature Timeout	Temperature does not reach specified temperature.	Temperature has not reached required level in expected time if ambient temperature is within operating specifications (15 – 30 degrees C).	Call Affymetrix Technical Support for service.
Improper Script	Script does not work.	User is attempting to run a FS400 script on FS450Dx	Download proper FS450Dx script and continue.
Valve Motion Error			Run **Home** script and run desired script again. If problem persists, contact Affymetrix for service.
Valve Not Homed			Run **Home** script and run desired script again. If problem persists, contact Affymetrix for service.
Valve Out of Position			Run **Home** script and run desired script again. If problem persists, contact Affymetrix for service.

MEANING OF ERROR MESSAGES

The following lists some of the common error messages and what they mean (Table 6.4).

Table 6.4
Common Error Messages

Error Message	Meaning
"Invalid Command"	The script contains a command that can not be executed because its command code is either undefined or has a format error.
"Improper Script"	The first command of the script is not the required FS450 command.
"Temperature Timeout"	The Re-attempt command timed out before the set point temperature was reached.
"Sensor Timeout"	The Await Sensors command timed out before the anticipated sensor pattern was seen.
"Valve not Homed"	The Home command did not result in the valve reaching its HOME position.
"Valve Motion Error"	The Valve command did not result in the valve reaching its target valve position.
"Valve out of Position"	According to the outer valve encoder, the valve did not reach a valid position when it was last rotated.
"Error while Filling"	While filling the cartridge, the AwaitMotor command terminated because of the step count not the expected sensor pattern, and that the same error had occurred several times consecutively.
"Error while Draining"	While draining the cartridge, the AwaitMotor command terminated because the step count was not the expected sensor pattern, and the same error had occurred several times consecutively.

Table 6.4 (Continued)
Common Error Messages

Error Message	Meaning
"Missing Fluid Error" **Examples:** **"Stage C" "WashA"** **"Sense/Threshold" "960/890"**	"Stage C" "WashA" "Sense/Threshold" "960/890" The Pump command completed its step count before the conductivity sensor determined that the cartridge contained a solution with conductivity below the set threshold value. The Missing Fluid Error (MFE) Display not only gives a visual notification of an error condition to the operator, but gives the operator information that enables him/her to determine the cause of the error. It does this by displaying information about the sensor value and the fluid that caused the error. It shows this internal information in a continuous loop until the machine is powered down or a script is started. For example: Missing Fluid Error for 4 seconds Stage A valvePos WashA for 4 seconds Sense/Threshold 820/600 for 4 seconds

OTHER PROBLEMS AND SOLUTIONS

Table 6.5 lists other problems, causes and solutions that may be encountered.

Table 6.5
Other Problems

Problem	Possible Cause	Solution
Air bubbles left in cartridge at the end of a hybridization-wash script.	Air bubble in wash line.	**Consult Chapter 5 for information specific to array type.**
Buffer leaking inside the fluidics station.	Loose tubing attachments inside the fluidics station.	Call Affymetrix Technical Support for service.
	Washblock requires replacement.	Call Affymetrix Technical Support for service.
	Salt buildup in the lines of the fluidics station.	Run the Clean or Prime script with fresh DI water to flush out salt blockage.
Cartridge needles of the fluidics station not engaging with the cartridge.	Possible defective septa on the cartridge.	Use another cartridge.
	Extra flashing on the cartridge.	Use another cartridge, or call Affymetrix Technical Support for service.
	Salt buildup on the cartridge needles.	Run the Clean script with fresh DI water to flush out salt blockage. Clean cartridge needles with a wet cotton swab.
	Cartridge holder aligned and attached to the fluidics station improperly.	Call Affymetrix Technical Support for service.
	Cartridge holder not properly engaged to the fluidics station.	Place the cartridge into the cartridge holder. Push the holder door shut, and firmly lift the lever to engage the cartridge needles.

Table 6.5 (Continued)
Other Problems

Problem	Possible Cause	Solution
Sample needles do not properly enter vial.	Bent sample needle	Replace sample needle.
	User may be pressing the needle lever down too quickly or with too much force.	Engage sample needle lever more slowly and/or with less force.

INSTRUMENT SPECIFICATIONS

Fluidics Station Dimensions:

(height, depth, width)
40.2 x 41.0 x 71.1 cm or 15 13/16 x 16 1/8 x 28 inches

Product Weight:

Approximately 80 pounds or 36.3 kg

Power Input:

100 to 240 V~, 3 A
300 watts or less. Main supply voltage fluctuations not to exceed 15% of the nominal supply voltage.

Temperature:

Operating: 15° to 30°C
Storage (non-operating):-10° to 60°C

Humidity:

Operating: 10-90% RH, non-condensing
Storage (non-operating):10% to 95% RH

Other:

Pollution degree, 2

Installation category, II

Electrical Supply

The electrical supply should meet the input specified on the instrument label. Voltage fluctuations should not exceed 15% nominal supply voltage.

Altitude

<2000 m

Chapter 7

Analysis Workflow

Chapter 7

Introduction

The purpose of this chapter is to outline the necessary steps to analyze data following the scanning of GeneChip® Human Mapping 500K Array Set 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 User's Guide* (GCOS) (P/N 701439)
- *GeneChip® Genotyping Analysis Software User's Guide* (GTYPE) (P/N 702083)

Software Requirements

- GeneChip® Operating Software (GCOS) 1.4 (client), GCOS 1.3 (server) or higher
- GeneChip® Genotyping Analysis Software (GTYPE) 4.0 or higher
- GeneChip® Human Mapping 500K Set library files: Mapping 250K_Nsp or Mapping 250K_Sty

Analysis Workflow

This section describes the workflow for generating genotyping calls using GeneChip Genotyping Analysis Software (GTYPE). This process is outlined in Figure 7.1. Please note that acquisition of raw data using GeneChip Operating Software (GCOS) precedes analysis in GTYPE. Refer to the *GeneChip® Operating Software User's Guide* for instructions on .dat and .cel file generation.

Significant improvements have been made to GCOS and the Data Transfer Tool (DTT) to support large .dat and .cel files associated with the 500K Array Set. For more details on these improvements, please consult:

- *Data Transfer Tool User's Guide* for more information about improved performance and enhanced data management flexibility.
- *GeneChip® Operating Software User's Guide* for more information about Auto-Archive of .dat files.

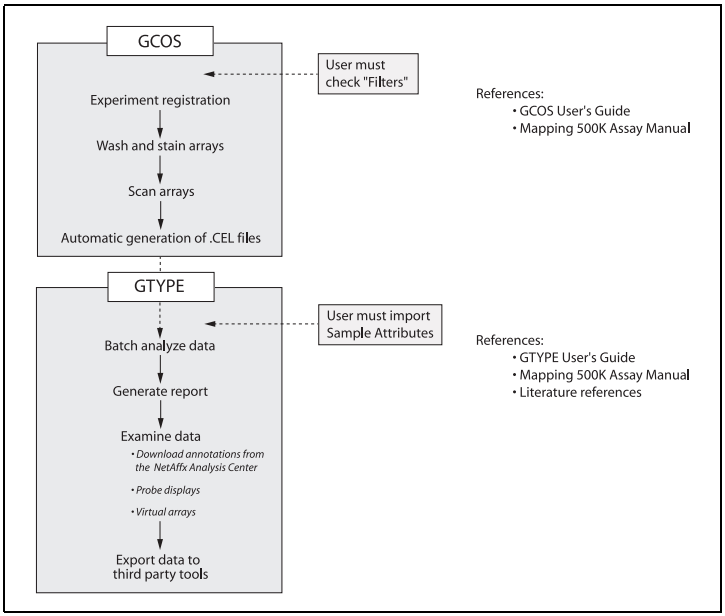


Figure 7.1
Data Analysis workflow for GeneChip® Human Mapping 250K Arrays using GCOS and GTYPE

Analysis

The following is an overview of the steps necessary to analyze data using GTYPE 4.0 or higher. GCOS 1.4 or higher must be installed on the same workstation in order to use GTYPE. Detailed instructions may be found in the *GeneChip® Genotyping Analysis Software User's Guide*.

Batch Analysis:

1. Open the Batch Analysis window. Then, select the appropriate .cel files and drag the files into the Batch Analysis window.

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 7.2). The filter settings can be adjusted by selecting the filter options in the Tools menu bar.

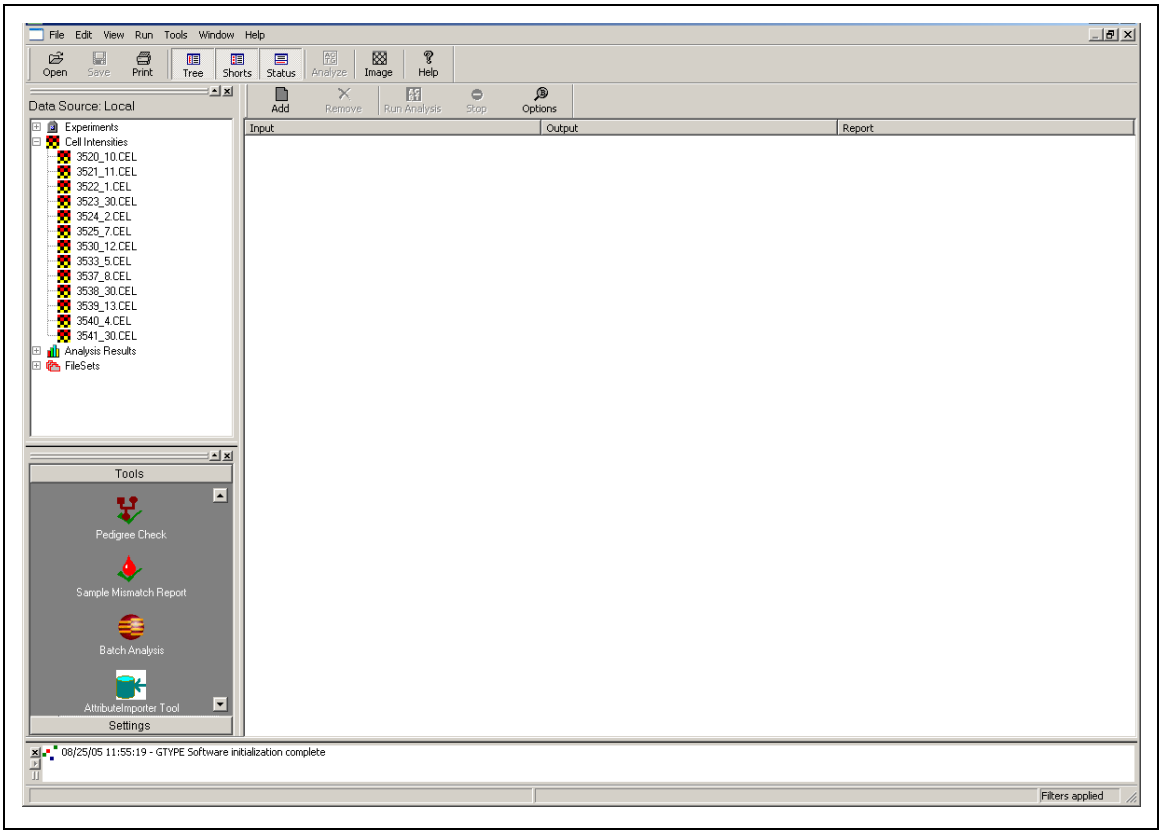


Figure 7.2
GTYPE main window with the Batch Analysis window and Filters Applied message

2. Click Analyze and the files will be analyzed, generating .chp files containing genotype calls.
3. After Batch Analysis is complete, a report will be displayed summarizing data from the samples. This .rpt file is saved in the GCOS data directory.

Single File Analysis:

1. In the Data Source window, browse to your .cel file of interest. Right-click that .cel file.
2. Click Analyze and a .chp file of genotyping calls will be generated. Further information on the use of .chp files may be found below.
3. Right-click on the generated .chp file in the Data Source window.
4. Click Report to display the .rpt file summarizing data from that sample. This .rpt file is saved in the GCOS data directory.

OUTPUT

NOTE

For customers interested in generating data using the new BRLMM algorithm for 500K data, please refer to www.Affymetrix.com for detailed information and documentation on the BRLMM algorithm.

Following analysis, .chp files are generated for each sample. The .chp files can be opened by double-clicking on the files in the GTYPE data file tree. Multiple .chp files can be opened simultaneously. Once opened the .chp files will display a Dynamic Model (DM) scatter plot in the upper portion of the window and a data table in the lower portion of the window as shown in Figure 7.3. In the data table a genotype call for each SNP along with a confidence score is displayed.

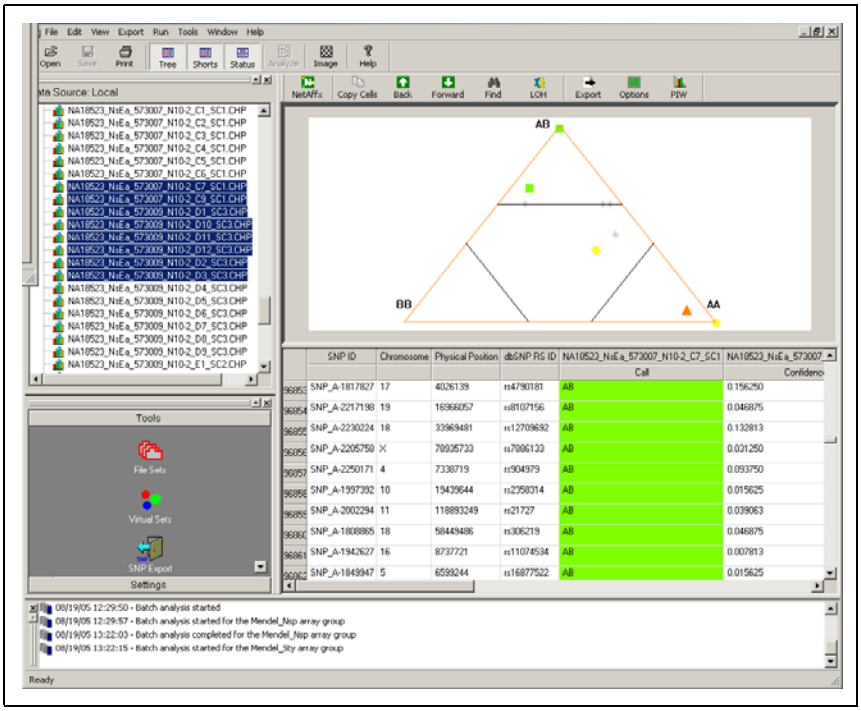


Figure 7.3
Dynamic Model Mapping Algorithm Table View

TIP

If the user-generated .chp files are not seen in the data file tree, right-click the "Analysis Results" bar and click "Refresh All," or make sure the Filter settings are set correctly.

REPORT

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

IMPORTANT !

The Mapping Algorithm Report has several important indicators that must be checked for each array in order to assess the quality of the data.

A saved Mapping Algorithm can be opened by:

1. Clicking the Open button in the main toolbar, or selecting File → Open from the main menu.

   The Open dialog box appears.

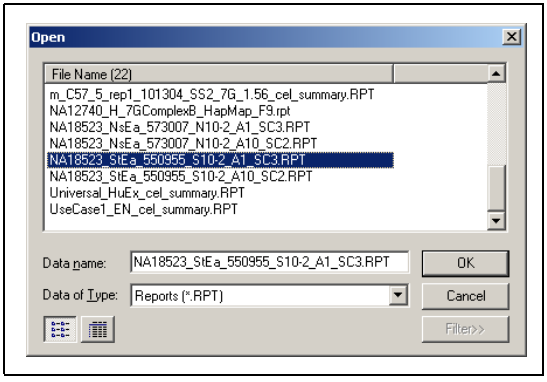


Figure 7.4
Open dialog box

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. Refer to Table 7.1 for the report components and description.

Table 7.1
Dynamic Model Mapping Algorithm Report Components

Report Component	Description
Total number of SNPs	Number of SNPs on array.
Total number of QC Probes	Number of QC SNPs on array.
Probe array type	Affymetrix probe array model number.
SNP Performance	A list of the analyzed samples, with the following information:
Sample	An identifier assigned to the analyzed samples.
Gender	Gender of the sample determined by the DM algorithm.
SNP Call Rate	Number of SNP calls/Total number of SNPs on the array.
AA Call Rate	The number of AA calls for the sample/Total number of SNPs on the array.
AB Call Rate	The number of AB calls for the sample/Total number of SNPs on the array.
BB Call Rate	The number of BB calls for the sample/Total number of SNPs on the array.
QC Performance	A list of the analyzed samples, with the following information:
Sample	An identifier assigned to the analyzed samples.
Median PM (QC Probe Set ID)	Median Intensity Value for perfect match cells for the QC probe sets.
MCR (DM Mapping only)	MPAM algorithm Call Rate. This is used to determine whether a sample is contaminated.
MDR (DM Mapping only)	MPAM algorithm Discrimination Rate. This is used to determine whether a sample is contaminated.
Common SNP Patterns (DM Mapping only)	Calls for a selected set of SNPs, displayed as a list of the analyzed samples with the allele calls for the common SNPs.

GTYPE ADDITIONAL FUNCTIONALITY

Additional functionality within GTYPE includes the ability to import sample attributes, create custom file sets, export batch SNP or linkage data, create virtual data sets, assess sample mismatch, and update SNP annotations from the NetAffx™ Analysis Center.

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 include information like father, mother, family and diseased state and must be imported before checking Mendelian Inheritance or importing into MERLIN or GeneHunter.

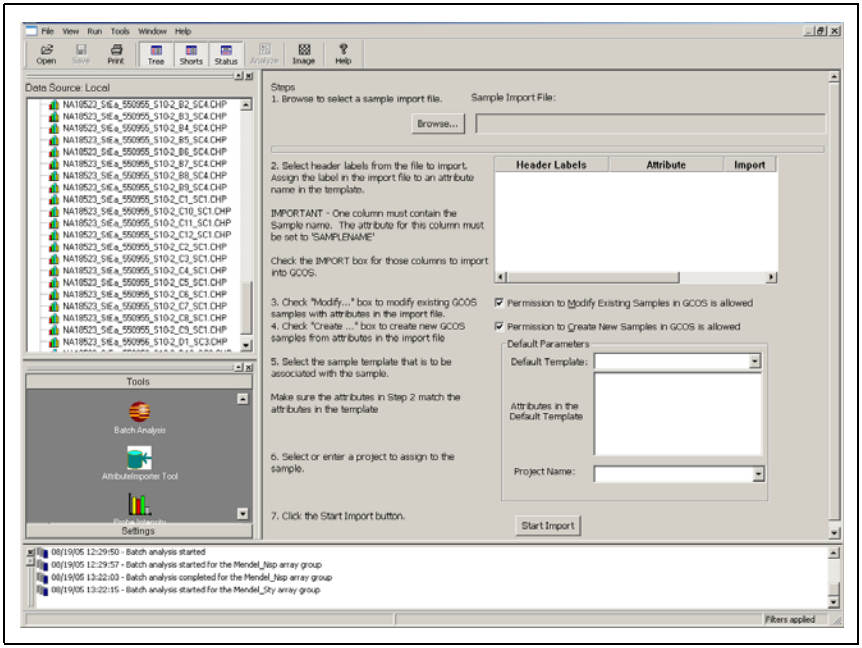


Figure 7.5
GTYPE AttributeImporter Tool

FILE SETS

File Sets is a new feature in GTYPE 4.0 that provides a way to custom group Mapping data files for the analysis of previously unassociated data at a single time. This may be helpful in situations where multiple users are working on a project or where experiments are repeated. Please note that File Sets may only be accessed within GTYPE and do not supersede or overwrite the GCOS file structure. Each File Set is shown as a node in the data tree.

You can use File Sets for any process where you need to select a file, for functions like:

- Linkage Export
- Pedigree Check
- SNP Export
- Batch Tool
- Sample Quality Tool

To group files into a File Set:

1. Right-click in the Data Tree and select File Sets from the shortcut menu; or from the main menu, select Tools → File Sets.
2. Select files for a set in the Data Tree, and drag them to the File Set Members box.
3. Enter a name for the File Set and click Save.

For more information regarding using File Sets, please consult the *GeneChip® Genotyping Analysis Software User's Guide*.

EXAMINING THE RAW PROBE DATA

Information on raw probe intensity can be obtained by selecting a SNP identifier from within the .chp file and choosing the Probe Intensity option in the Run menu. Probe intensity data is displayed for each probe in bar, line, or trace graph format.

EXPORT

SNP call data can be exported for use in third party software products as either a tab delimited file or linkage formats compatible with MERLIN, GeneHunter, or Haploview software (new for GTYPE 4.0).

If pedigree information is included in your experiment file, use GTYPE to export the genotype calls and pedigree information to a file in either MERLIN, GeneHunter, or Haploview format. For more information on these formats, please refer to the *GeneChip® Genotyping Analysis Software User's Guide*.

NOTE

Before using the linkage analysis export tool, the following must be done:

- **Assign a sample template with pedigree attributes to the samples.**
- **Import sample attribute data.**

To export data for linkage analysis:

1. Click the SNP Export button in the Genotyping Views shortcut bar, or select Tools → Genotype → SNP Export... from the menu bar.
2. Click the MERLIN, GeneHunter, or Haploview tab to select the export format. Specify sample attributes containing pedigree information.

NOTE

For information on sample attribute values for these formats, please consult the *GeneChip® Genotyping Analysis Software User's Guide*.

3. Select the file set, project, sample, experiments, or analysis data in the data tree and drag them to the Samples box.
4. (Optional) Choose any filters that need to be applied to the data. The SNP filters allow you to filter out SNPs that do not meet the particular requirements of your experiment from the export. You can filter by:
   - Allele Frequency
   - Hardy-Weinberg Probability Value
   - Mendelian error rate

   When filtering on Mendelian error, you have the option to remove the erroneous SNPs from the export files, or to set calls for erroneous SNPs to NoCalls for families with inheritance errors. You may want to use the second option if exporting multiple families at once.
5. Select output file names and choose the path to save the files. Click Export to export your data.

NOTE **Consult the *GeneChip® Genotyping Analysis Software User's Guide* for directions specific to each format type.**

To batch export genotyping calls:

The Tab Delimited export tool allows batch export of genotyping calls from large data sets simultaneously into tab delimited files. This feature can be combined with File Sets to provide a flexible method of analyzing custom groupings of large data sets.

The data is exported in a tab-delimited text file with the following columns:

- Affymetrix SNP ID
- Genotype

1. Click the SNP Export button in the Genotyping Views shortcut bar, or select Tools → Genotype → SNP Export… from the menu bar.
2. Choose the Tab Delimited tab.

3. Select the file set, project, sample, experiments, or analysis data in the data tree and drag them to the Samples box.
4. (Optional) Choose any filters you wish to apply to the data. The SNP filters allow you to filter out SNPs that do not meet particular requirements from the export. You can filter by:
   - Allele Frequency
   - Hardy-Weinberg Probability Value
   - Mendelian error rate
5. Select output file names and choose the path to save the files. Click Export to export your data.

   During export the SNP Statistics Report is also generated.

NOTE **Consult the *GeneChip® Genotyping Analysis Software User's Guide* for further directions.**

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 Dynamic Model Mapping Analysis window.

To use the NetAffx™ annotation option for the first time:

1. Download the NetAffx annotations you wish to display.
2. Select the NetAffx annotations to display.

NOTE

The first time you open a new array type, you will be prompted to download current annotations from the NetAffx™ Analysis Center. Click "Yes" if you want to see these annotations.

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

The NetAffx annotation download functionality has been enhanced in GTYPE. Please note that due to the increased speed of download, a dialogue box representing download progress is no longer shown.

You may need to initialize NetAffx Analysis Center 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 *GeneChip® Genotyping Analysis Software User's Guide*.

Assessing Data Quality

The purpose of this section is to help researchers establish guidelines for evaluating results generated from Mapping experiments. The Mapping Algorithm Report in GTYPE has a number of parameters that must be checked for each array in order to assess the quality of the data, and to identify outlier samples (Table 7.2). It is important to check these parameters, and to create a running log for each project. The Reference Genomic DNA 103, included in the GeneChip® Mapping 250K Assay Kits can serve as a positive control to ensure that all steps of the assay are being performed correctly.

Table 7.2
Dynamic Model Mapping Algorithm Report Metrics

Metric	Description
Call Rate (SNP Call)	Good first pass evaluation. If the genomic DNA sample is of equivalent quality and purity to the Reference Genomic DNA 103, then the Call Rate should be similar when analyzed at the same algorithm settings.
Reference Genomic DNA 103 Call rate	Process control to show that assay steps are being performed correctly.
MDR – MCR	Difference can be used to identify sample contamination.
Shared SNPs	Evaluate possible sample mix ups.
Oligonucleotide controls	Help evaluate hybridization, fluidics and scanning steps.

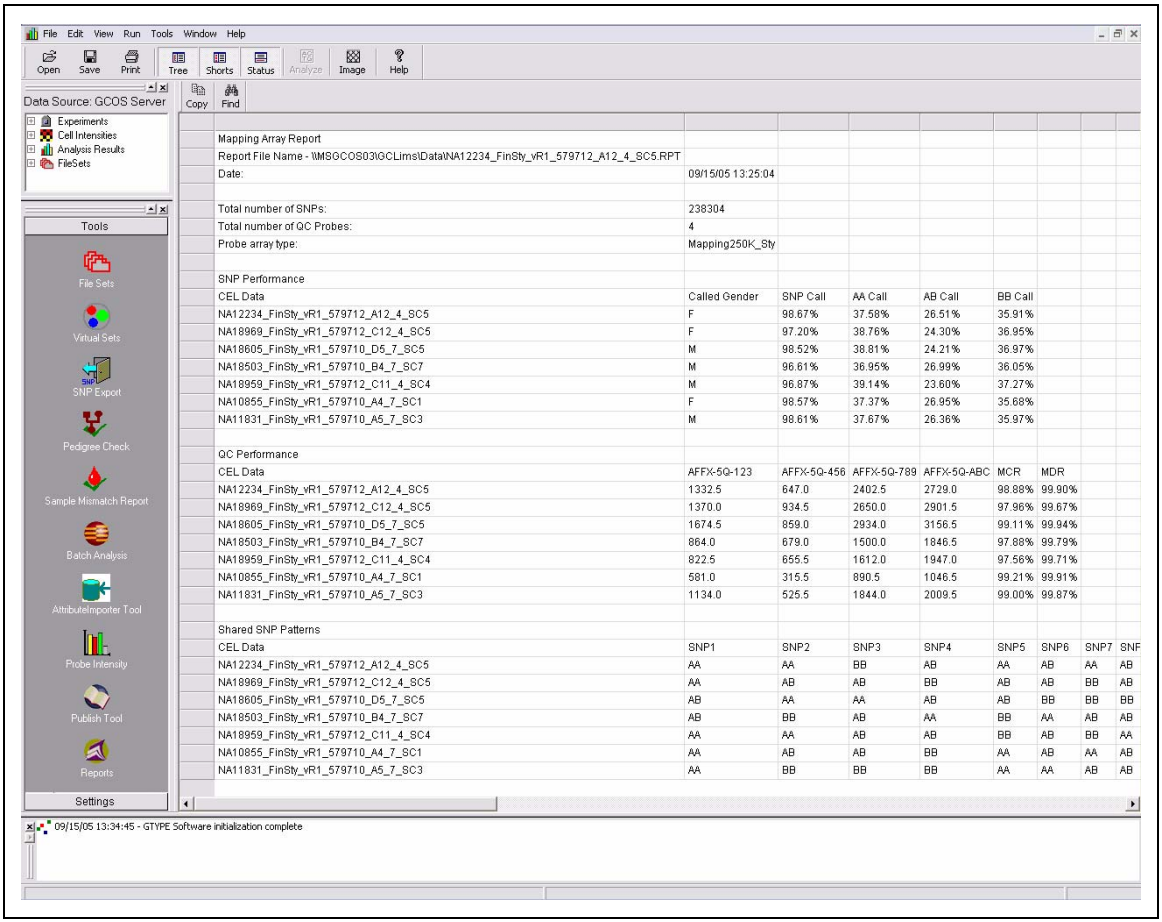
Evaluation of a particular sample should be based on the examination of all samples and array performance metrics.

CALL RATE

Call Rate is displayed in the Dynamic Mapping Algorithm report (Figure 7.6) in the SNP Call column. It is an indicator of the overall performance of the assay. Using the Reference Genomic DNA 103 sample as the standard, a Call Rate in excess of 93% at a confidence score of 0.33 indicates that all steps, from restriction digestion through scanning, worked as expected. A reduced Call Rate may result if an error in any of the assay steps occurs or if lower quality DNA samples are processed. It is also common to observe lower Call Rates in circumstances where a new operator is learning the assay or the number of samples processed at one time increases. In these later examples, it may be prudent to budget time for additional practice for the operator in order to increase proficiency with the assay and achieve higher performance. Some other factors that can lead to a reduced Call Rate include:

- Deviation from the assay protocol
- Contaminated DNA
- Expired reagents
- Inaccurate quantitation

For a sample with a lower Call Rate, it is important to take into consideration the reasons for the lower Call Rate as well as the degree to which accuracy is compromised. It may be necessary to repeat target preparation for that sample depending on the degree to which the lower Call Rate and decrease in accuracy affects the overall experimental goals. Please refer to Chapter 8 for detailed troubleshooting tips.



Mapping Array Report									
Report File Name - \\MSGCOS03\GCLims\Data\NA12234_FinSty_vR1_579712_A12_4_SC5.RPT									
Date:	09/15/05 13:25:04								
Total number of SNPs:	238304								
Total number of QC Probes:	4								
Probe array type:	Mapping250K_Sty								
SNP Performance									
CEL Data	Called Gender	SNP Call	AA Call	AB Call	BB Call				
NA12234_FinSty_vR1_579712_A12_4_SC5	F	98.67%	37.58%	26.51%	35.91%				
NA18969_FinSty_vR1_579712_C12_4_SC5	F	97.20%	38.76%	24.30%	36.95%				
NA18605_FinSty_vR1_579710_D5_7_SC5	M	98.52%	38.81%	24.21%	36.97%				
NA18503_FinSty_vR1_579710_B4_7_SC7	M	96.61%	36.95%	26.99%	36.05%				
NA18959_FinSty_vR1_579712_C11_4_SC4	M	96.87%	39.14%	23.60%	37.27%				
NA10855_FinSty_vR1_579710_A4_7_SC1	F	98.57%	37.37%	26.95%	35.68%				
NA11831_FinSty_vR1_579710_A5_7_SC3	M	98.61%	37.67%	26.36%	35.97%				
QC Performance									
CEL Data	AFFX-5Q-123	AFFX-5Q-456	AFFX-5Q-789	AFFX-5Q-ABC	MCR	MDR			
NA12234_FinSty_vR1_579712_A12_4_SC5	1332.5	647.0	2402.5	2729.0	98.88%	99.90%			
NA18969_FinSty_vR1_579712_C12_4_SC5	1370.0	934.5	2650.0	2901.5	97.96%	99.67%			
NA18605_FinSty_vR1_579710_D5_7_SC5	1674.5	859.0	2934.0	3156.5	99.11%	99.94%			
NA18503_FinSty_vR1_579710_B4_7_SC7	864.0	679.0	1500.0	1846.5	97.88%	99.79%			
NA18959_FinSty_vR1_579712_C11_4_SC4	822.5	655.5	1612.0	1947.0	97.56%	99.71%			
NA10855_FinSty_vR1_579710_A4_7_SC1	581.0	315.5	890.5	1046.5	99.21%	99.91%			
NA11831_FinSty_vR1_579710_A5_7_SC3	1134.0	525.5	1844.0	2009.5	99.00%	99.87%			
Shared SNP Patterns									
CEL Data	SNP1	SNP2	SNP3	SNP4	SNP5	SNP6	SNP7	SNP	
NA12234_FinSty_vR1_579712_A12_4_SC5	AA	AA	BB	AB	AA	AB	AA	AB	
NA18969_FinSty_vR1_579712_C12_4_SC5	AA	AB	AB	BB	AB	AB	BB	AB	
NA18605_FinSty_vR1_579710_D5_7_SC5	AB	AA	AA	AB	AB	BB	BB	BB	
NA18503_FinSty_vR1_579710_B4_7_SC7	AB	BB	AB	AA	BB	AA	AB	AB	
NA18959_FinSty_vR1_579712_C11_4_SC4	AA	AA	AB	AB	BB	AB	BB	AA	
NA10855_FinSty_vR1_579710_A4_7_SC1	AA	AB	AB	BB	AA	AB	AA	AB	
NA11831_FinSty_vR1_579710_A5_7_SC3	AA	BB	BB	BB	AA	AA	AB	AB	

Figure 7.6
Dynamic Model 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 should be used to evaluate DNA quality. Direct comparison to the Reference Genomic DNA 103 control in a 2% agarose gel is one way to accomplish this. If an alternate genomic DNA preparation method is used, it is highly recommended that a small pilot experiment 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 and before PCR cleanup, the PCR yield after cleanup, and a gel image after fragmentation can be used to identify problematic steps. Consult Chapter 8, *Troubleshooting* for further information.

At a minimum, a PCR negative control (water instead of DNA template) should be incorporated into each group of samples processed. The Reference Genomic DNA 103 is included in the assay kit as a positive process control.

DETECTING SAMPLE CONTAMINATION

Monitoring sample contamination is a critical component of sample processing in SNP genotyping. While detection of mixed or contaminated samples is relatively straightforward with multi-allelic markers such as microsatellite markers, it can be more of a challenge for bi-allelic markers. The presence of contamination in a sample reduces genotyping accuracy and therefore genetic power. Guidelines are described in this manual that aid in reducing contamination (i.e., lab set-up) as well as detecting contamination through gel electrophoresis (i.e., process negative controls and PCR negative controls) and analysis metrics in GTYPE (GTYPE Report metrics).

Because of the importance of detecting mixed or contaminated samples, GTYPE has an additional algorithm to supplement the Dynamic Model algorithm to help with sample contamination

detection. The MPAM calling algorithm, used to make genotype calls for the Mapping 10K array, is employed in this analysis to make genotype calls on a subset of ~8,000 SNPs chosen for each of the GeneChip® Mapping 250K Nsp and 250K Sty Arrays. These SNPs were chosen based upon the ability to be a suitable detector of sample contamination across large data sets. Using this subset also allows the software to quickly derive contamination metrics without the additional computational burden of having to make an extra set of calls on the entire array.

In a pure sample, the proportion of labeled DNA target for two alleles of any SNP will typically be present at one of three allelic ratios, 100:0, 50:50 or 0:100. The models used to make genotype calls from probe hybridization data apply this information to determine the genotype call for each SNP, and assign a no call if the observed data fall too far from these predicted ratios. If a mixed or contaminated DNA sample is genotyped, the assumption of only three possible allelic ratios will be violated for many SNPs, resulting in lower Call Rates. However, whether or not the sample is contaminated, the MPAM Detection Rate (MDR) will remain high as it is based only on the extent to which PM probes are brighter than MM probes. Therefore, a reduction in Call Rate (MCR < 0.94), accompanied by no decrease in MDR (MDR > 0.99), is a characteristic indicator of sample contamination (Figure 7.7 and Figure 7.8). Note that this contamination metric does not apply if the MDR is < 99%.

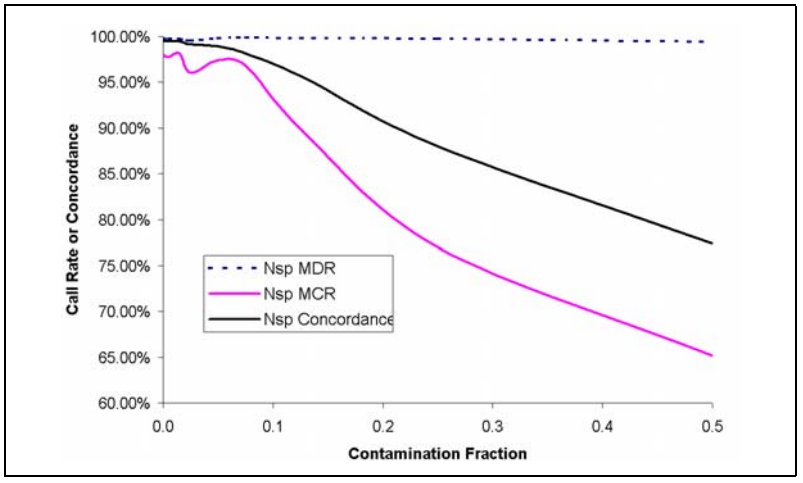


Figure 7.7
The effect of genomic DNA contamination on MPAM Detection Rate (MDR), Concordance Rate, and MPAM Call Rate (MCR) (Nsp).

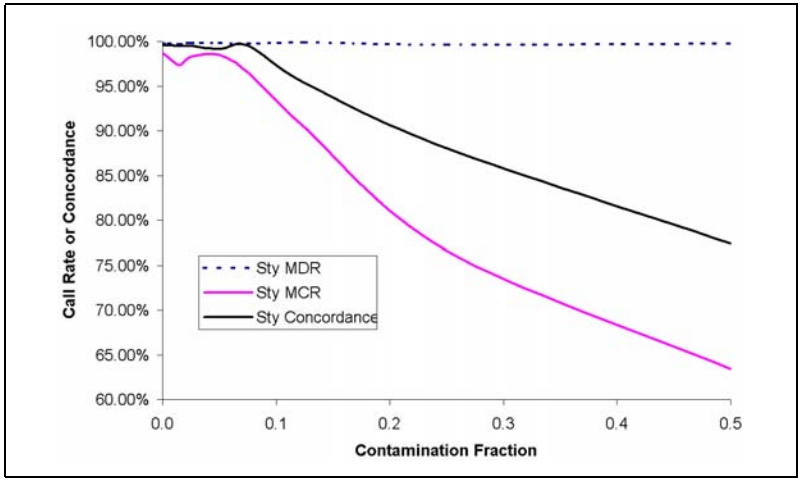


Figure 7.8
The effect of genomic DNA contamination on MPAM Detection Rate (MDR), Concordance Rate, and MPAM Call Rate (MCR) (Sty).

TIP

If you suspect a sample has DNA contamination, you may want to consider testing it with microsatellite markers.

OLIGONUCLEOTIDE CONTROLS

The oligonucleotide control reagent Box (GeneChip® Mapping 250K Assay Kit Box 3) contains oligonucleotide B2 and 4 hybridization control oligonucleotides. Each hybridization control oligonucleotide is present in the hybridization mix at determined concentrations ranging from 0.033 pM to 1.0 pM. The median 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 linear relative to the spike in controls. If they are not detected or do not form a roughly linear response this may indicate a problem in the washing and staining procedure. See Chapter 8, *Troubleshooting* for further information.

SAMPLE MISMATCH REPORT

To prevent mistakes that can commonly occur when working with large genotyping data sets, GTYPE incorporates a new function to prevent sample mismatch. The Sample Mismatch Report identifies .chp files that are potentially labeled incorrectly, resulting from duplicate files, sample labeling errors, or other problems. This function is particularly powerful as a method to analyze large data sets in batch and may be used in conjunction with File Sets for additional flexibility. The Sample Mismatch Report utilizes a set of 50 SNP probe sets that are shared across the Mapping 250K Nsp and Sty arrays. The Sample Mismatch Report also allows for cross-checking previous generations of Mapping arrays.

The Sample Mismatch Report:

- Identifies .chp files with the same SNP and gender calls.
- Identifies .chp files with Sample IDs which agree or disagree with the experimental information. There are two types of errors:
  - .chp files with the same sample IDs, but a different common SNP pattern or gender call.
  - .chp files with different sample IDs but identical shared SNP patterns.

The Sample Mismatch Report performs a pair-wise comparison of the

SNPs and gender calls for each .chp file with every other .chp file in the analysis group. The report shows every .chp file pair with significant SNP overlap. It also shows pairs with the same sample IDs but with significantly different genotyping calls.

The Sample Mismatch Report calculates the Mismatch metric for every pair of .chp files. The Mismatch metric is the averaged IBS (Identity by State), a measure of the number of alleles shared by two individuals. This value increases with the degree of relatedness.

- Matched samples, replicates, or Identical twins should have Mismatch values ~1.95 – 2.
- Sib pairs and parent-offspring should have Mismatch values ranging from 1.3 - 1.8.
- Unrelated pairs should have Mismatch values between 0.9 – 1.3.

For more information about the calculation of the IBS metric, please consult the *GeneChip® Genotyping Analysis Software User's Guide*.

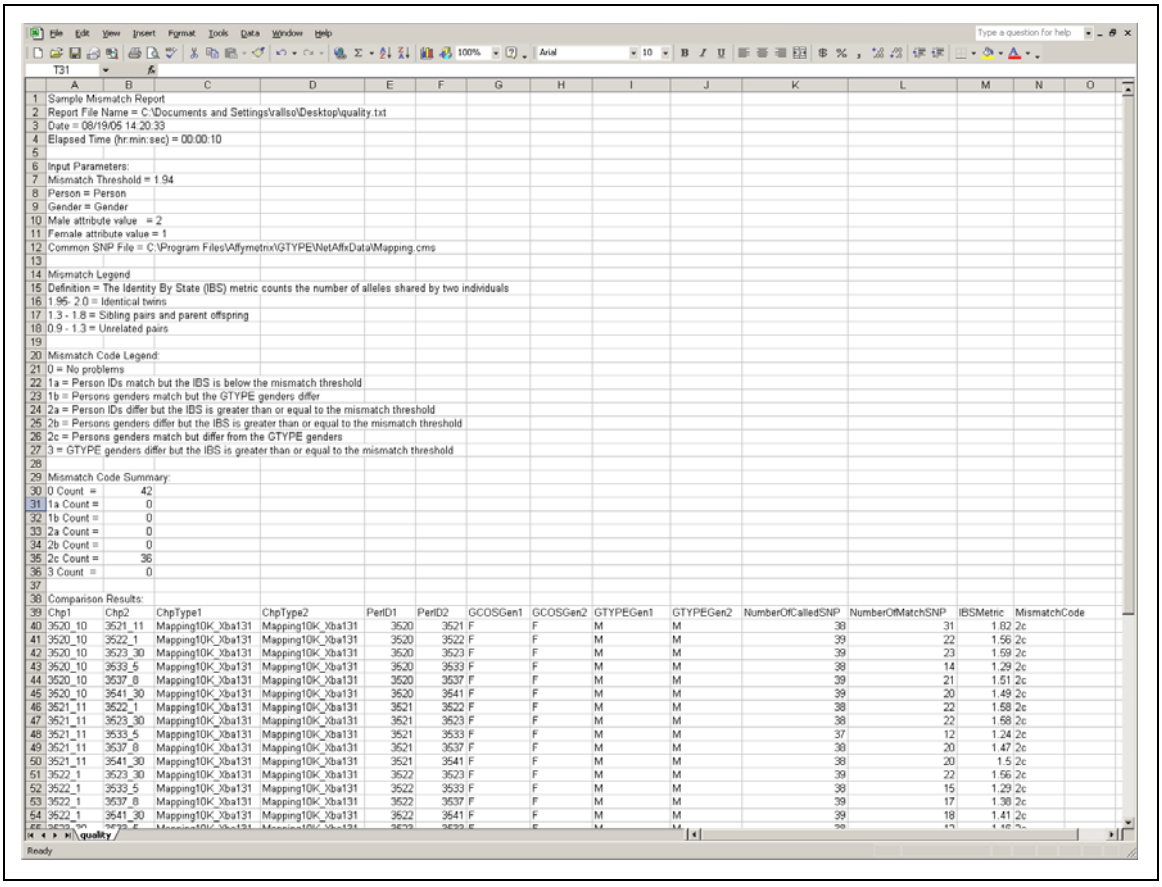
To generate a Sample Mismatch Report:

1. Click the Mismatch Report button in the Genotyping Views shortcut bar; or select Tools → Sample Mismatch Report from the menu bar.
2. Drag and drop files from the Data Tree into the Mismatch Report dialog box.
3. Set the Mismatch Threshold. Default is 1.95.
4. (optional) If you defined Sample identifiers as sample attributes, you can uses these attributes to identify mismatch samples. Click the Define Sample Attributes button to define the sample sample attribute. You can also specify a gender attribute to detect gender misregistrations.

The Sample Mismatch Report (Figure 7.9) is a tab-delimited text file providing information on the experiment, input parameters, a legend of the codes used in the Sample Mismatch Report, as well as a list of data compared showing assigned and called gender, the Mismatch score, and any problems detected in the pair-wise analysis.

For more information on the Sample Mismatch Report and Mismatch score, please consult the *GeneChip® Genotyping Analysis Software User's*

Guide.



Sample Mismatch Report
Report File Name = C:\Documents and Settings\vallso\Desktop\quality.txt
Date = 08/19/05 14:20:33
Elapsed Time (hr:min:sec) = 00:00:10

Input Parameters:
Mismatch Threshold = 1.94
Person = Person
Gender = Gender
Male attribute value = 2
Female attribute value = 1
Common SNP File = C:\Program Files\Affymetrix\GTYPE\NetAffxData\Mapping.cms

Mismatch Legend
Definition = The Identity By State (IBS) metric counts the number of alleles shared by two individuals
1.95- 2.0 = Identical twins
1.3 - 1.8 = Sibling pairs and parent offspring
0.9 - 1.3 = Unrelated pairs

Mismatch Code Legend:
0 = No problems
1a = Person IDs match but the IBS is below the mismatch threshold
1b = Persons genders match but the GTYPE genders differ
2a = Person IDs differ but the IBS is greater than or equal to the mismatch threshold
2b = Persons genders differ but the IBS is greater than or equal to the mismatch threshold
2c = Persons genders match but differ from the GTYPE genders
3 = GTYPE genders differ but the IBS is greater than or equal to the mismatch threshold

Mismatch Code Summary:

0 Count =	42
1a Count =	0
1b Count =	0
2a Count =	0
2b Count =	0
2c Count =	36
3 Count =	0

Comparison Results:

Chp1	Chp2	ChpType1	ChpType2	PerID1	PerID2	GCOSGen1	GCOSGen2	GTYPEGen1	GTYPEGen2	NumberOfCalledSNP	NumberOfMatchSNP	IBSMetric	MismatchCode
3520_10	3521_11	Mapping10K_Xba131	Mapping10K_Xba131	3520	3521	F	F	M	M	38	31	1.82	2c
3520_10	3522_1	Mapping10K_Xba131	Mapping10K_Xba131	3520	3522	F	F	M	M	39	22	1.56	2c
3520_10	3523_30	Mapping10K_Xba131	Mapping10K_Xba131	3520	3523	F	F	M	M	39	23	1.59	2c
3520_10	3533_5	Mapping10K_Xba131	Mapping10K_Xba131	3520	3533	F	F	M	M	38	14	1.29	2c
3520_10	3537_8	Mapping10K_Xba131	Mapping10K_Xba131	3520	3537	F	F	M	M	39	21	1.51	2c
3520_10	3541_30	Mapping10K_Xba131	Mapping10K_Xba131	3520	3541	F	F	M	M	39	20	1.49	2c
3521_11	3522_1	Mapping10K_Xba131	Mapping10K_Xba131	3521	3522	F	F	M	M	38	22	1.58	2c
3521_11	3523_30	Mapping10K_Xba131	Mapping10K_Xba131	3521	3523	F	F	M	M	38	22	1.58	2c
3521_11	3533_5	Mapping10K_Xba131	Mapping10K_Xba131	3521	3533	F	F	M	M	37	12	1.24	2c
3521_11	3537_8	Mapping10K_Xba131	Mapping10K_Xba131	3521	3537	F	F	M	M	38	20	1.47	2c
3521_11	3541_30	Mapping10K_Xba131	Mapping10K_Xba131	3521	3541	F	F	M	M	38	20	1.5	2c
3522_1	3523_30	Mapping10K_Xba131	Mapping10K_Xba131	3522	3523	F	F	M	M	39	22	1.56	2c
3522_1	3533_5	Mapping10K_Xba131	Mapping10K_Xba131	3522	3533	F	F	M	M	38	15	1.29	2c
3522_1	3537_8	Mapping10K_Xba131	Mapping10K_Xba131	3522	3537	F	F	M	M	39	17	1.38	2c
3522_1	3541_30	Mapping10K_Xba131	Mapping10K_Xba131	3522	3541	F	F	M	M	39	18	1.41	2c

Figure 7.9
Sample Mismatch Report

B2 OLIGO PERFORMANCE

The B2 oligo is a component of the Oligo Control Reagent, 0100 (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 7.10) and throughout the array
- The array name, located in the lower left corner of the array (Figure 7.11)

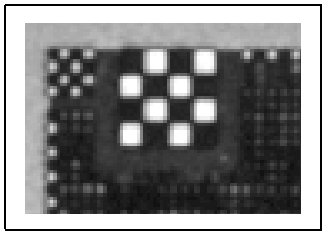


Figure 7.10
Example of corner checkerboard pattern

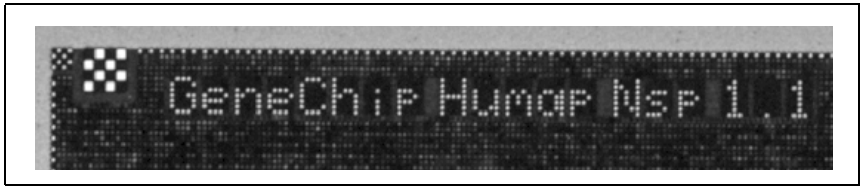


Figure 7.11
Array name (image has been rotated for display)

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.

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 250K Assay Kits, 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.

NOTE **Microsoft Excel is limited to a maximum of 65,536 rows. Because your data will exceed this number, Microsoft Excel will give you a warning when you paste in your data.**

1. Open the reference and experimental Genotype calls in GTYPE.
   - **A.** Sort the calls in ascending order on the SNP ID.
   - **B.** Check that the SNP IDs match.
2. Copy and paste the calls from GTYPE and paste into Microsoft Excel. Create a header row.

Table 7.3
Columns used to calculate concordance

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

3. 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 the column.
4. 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="NoCall",C2="NC")”.
   - **B.** Auto fill down the column.
5. 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 the column.
6. Count the number of SNPs that had a genotype call in both experiments.
   - **A.** Within the spreadsheet, enter the following formula into a cell: “=COUNTIF(E2:65536,"FALSE")”.
   - **B.** Press return.
7. Count the number of SNPs that have a genotype call in both experiments and are concordant.
   - **A.** Elsewhere within the spreadsheet, enter the following formula into a cell: “=COUNTIF(F2:65536,"TRUE")”.
   - **B.** Press return.
8. The Concordance rate can be calculated by dividing the value from step 7 by the value from step 6.

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 Filtering

For many genotyping applications, loss of accuracy can result in a significant decrease in genetic power. This loss of accuracy can be caused by systematic or sporadic errors that occur due to stochastic, sample or experimental factors. To filter out errors and exclude these SNPs in downstream analysis, a two-tiered filtering process is recommended. In the first filter, samples are included only if the call rate is greater than 93% at a 0.33 confidence score when using high quality DNA (see Chapter 3). Those filtered samples are then evaluated in a secondary SNP filter where any SNP with less than an 85% call rate across samples is eliminated from the analysis. These two filters together should result in a high quality set of samples and SNPs for use in downstream analysis.

Studies on multiple 500K data sets have shown that SNPs with a lower per SNP call rate tend to have a higher error rate, and disproportionately contribute to the overall error rate in the experiment.

Consider taking additional 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. GTYPE has functionality to identify SNPs showing Mendelian or Hardy Weinberg errors in user-defined samples. These genotyping errors can be filtered out upon data export. Please consult the *GeneChip® Genotyping Analysis Software User's Guide* for more information.

Adjust the Default Value to Increase or Decrease Accuracy and Call Rate

GTYPE 4.0 software provides flexible options to enable a trade off between Call Rate and genotyping accuracy. The Dynamic Model Mapping Algorithm uses the Wilcoxon Signed Rank Test to assign a confidence score measuring the reliability of a genotype call (see Appendix D in the *GeneChip® Genotyping Analysis Software User's Guide*)[1,2].

We have chosen a confidence score value of 0.33 as the default because it gives a good compromise between accuracy and Call Rate, especially when the 85% per sample filter is applied. In cases where you see a lower Call Rate, you may wish to consider reanalyzing the data at the more stringent confidence score (e.g., 0.26), which will decrease the Call Rate for the sample, but may increase the accuracy of the genotypes.

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 (e.g., out of Hardy Weinberg equilibrium) have been removed through a SNP selection process[1]. Overall the accuracy has been shown to be approximately 99.5% on the Mapping 500K Array Set based upon internal data.

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. GTYPE has functionality to identify SNPs showing Mendelian or Hardy Weinberg errors in user defined samples. These genotyping errors can be filtered out upon data export. Please consult the *GeneChip® Genotyping Analysis Software User's Guide* for more information.

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[1] *Matsuzaki H., Dong S., Loi H., Di X., Liu G., Hubbell E., Law J., Berntsen T., Chadha M., Hui H., Yang G., Kennedy G., Webster T., Cawley S., Walsh P., Jones K., Fodor S., Mei R. Genotyping over 100,000 SNPs on a pair of oligonucleotide arrays. Nat Methods 1:109-111 (2004).*

[2] *Di X., Matsuzaki H., Webster T.A., Hubbell E., Liu G., Dong S., Bartell D., Huang J., Chiles R., Yang G., Shen M.M., Kulp D., Kennedy G.C., Mei R., Jones K.W., Cawley S. Dynamic model based algorithms for screening and genotyping over 100K SNPs on oligonucleotide microarrays. Bioinformatics 21:1958-63 (2005).*

[1] *See SNP Selection Criteria for the GeneChip Human Mapping 10K Array Xba 131 Technical Note. A similar process was used for the Mapping 500K SNP selection.*

Chapter 8

Troubleshooting

Chapter 8

Assay Recommendations

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 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. For example, always use AccuGENE® water from Cambrex, and ligase and restriction enzymes from New England Biolabs.

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°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°C directly after use. Take care when pipetting enzymes stored in glycerol, which is viscous. Do not store at –80°C.

4. Fragmentation Reagent (DNAse I) activity can decline over time after dilution on ice, and so the reagent should be added to samples as quickly as possible.
5. The use of master mixes prepared with an excess (15% for 96-well plate protocol; 5% for low throughput protocol) 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. We highly recommend the PCR thermal cyclers be calibrated regularly. 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 200 to 1,100 bp size range. See Figure 4.3 on page 73.

   Following fragmentation, run samples on a 4% agarose gel. Successful fragmentation is confirmed by the presence of a smear of less than 200 bp in size, shown in Figure 4.7 on page 106.
8. Run controls in parallel with each group of samples.

   Substitute 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 successful 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, washing, staining, and sensitivity of the array. The oligonucleotide control reagents contain oligo B2 which is used for grid alignment.

10. For the 96-well plate protocol, we highly recommend all mutlichannel pipettes be calibrated regularly.

11. For the 96-well plate protocol, we recommend using a team approach to sample processing for greater efficiency. This approach is described on page 37.

Important Differences Between GeneChip® Mapping Arrays and GeneChip® Expression Arrays

12. For laboratories that also run GeneChip Expression arrays it is important to check the temperature setting on the Hybridization Oven 640. For the GeneChip® Mapping 250K Nsp Array and the GeneChip® Mapping 250K Sty Array, ovens should be set to 49°C. The temperature for hybridization on expression arrays is 45°C.

    We also recommend that your hybridization ovens be serviced at least once per year to ensure that they are operating within manufacture specifications

13. 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 500K Set. Also, care should be taken to ensure the fluidics station is properly maintained and primed with the correct buffers prior to use.

14. Both the GeneChip Mapping 500K and Expression protocols use the same stain reagents for each staining step. However, after the last wash the Mapping 250K Array is filled with Array Holding Buffer.

15. The arrays in the GeneChip® Mapping 500K Set are scanned once at 570 nm on the GeneChip® Scanner 3000 7G.

Troubleshooting Guide for the GeneChip® Mapping 500K Assay

Problem	Likely Cause	Solution
Faint/absent bands on PCR gel		
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.	Use restriction enzyme to digest a known good DNA sample. Run gel to confirm restriction enzyme activity.
	Failed adaptor ligation reaction.	Confirm enzyme activity.
		Ligase buffer contains ATP and should be defrosted/ held at 4°C. Mix ligase buffer thoroughly before use to ensure precipitate is re-suspended. Avoid multiple freeze-thaw cycles. Try a fresh tube of buffer.
	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.
		Thoroughly mix PCR reaction.
	Ligation mix not diluted prior to PCR reaction.	Ligation mixture diluted 1:4 with molecular biology grade water to remove potential inhibitors and maintain optimal pH and salt concentration.
	Incorrect concentration of nucleotides.	Check dNTP stock concentration and vendor.
	Used Nsp adaptor for Sty digest, or vice versa.	Repeat Ligation step with correct adaptors.

Problem	Likely Cause	Solution
Faint/absent bands on PCR gel (continued)		
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.
	Insufficient starting material.	250 ng genomic DNA should be used. Confirm concentration using calibrated spectrophotometer.
	Sample DNA contains enzymatic or chemical inhibitors.	Ensure genomic DNA is purified and diluted in Low EDTA (0.1mm) TE buffer.
		Use recommended procedure to ethanol precipitate genomic DNA to remove inhibitors.
	Degraded sample DNA.	Confirm quality of genomic DNA sample.
Low PCR yield		
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 is lost due to reduced temperature.	All purification steps must be carried out at 25 to 35°C.
	Pipette used for quantitation not calibrated.	Calibrate Pipette. Proceed with assay.
White precipitate is seen after the PCR reaction		
	Precipitation from assay reagents.	Add 0.1 M EDTA, as per protocol, before PCR product purification. This precipitate should no longer be present after purification.

Problem	Likely Cause	Solution
Fragmented PCR product is not the correct size		
PCR product is still visible in 200-1,100 bp size region	Incomplete fragmentation due to underestimated DNA concentration.	Ensure spectrophotometer is properly calibrated so only 90 µg of DNA is added to the fragmentation reaction.
	Failed or incomplete fragmentation due to reduced DNase activity.	Check that you have entered the correct activity of DNase into the formula for calculating amount of DNase to add to fragmentation reaction. (See *Dilute the Fragmentation Reagent* on page 102; amount is the same for high and low throughput.)
		Ensure fragmentation reagent (DNase I) is kept at –20°C. Do not reuse diluted working stock.
.CEL file can not be generated		
GCOS 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.
.dat 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.
Low SNP call rates		
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/µL on the label and check dilution formula (*Dilute the Fragmentation Reagent* on page 102; amount is the same for high and low throughput). Work quickly and on ice; transfer reaction tubes to pre-heated thermal cycler (37°C). Mix thoroughly.
Extremely low call rate Sample hybridization is absent on .cel and .dat images but B2 grid is bright.	Labeling reaction suboptimal.	Use a new vial of Terminal Dideoxynucleotidyl Transferase. Verify the labeling reagents and repeat labeling.

Problem	Likely Cause	Solution
Positive control has good call rates but samples are lower than expected.	Genomic DNA not optimal.	Ensure DNA samples are of high quality (i.e., run in a 1 to 2% gel and compare to Reference 103 DNA control). Use positive control sample as a reference guide for assay procedures. Prepare master mixes for samples and controls.
MDR high, MCR low.	Mixed or contaminated genomic DNA sample (page 197).	Use uncontaminated stock of DNA sample.
Very low call rates	Mixed up Nsp arrays and Sty sample, or vice versa.	Do Nsp and Sty work on separate days.

OD Troubleshooting Guidelines

Refer to the tables below when troubleshooting OD readings.

Table 8.1
PROBLEM: Average Sample OD is greater than 0.7 (3.5 µg/µL)

If the average sample OD of three independent measurements is greater than 0.7 (calculated concentration greater than 3.5 µg/µL), a problem exists with either the elution of PCR products or the OD reading. The limit on PCR yield is approximately 3.5 µg/µL, as observed in practice and as predicted by the mass of dNTPs in the reaction.

Possible causes include:

- The purified PCR product was eluted in a volume less than 45 µL.
- The purified PCR product was not mixed adequately before making the 1:100 dilution.
- The diluted PCR product was not mixed adequately before taking the OD reading.
- The water blank reading was not subtracted from each sample OD reading.
- The spectrophotometer plate reader may require calibration.
- Pipettes may require calibration.
- There may be air bubbles or dust in the OD plate.
- There may be defects in the plastic of the plate.
- The settings on the spectrophotometer plate reader or the software may be incorrect.
- OD calculations may be incorrect and should be checked.

Reliance on any single OD reading may give an outlier result. You should make three independent dilutions and take three independent OD readings per dilution.

Table 8.2
PROBLEM: Average Sample OD is Less Than 0.5 (2.5 µg/µL)

If the average sample OD of three independent measurements is less than 0.5 (calculated concentration less than 2.5 µg/µL), a problem exists with either the genomic DNA, the PCR reaction, the elution of purified PCR products, or the OD readings.

Possible problems with input genomic DNA that would lead to reduced yield include:

- The presence of inhibitors (heme, EDTA, etc.).
- Severely degraded genomic DNA.
- Inaccurate concentration of genomic DNA.

  NOTE: Check the OD reading for the PCR products derived from RefDNA 103 as a control for these issues.

To prevent problems with the PCR reaction that would lead to reduced yield:

- Use the recommended reagents and vendors (including AccuGENE® water) for all PCR mix components.
- Thoroughly mix all components before making the PCR Master Mix.
- Pipette all reagents carefully, particularly the PCR Primer, when making the master mix.
- Check all volume calculations for making the master mix.
- Store all components and mixes on ice when working at the bench. Do not allow reagents to sit at room temperature for extended periods of time.
- Be sure to use the recommended PCR plates. Plates from other vendors may not fit correctly in the thermal cycler block. Differences in plastic thickness and fit with the thermal cycler may lead to variance in temperatures and ramp times.
- Be sure to use the correct cycling mode when programming the thermal cycler (*maximum mode* on the GeneAmp® PCR System 9700; *calculated mode* on the MJ Tetrad PTC-225).
- Be sure to use silver or gold-plated silver blocks on the GeneAmp® PCR System 9700 (other blocks are not capable of maximum mode, which will affect ramp times).
- Use the recommended plate seal. Make sure the seal is tight and that no significant evaporation occurs during the PCR.

NOTE: The Mapping 500K PCR reaction amplifies a size range of fragments that represents 15-20% of the genome. The Mapping 500K arrays are designed to detect the SNPs that are amplified in this complex fragment population. Subtle changes in the PCR conditions may not affect the PCR yield, but may shift the amplified size range up or down very slightly. This can lead to reduced amplification of SNPs that are assayed on the array set, subsequently leading to lower call rates.

Table 8.2
PROBLEM: Average Sample OD is Less Than 0.5 (2.5 µg/µL)

Troubleshooting Possible Problems with the Elution or OD Readings – possible causes include:

- The purified PCR product was eluted in a volume greater than 45 µL.
- The purified PCR product was not mixed adequately before making the 1:100 dilution.
- The diluted PCR product was not mixed adequately before taking the OD reading.
- The water blank reading was not subtracted from each sample OD reading.
- The spectrophotometer plate reader may require calibration.
- Pipettes may require calibration.
- There may be air bubbles or dust in the OD plate.
- There may be defects in the plastic of the plate.
- The settings on the spectrophotometer plate reader or the software may be incorrect.
- OD calculations may be incorrect and should be checked.

Reliance on any single OD reading may give an outlier result. You should make three independent dilutions and take three independent OD readings per dilution.

Table 8.3
PROBLEM: OD260/OD280 ratio is not between 1.8 and 2.0

Possible causes include:

- The PCR product may be not be sufficiently purified. Be sure to perform three water washes and check to be sure the vacuum manifold is working properly.
- An error may have been made while taking the OD readings.

Table 8.4
PROBLEM: The OD320 measurement is significantly larger than zero (0 $\pm$ 0.005)

Possible causes include:

- Precipitate may be present in the eluted samples. Be sure to add diluted EDTA to PCR products before purification.
- There may be defects in the OD plate.
- Air bubbles in the OD plate or in solutions.

When to Contact Technical Support

Under any of the following conditions, unplug the instrument from the power source and contact Affymetrix Technical Support:

- when the power cord is damaged or frayed;
- if any liquid has penetrated the instrument;
- if, after service or calibration, the instrument does not perform to the specifications stated in *Instrument Specifications* on page 178.

If the instrument must be returned for repair, call Affymetrix Technical Support.

IMPORTANT **Make sure you have the model and serial number.**

Affymetrix, Inc.
3420 Central Expressway
Santa Clara, CA 95051
USA

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

Affymetrix UK Ltd
Voyager, Mercury Park,
Wycombe Lane, Wooburn Green,
High Wycombe HP10 0HH
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E-mail: supporteurope@affymetrix.com
UK and Others Tel: +44 (0) 1628 552550
France Tel: 0800919505
Germany Tel: 01803001334
Fax: +44 (0) 1628 552585

Affymetrix Japan, K. K.
Mita NN Bldg
16 Floor, 4-1-23 Shiba,
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Japan

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Appendix A

Reagents, Equipment, and Consumables Required for 96-Well Plate Protocol

Appendix A

Reagents, Equipment, and Consumables Required for 96-Well Plate Protocol

ABOUT THIS APPENDIX

This appendix includes the vendor and part number information for the reagents, equipment and consumables that have been validated for use with the GeneChip® Mapping 500K 96-well plate protocol.

IMPORTANT !

Use only the PCR plate, adhesive film and thermal cyclers listed in Table A.7. Using other PCR plates and film that are incompatible with the thermal cycler can result in crushed tubes, loss of sample, or poor results.

The following lists of reagents, equipment and consumables are included in this appendix:

Reagents

AFFYMETRIX REAGENTS REQUIRED

Table A.1
Affymetrix GeneChip® Mapping 250K Nsp Assay Kit – 100 Reactions

Kit Contents	Part Number
• Adaptor Nsp, 50 µM	900753 (100 reactions)
• DNA Labeling Reagent, 30 mM	
• Fragmentation Buffer	
• Fragmentation Reagent	
• Oligo Control Reagent, 0100	
• PCR Primer 002, 100 µM	
• Terminal Deoxynucleotidyl Transferase	
• 5X Terminal Deoxynucleotidyl Transferase Buffer	
• Reference Genomic DNA 103 (use as a positive control)	

Table A.2
Affymetrix GeneChip® Mapping 250K Sty Assay Kit – 100 Reactions

Kit Contents	Part Number
• Adaptor Sty, 50 µM	900754 (100 reactions)
• DNA Labeling Reagent, 30 mM	
• Fragmentation Buffer	
• Fragmentation Reagent	
• Oligo Control Reagent, 0100	
• PCR Primer 002, 100 µM	
• Terminal Deoxynucleotidyl Transferase	
• 5X Terminal Deoxynucleotidyl Transferase Buffer	
• Reference Genomic DNA 103 (use as a positive control)	

NEW ENGLAND BIOLABS REAGENTS REQUIRED

Table A.3
New England Biolabs Reagents Required

Reagent	Description	Part Number
Nsp I, 125 µL vial	10,000 U/mL containing: • Bovine Serum Albumin (BSA); NEB P/N B9001S • NE Buffer 2; NEB P/N B7002S The BSA and NE Buffer can be ordered separately using these part numbers.	R0602L
Sty I, 300 µL vial	10,000 U/mL containing: • Bovine Serum Albumin (BSA); NEB P/N B9001S • NE Buffer; NEB P/N B7003S The BSA and NE Buffer can be ordered separately using these part numbers.	R0500S
T4 DNA Ligase, 250 µL vial	Contains: • T4 DNA Ligase • T4 DNA Ligase Buffer; NEB P/N B202S	M0202L

OTHER REAGENTS REQUIRED

Table A.4
Other Reagents Required for the Mapping 500K 96-Well Plate Protocol

Reagent	Vendor	Description	Part Number
TITANIUM™ DNA Amplification Kit	Clontech	Contains: • 50X TITANIUM™ *Taq* DNA Polymerase • 10X TITANIUM™ *Taq* PCR Buffer • GC-Melt • dNTPs	639240 (300 reactions-enough for 100 samples)
DNA Amplification Clean-Up Kit	Clontech	Each kit includes RB Buffer	1 Plate: 636974 4 Plates: 636975
TITANIUM™ *Taq* DNA Polymerase (50X) and TITANIUM™ *Taq* PCR Buffer	Clontech	Conains: • 50X Clontech TITANIUM™ *Taq* DNA Polymerase • 10X Clontech TITANIUM™ *Taq* PCR Buffer	P/N 639209
GC-Melt	Clontech	5 M	639238
dNTPs*	Takara	mixture of dATP, dCTP, dGTP, dTTP at 2.5 mM each	4030
	Fisher Scientific		TAK 4030
Denhart's Solution	Sigma-Aldrich		D2532
DMSO	Sigma-Aldrich		D5879
EDTA	Ambion	0.5 M, pH 8.0 (dilute to 0.1 M before use)	9260G
Herring Sperm DNA (HSDNA)	Promega		D1815
Human Cot-1 DNA®	Invitrogen		15279-011
MES Hydrate SigmaUltra	Sigma-Aldrich		M5287
MES Sodium Salt	Sigma-Aldrich		M5057
Reduced EDTA TE Buffer	TEKnova	10 mM Tris HCL, 0.1 mM EDTA, pH 8.0	T0223
RB Buffer	Contained in the DNA Amplification Clean-Up Kit listed above.		
Tetramethyl Ammonium Chloride (TMACL; 5M)	Sigma-Aldrich	5M	T3411

Table A.4
Other Reagents Required for the Mapping 500K 96-Well Plate Protocol

Reagent	Vendor	Description	Part Number
Tween-20, 10%	Pierce	10%, diluted to 3% in molecular biology-grade water	28320 (Surfact-AmpsΩ®)
Water, AccuGENE®	Cambrex	AccuGENE® Molecular Biology-Grade Water, 1 L	51200

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.

Equipment and Software Required

The 96-well plate protocol has been optimized using the following equipment and software.

AFFYMETRIX EQUIPMENT AND SOFTWARE REQUIRED

Table A.5
Affymetrix Equipment and Software Required

Item	Part Number
GeneChip® Fluidics Station 450*	00-0079
GeneChip® Hybridization Oven 640*	800139
GeneChip® Scanner 3000 7G*	00-0205
GeneChip® Operating Software version 1.4*	690031
GeneChip® Genotyping Analysis Software 4.0*	690051

** 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.*

OTHER EQUIPMENT REQUIRED

Table A.6
Other Equipment Required to Run the GeneChip® Mapping 500K 96-Well Plate Protocol

Equipment		Quantity	Manufacturer/ Distributor	Part Number	Laboratory Location
Cooler	StrataCooler® Lite Benchtop	2	Stratagene	400012	Pre-PCR and Main Lab
	StrataCooler® II Benchtop			400002 (blue) 400008 (red)	
Cooling chamber	single gold block	2 double and 1 single	Diversified Biotech	CHAM-1000	1 single in Pre-PCR; enough for 4 plates in Main Lab
	double gold block			CHAM-1020	
Heat block (required only if using tubes for *Stage 9: Target Hybridization*)		2	Fisher Scientific	11-718-9	Main Lab
Ice bucket	—	2	—	—	Pre-PCR and Main Lab
Jitterbug™, 115V AC		1	Boekel Scientific	130000	Main Lab
Manifold, QIAvac multiwell unit		1	QIAGEN	9014579	Main Lab
Microcentrifuge, PicoFuge® (maximum rotation 6000 rpm)		2	Stratagene	400550	Pre-PCR and Main Lab
Pipet-Lite™, Magnetic-Assist single channel P10		2	Rainin	L-10	Main Lab
Pipet-Lite™, Magnetic-Assist single channell P20		2	Rainin	L-20	Pre-PCR and Main Lab
Pipet-Lite™, Magnetic-Assist single channel P100		2	Rainin	L-100	Main Lab
Pipet-Lite™, Magnetic-Assist single channel P200		2	Rainin	L-200	Pre-PCR and Main Lab
Pipet-Lite™, Magnetic-Assist single channel P1000		2	Rainin	L-1000	Main Lab
Pipette, 12-channel P20 (accurate to within ± 5%)		2	Rainin	P/N L12-20	Pre-PCR and Main Lab

Table A.6
Other Equipment Required to Run the GeneChip® Mapping 500K 96-Well Plate Protocol

Equipment	Quantity	Manufacturer/ Distributor	Part Number	Laboratory Location
Pipette, 12-channel P100	2	Rainin	P/N L12-100	Pre-PCR and Main Lab
Pipette, 12-channel P200	2	Rainin	P/N L12-200	Pre-PCR and Main Lab
Plate, UV Star Transparent, 96-well	3	E&K Scientific	EK-25801	Main Lab
Plate Centrifuge, multipurpose	2	Eppendorf	5804 or 5810	Pre-PCR and Main Lab
Plate stand (optional)	1	Diversified Biotech	WPST-1000	Main Lab
Spectrophotometer, high throughput microplate spectrophotometer	1	Molecular Devices	SpectraMax Plus^{384}	Main Lab
Thermal Cyclers – see Table A.7 on page 232				
Timers (required only if using tubes for *Stage 9: Target Hybridization*)	4	—	—	Main Lab
Vacuum regulator Protocol requires 600 mb vacuum. If your lab does not have an internally regulated vacuum source, this regulator is strongly recommended.	1	QIAGEN	19530	Main Lab
Vortexer, for plates and tubes	2	VWR	58816-12	Pre-PCR and Main Lab

THERMAL CYCLERS, PCR PLATES AND PLATE SEALS

The 96-well plate protocol has been optimized using the following thermal cyclers, PCR plate and adhesive film.

IMPORTANT !

Use only the PCR plate, adhesive film and thermal cyclers listed in Table A.7. Using other PCR plates and film that are incompatible with the thermal cycler can result in crushed tubes, loss of sample, or poor results.

Table A.7
Thermal Cyclers, PCR Plates and Plate Seals Optimized for Use With the Mapping 500K 96-Well Plate Protocol

Area	Thermal Cyclers Validated for Use	Plate	Cover
Pre-PCR	2720 Thermal Cycler or GeneAmp® PCR System 9700 by Applied Biosystems MJ Tetrad PTC-225 by Bio-Rad	Multiplate 96-Well Unskirted PCR Plates Bio-Rad, P/N MLP-9601	MicroAmp® Clear Adhesive Films Applied Biosystems, P/N 4306311
PCR and Post-PCR	GeneAmp® PCR System 9700 by Applied Biosystems (silver block or gold-plated silver block) MJ Tetrad PTC-225 by Bio-Rad	Multiplate 96-Well Unskirted PCR Plates Bio-Rad, P/N MLP-9601	MicroAmp® Clear Adhesive Films Applied Biosystems, P/N 4306311

Consumables Required

GENECHIP® ARRAYS REQUIRED

The following GeneChip® arrays are required:

Table A.8
Arrays Required for the Mapping 500K 96-Well Plate Protocol

Array	Part Number
GeneChip® Human Mapping 250K Nsp Array* (minimum order is 200 arrays)	520330 (1 array)
GeneChip® Human Mapping 250K Nsp Array*	900768 (30 arrays)
GeneChip® Human Mapping 250K Sty Array* (minimum order is 200 arrays)	520331 (1 array)
GeneChip® Human Mapping 250K Sty Array*	900770 (30 arrays)

** Denotes critical equipment. Vendors not listed here have not been tested and verified at Affymetrix.*

OTHER CONSUMABLES REQUIRED

Table A.9
Other Consumables Required for the Mapping 500K 96-Well Plate Protocol

Item	Manufacturer/ Distributor	Part Number	Laboratory Location
Gel, Reliant® Gel System, precast agarose gel (2% SeaKem Gold, TBE)	Cambrex	54939	Main Lab
DNA Marker	Bionexus	All Purpose Hi-Lo, BN2050	Main Lab
Gel Loading Solution	Sigma-Aldrich	G2526	Main Lab

Table A.9
Other Consumables Required for the Mapping 500K 96-Well Plate Protocol

Item	Manufacturer/ Distributor	Part Number	Laboratory Location
Pipette tips As needed for pipettes listed in Table A.6.	Rainin	GP-L10F GP-L200F GP-L1000F RT-L10F RT-L200F RT-L1000F GP = refill RT = with rack	Pre-PCR and Main Lab
Plate seals – see Table A.7 on page 232			Pre-PCR and Main Lab
Plates, 96-well PCR – see Table A.7 on page 232			Pre-PCR and Main Lab
Plates, 96-well UV Star, 370 µL/well	E&K Scientific	25801	Main Lab
Solution Basin, 100 mL sterile, multichannel	Labcor	730-014	Main Lab
Solution Basin, 55 mL sterile, multichannel	Labcor	730-004	Pre-PCR and Main Lab
Solution Basin lid, 55 mL	Labcor	730-021	Pre-PCR and Main Lab
Tough-Spots®	Diversified Biotech	SPOT-1000	Main Lab
	USA Scientific	9185-1000	
Tubes, strip of 12, thin wall (0.2 mL)	CLP Direct	3426.12	Pre-PCR and Main Lab
	ISC BioExpress	T-3114-1	
Tube, centrifuge 15 mL	VWR	20171-020	Main Lab
Tube, centrifuge 50 mL	VWR	21008-178	Main Lab
Tube, Eppendorf 2.0 mL	VWR	20901-540	Pre-PCR
Tube, Eppendorf Safe-Lock, 1.5 mL (required only if using tubes for *Stage 9: Target Hybridization*)	VWR	21008-959	Main Lab
Tube, Falcon, 50 mL	VWR	21008-940	Pre-PCR

Supplier Contact List

Table A.10
Supplier Contact List

Supplier	Web Site
Affymetrix	www.affymetrix.com
Ambion	www.ambion.com
Applied Biosystems	home.appliedbiosystems.com
Bionexus Inc.	www.bionexus.net
Bio-Rad	www.bio-rad.com
Boekel Scientific	www.boekelsci.com
Cambrex	www.cambrex.com
CLP Direct	www.clpdirect.com
Clontech	www.clontech.com/clontech
Diversified Biotech	www.divbio.com
E&K Scientific	www.eandkscientific.com
Eppendorf	www.eppendorf.com
Fisher Scientific	www.fisherscientific.com
Invitrogen Life Technologies	www.invitrogen.com
ISC BioExpress	www.bioexpress.com
Labcor	www.labcorproducts.com
Molecular Devices	www.moleculardevices.com
New England Biolabs	www.neb.com
Pierce Biotechnology	www.piercenet.com
Promega	www.promega.com
QIAGEN	www.qiagen.com
Rainin	www.rainin.com
Sigma-Aldrich	www.sigma-aldrich.com

Table A.10
Supplier Contact List

Supplier	Web Site
Stratagene	www.stratagene.com
Takara Bio Inc.	www.takara-bio.com
Teknova	www.teknova.com
VWR	www.vwrsp.com

Appendix B

Thermal Cycler Programs

Appendix B

Thermal Cycler Programs

ABOUT THIS APPENDIX

This appendix includes the thermal cycler programs required for the GeneChip® Mapping 500K protocols.

Before you begin processing samples, enter and save the programs into the appropriate thermal cyclers.

500K DIGEST

Required for the 96-well plate and the low throughput protocol.

500K Digest Program	
Temperature	**Time**
37°C	120 minutes
65°C	20 minutes
4°C	Hold

500K LIGATE

Required for the 96-well plate and the low throughput protocol.

500K Ligate Program	
Temperature	**Time**
16°C	180 minutes
70°C	20 minutes
4°C	Hold

500K PCR

Required for the 96-well plate and the low throughput protocol.

For the GeneAmp® PCR System 9700

You must use GeneAmp PCR System 9700 thermal cyclers with silver or gold-plated silver blocks. Do not use GeneAmp® PCR System 9700 thermal cyclers with aluminum blocks.

Ramp speed: Max Volume: 100 µL

500K PCR Program for GeneAmp® PCR System 9700		
Temperature	**Time**	**Cycles**
94°C	3 minutes	1X
94°C	30 seconds	30X
60°C	45 seconds	
68°C	15 seconds	
68°C	7 minutes	1X
4°C	HOLD (Can be held overnight)	

For the MJ Tetrad PTC-225

Use: *Heated Lid* and *Calculated Temperature* Volume: 100 µL

500K PCR Program for MJ Tetrad PTC-225		
Temperature	**Time**	**Cycles**
94°C	3 minutes	1X
94°C	30 seconds	30X
60°C	30 seconds	
68°C	15 seconds	
68°C	7 minutes	1X
4°C	HOLD (Can be held overnight)	

500K FRAGMENT

Required for the 96-well plate and the low throughput protocol.

500K Fragment Program	
Temperature	**Time**
37°C	35 minutes
95°C	15 minutes
4°C	Hold

500K LABEL

Required for the 96-well plate and the low throughput protocol.

500K Label Program	
Temperature	**Time**
37°C	4 hours
95°C	15 minutes
4°C	Hold
Samples can remain at 4 °C overnight.	

500K HYB

Required for the 96-well plate protocol only if denaturning samples on a thermal cycler.

500K Hyb Program	
Temperature	**Time**
95°C	10 minutes
49°C	Hold

Appendix C

Low Throughput Protocol

Appendix C

Introduction

The Affymetrix GeneChip® Mapping 500K Assay, in conjunction with the GeneChip® Mapping 500K Set, is designed to detect greater than 500,000 Single Nucleotide Polymorphisms (SNPs) in samples of genomic DNA. The Mapping 500K Set is comprised of two arrays and two assay kits. Each array and its corresponding assay kit are processed independently of the other array and assay kit. The protocol starts with 250 ng of genomic DNA per array and will generate SNP genotype calls for approximately 250,000 SNPs for each array of the two array set. An overview of the assay is shown in Figure C.1. The assay utilizes a strategy that reduces the complexity of human genomic DNA up to 10 fold by first digesting the genomic DNA with the Nsp I or Sty I restriction enzyme and then ligating sequences onto the DNA fragments. The complexity is further reduced by a PCR procedure optimized for fragments of a specified size range. Following these steps the PCR products (amplicons) are fragmented, end-labeled, and hybridized to a GeneChip array.

IMPORTANT ! **Arrays from different enzyme fractions should not be processed by the same technician on the same day.**

ASSAY OVERVIEW

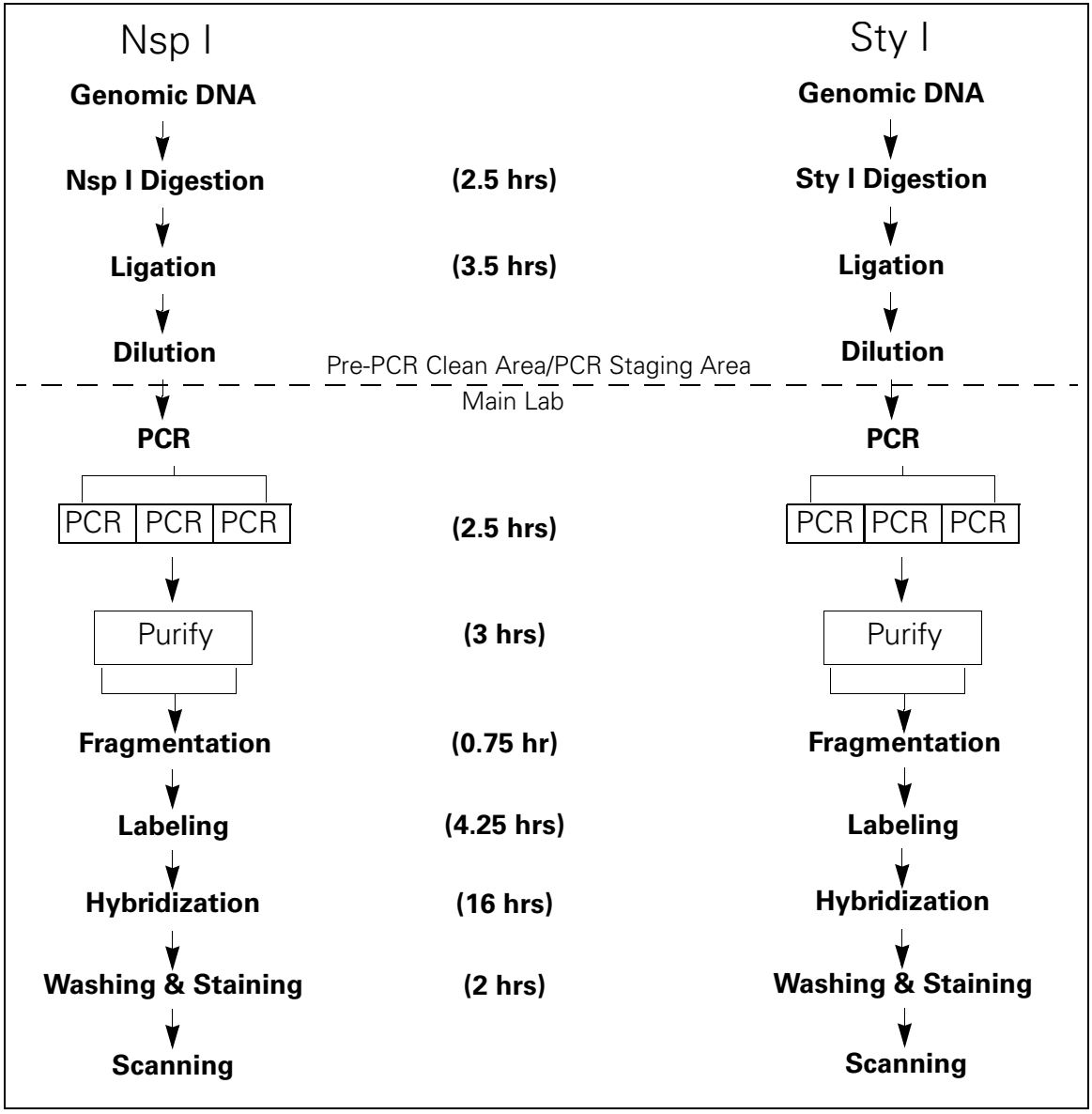

Figure C.1
GeneChip® Mapping 500K Assay Overview

BEFORE YOU BEGIN

The Mapping 500K low throughput protocol includes the thermal cycler programs listed below. Before you begin processing samples, enter and store these programs on the thermal cyclers that will be used for this protocol.

Thermal cycler program details are listed in Appendix B, Thermal Cycler Programs.

Table C.1
Thermal Cycler Programs Required for the Low Throughput Protocol

Program Name	# of Thermal Cyclers Required (based on 96 sample throughput)	Laboratory
500K Digest	1	Pre-PCR Clean Area
500K Ligate	1	Pre-PCR Clean Area
500K PCR	3	Main Lab
500K Fragment	1	Main Lab
500K Label	1	Main Lab

Genomic DNA Preparation

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). Diluted genomic DNA can be aliquoted at this point. For high throughput assays (2 or more 96-well plates), make multiple replicates of each plate.

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

REAGENTS

- Reduced EDTA TE Buffer (10 mM Tris HCL, 0.1 mM 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 in the pre-PCR clean room (or area for the DNA template and free of PCR products) to minimize cross contamination between samples. 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

REAGENTS AND EQUIPMENT

- 250 ng Genomic DNA per array working stock: 50 ng/µL
- Sty I (10,000 U/mL): New England Biolabs (NEB), P/N R0500S containing:
  - NE Buffer 3: New England Biolab (NEB), (P/N B7003S to order separately)
  - BSA (Bovine Serum Albumin): New England Biolab (NEB), (P/N B9001S to order separately)
- Nsp I (10,000 U/mL): New England Biolab (NEB), P/N R0602L containing:
  - NE Buffer 2: New England Biolab (NEB), (P/N B7002S to order separately)
  - BSA (Bovine Serum Albumin): New England Biolab (NEB), (P/N B9001S to order separately)
- H_2O (Molecular Biology Grade Water): BioWhittaker Molecular Applications/Cambrex, P/N 51200
- 96-well plate: Bio-Rad[1], P/N MLP-9601; or Applied Biosystems, P/N 403083
- 96-well Clear Adhesive Films: Applied Biosystems, P/N 4306311
- Thermal cycler (any Pre-PCR Clean Room thermocycler)
- 8-Tube Strips, thin-wall (0.2 mL): Bio-Rad[1], P/N TBS-0201; Strip of 8 caps: Bio-Rad[1], P/N TCS-0801

[1] *This product was formerly an MJ Research product and is currently available from Bio-Rad.*

IMPORTANT !

Program the thermal cycler in advance. Switch on the thermal cycler 10 minutes before reactions are ready so that the lid is heated. See Appendix B for a list of thermal cycler programs.

WARNING ▲

PCR tubes must be compatible and qualified with either MJ Research DNA Engine Tetrad®, or ABI GeneAmp® 9700. For example, individual tubes: Bio-Rad P/N TWI-0201.

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

NOTE

Reference Genomic DNA 103 is supplied in both the Sty and Nsp GeneChip® Mapping 250K Assay Kits (Nsp 30 Rxn Kit P/N 900766, Nsp 100 Rxn Kit P/N 900753, Sty 30 Rxn Kit P/N 900765, and Sty 100 Rxn Kit P/N 900754). This DNA can be used as a positive control.

CAUTION

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

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.

DIGESTION PROCEDURE

PRE-PCR CLEAN AREA

1. Depending on the restriction enzyme used, prepare the following Digestion Master Mix ON ICE (for multiple samples make a 5% excess):

Table C.2

Nsp I		
Reagent Stock	**1 Sample**	**Final Conc. in Sample**
H_2O	9.75 µL	
NE buffer 2 (10X)	2 µL	1X
BSA (10X (1 mg/mL))	2 µL	1X
Nsp I (10 U/µL)	1 µL	0.5 U/µL
Total	14.75 µL	

Sty I		
Reagent Stock	**1 Sample**	**Final Conc. in Sample**
H_2O	9.75 µL	
NE buffer 3 (10X)	2 µL	1X
BSA (10X (1 mg/mL))	2 µL	1X
Sty I (10 U/µL)	1 µL	0.5 U/µL
Total	14.75 µL	

TIP

Avoid processing samples with both Nsp and Sty enzymes on the same day. Best practice is to process samples for either Nsp or Sty on a given day.

NOTE

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

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 Restriction Enzyme.
3. Aliquot 14.75 µL of the Digestion Master Mix to each well of the 96-well plate containing genomic DNA.

Table C.3

Reagent Stock	Volume/Sample
Genomic DNA (50 ng/µL)	5 µL
Digestion Master Mix	14.75 µL
Total Volume	19.75 µL

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. Be sure to change tips between samples.**

4. Cover the plate with a plate cover and seal tightly, vortex at medium speed for 2 seconds, and spin at 2,000 rpm for 1 minute.
5. Put the plate on a thermal cycler and run the *500K Digest* program:

500K Digest Program	
Temperature	**Time**
37°C	120 minutes
65°C	20 minutes
4°C	Hold

NOTE **Store sample 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
- Molecular Biology Grade Water: Bio Whittaker Molecular Applications/Cambrex, P/N 51200
- Adaptor Nsp (50 µM): Affymetrix, P/N 900596 for 30 Rxns and P/N 900697 for 100 Rxns. Available in the Nsp and Sty GeneChip® Mapping 250K Assay Kits:
  - Nsp 30 Rxn Kit P/N 900766
  - Nsp 100 Rxn Kit P/N 900753
- Adaptor Sty (50 µM): Affymetrix, P/N 900597 for 30 Rxns and P/N 900698 for 100 Rxns. Available in the GeneChip® Mapping 250K Assay Kits:
  - Sty 30 Rxn Kit P/N 900765
  - Sty 100 Rxn Kit P/N 900754
- Thermal cycler (any Pre-PCR Clean Room thermocycler)

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

LIGATION PROCEDURE

PRE-PCR CLEAN AREA

IMPORTANT **Program the thermal cycler in advance. Switch on the thermal cycler 10 minutes before reactions are ready so that the lid is heated.**

IMPORTANT **Ligase buffer contains ATP and should be thawed/held at 4°C. Mix ligase buffer thoroughly before use to ensure precipitate is re-suspended. Avoid multiple freeze-thaw cycles, according to vendor's instructions.**

1. Depending on the restriction enzyme used, prepare the following Ligation Master Mix ON ICE (for multiple samples make a 5% excess):

Table C.4

Nsp I		
Reagent	**1 Sample**	**Final Conc. in Sample**
Adaptor Nsp I (50 µM)	0.75 µL	1.5 µM
T4 DNA Ligase buffer (10X) *	2.5 µL	1X
T4 DNA Ligase (400 U/µL)	2 µL	32 U/µL
Total	5.25 µL	

* Contains ATP and DTT. Keep on ice.

Sty I		
Reagent	**1 Sample**	**Final Conc. in Sample**
Adaptor Sty I (50 µM)	0.75 µL	1.5 µM
T4 DNA Ligase buffer (10X) *	2.5 µL	1X
T4 DNA Ligase (400 U/µL)	2 µL	32 U/µL
Total	5.25 µL	

* Contains ATP and DTT. Keep on ice.

PCR STAGING AREA

2. Aliquot 5.25 µL of the Ligation Master Mix into each digested DNA sample.

Table C.5

Reagent	Volume/Sample
Digested DNA	19.75 µL
Ligation mix*	5.25 µL
Total	25 µL

* Contains ATP and DTT. Keep on ice.

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. Be sure to change tips between samples.

3. Cover the plate with plate cover and seal tightly, vortex at medium speed for 2 seconds, and spin at 2,000 rpm for 1 minute.
4. Place the plate in a thermal cycler and run the *500K Ligate* program:

500K Ligate Program	
Temperature	**Time**
16°C	180 minutes
70°C	20 minutes
4°C	Hold

NOTE

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

5. Dilute each DNA ligation reaction by adding 75 µL of molecular biology-grade H_2O as described below:

Table C.6

Reagent	Volume/Sample
Ligated DNA	25 µL
H_2O	75 µL
Total	100 µL

IMPORTANT !

It is crucial to dilute the ligated DNA with molecular biology grade water prior to PCR.

STEP 4: PCR

REAGENTS AND EQUIPMENT

- G-C Melt (5 M): Clontech, P/N 639238
- H_2O (Molecular Biology Grade Water): BioWhittaker Molecular Applications/Cambrex, P/N 51200
- dNTP (2.5 mM each): Takara, P/N 4030; or Fisher Scientific, P/N TAK 4030[1]
- PCR Primer 002 (100 µM): Affymetrix, P/N 900595 for 30 Rxns and P/N 900702 for 100 Rxns. Available in the Nsp and Sty GeneChip® Mapping 250K Assay Kits:
  - Nsp 30 Rxn Kit P/N 900766
  - Nsp 100 Rxn Kit P/N 900753
  - Sty 30 Rxn Kit P/N 900765
  - Sty 100 Rxn Kit P/N 900754
- Clontech TITANIUM™ Taq DNA Polymerase (50X): Clontech, P/N 639209
  - 50X Clontech TITANIUM™ Taq DNA Polymerase
  - 10X Clontech TITANIUM™ Taq PCR Buffer
- 2% TBE Gel: BMA Reliant precast (2% SeaKem® Gold): Cambrex Bio Science, P/N 54939
- All Purpose Hi-Lo DNA Marker: Bionexus, Inc., P/N BN2050, or Direct Load Wide Range DNA Marker: Sigma, P/N D7058
- Gel Loading Solution: Sigma, P/N G2526
- Tubes:
  - Individual tubes: Bio-Rad, P/N TWI-0201
  - 8-Tube Strips, thin-wall (0.2 mL): Bio-Rad, P/N TBS-0201; Strip of 8 caps: Bio-Rad, P/N TCS-0801
- Plate:

[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.*

  - 96-well plate: Bio-Rad, P/N MLP-9601
- 96-well Clear Adhesive Films: Applied Biosystems, P/N 4306311
- PCR Thermal Cycler (this assay has only been optimized for the following two thermal cyclers)[1]:
  - GeneAmp® PCR System 9700 with gold-plated block, Applied Biosystems, or
  - DNA Engine Tetrad® PTC-225, MJ Research[2]

IMPORTANT !

- **Program the thermal cycler in advance (see page 261) and switch on the thermal cycler 10 minutes before reactions are ready, so that the lid is heated.**
- **Make sure the ligated DNA from the ligation step was diluted to 100 µL with water.**
- **Prepare PCR Master Mix in Pre-PCR Clean room.**
- **Set up PCRs in PCR Staging Area.**
- **Prepare 3 PCRs for each sample and for each restriction enzyme (3 PCRs are required for each array).**

[1] *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. You should obtain authorization from Roche or ABI (see PCR licensing information in the MJ Research User Manual), if you are not already licensed. 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.*

[2] *In 2004 MJ GeneWorks, Inc. and its subsidiary MJ Research, Inc. were purchased by Bio-Rad Laboratories, Inc. This model is no longer available. Bio-Rad has indicated that the DNA Engine Tetrad 2 gives similar performance with the same progams. Affymetrix has not tested the newer version.*

PCR PROCEDURE

IMPORTANT !

The PCR reaction is sensitive to the concentration of primer used. It is critical that the correct amount of primer is added to the PCR reaction to achieve the correct distribution of fragments (200 to 1100 base pairs) in the products. Check the PCR reactions on a gel to ensure that the distribution is correct (see Figure C.2).

PRE-PCR CLEAN ROOM

1. Prepare the following PCR Master Mix ON ICE (3 PCR reactions per sample) for Nsp I or Sty I ligation reactions and vortex at medium speed for 2 seconds (for multiple samples make a 5% excess):

Table C.7

Stock Reagent	1 PCR	3 PCR	Final Conc. in Sample
H_2O	39.5 µL	118.5 µL	
Clontech TITANIUM™ Taq PCR Buffer (10X)	10 µL	30 µL	1X
G-C Melt (5 M)	20 µL	60 µL	1 M
dNTP (2.5 mM each)	14 µL	42 µL	350 µM (each)
PCR Primer 002 (100 µM)	4.5 µL	13.5 µL	4.5 µM
Clontech TITANIUM™ Taq DNA Polymerase (50X)	2 µL	6 µL	1X
Total	90 µL	270 µL	

NOTE

90 µg of PCR product is needed for fragmentation.

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

2. Transfer 10 µL of each diluted ligated DNA from the 96-well plate into the corresponding three individual wells of the PCR plates using an 8- or 12-channel pipette. Be sure to change tips between samples.
3. Add 90 µL PCR Master mix to obtain a total volume of 100 µL. Final volume for each PCR is listed in the table below.

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

Table C.8

Reagent	Volume/PCR
PCR Master Mix	90 µL
Diluted ligated DNA (from Ligation step)	10 µL
Total	100 µL

NOTE **Three PCR reactions are needed to produce sufficient product for hybridization to one array (each reaction = 100 µL).**

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

MAIN LAB

IMPORTANT ! **Program the thermal cycler in advance. Switch on the thermal cycler 10 minutes before reactions are ready so that the lid is heated.**

IMPORTANT ! **PCR protocols for DNA Engine Tetrad and Applied Biosystems thermal cyclers are different as listed on the following pages.**

5. Run the *500K PCR* program on either an MJ Research DNA Tetrad Engine® or GeneAmp® PCR System 9700.

MJ Research DNA Engine Tetrad® 500K PCR Program

Specify 100 µL volume and use Heated Lid and Calculated Temperature.

500K PCR Program for MJ Tetrad PTC-225		
Temperature	**Time**	**Cycles**
94°C	3 minutes	1X
94°C	30 seconds	30X
60°C	30 seconds	
68°C	15 seconds	
68°C	7 minutes	1X
4°C	HOLD	

GeneAmp® PCR System 9700 Program

Specify 100 µL volume and maximum mode.

500K PCR Program for Geneamp® PCR System 9700		
Temperature	**Time**	**Cycles**
94°C	3 minutes	1X
94°C	30 seconds	30X
60°C	45 seconds	
68°C	15 seconds	
68°C	7 minutes	1X
4°C	HOLD	

6. 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.**

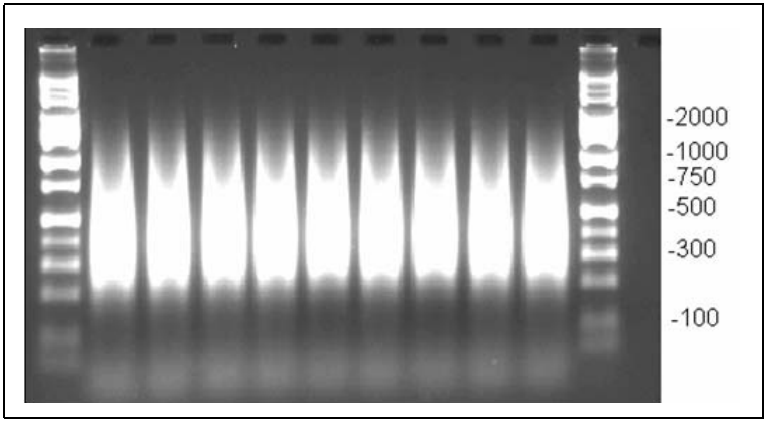

Figure C.2
Typical example of PCR products run on 2% TBE agarose gel at 120V for 1 hour, with average size between 200 and 1,100 bp

STEP 5: PCR Purification and Elution with Clontech Clean-Up Plate

REAGENTS AND EQUIPMENT

- DNA Amplification Clean-Up Kit, to be used with Affymetrix DNA products: Clontech, P/N 636974 (1 plate) or P/N 636975 (4 plates) (each kit also contains RB Buffer)
- Manifold - QIAvac multiwell unit: QIAGEN, P/N 9014579[1]
- EDTA (0.5 M, pH 8.0): Ambion, P/N 9260G
- Molecular Biology Grade Water: Bio Whittaker Molecular Applications/Cambrex, P/N 51200
- 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: QIAGEN, P/N 19530*

  * The Clontech protocol requires ~600 mb 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 Clontech Clean-Up Plate Handbook for the general procedure and ordering information.

1. Connect a vacuum manifold to a suitable vacuum source able to maintain ~600 mbar, e.g., QIAvac Multiwell Unit (QIAGEN). Place a waste tray inside the base of the manifold.
2. Place a Clean-Up Plate on top of the manifold. Cover wells that are not needed with PCR plate cover.

NOTE **To cover the unused wells, a PCR plate cover or an aluminum foil lid can be placed on top of the Clean-Up Plate. Apply pressure to make the cover stick to the plate. Cut the adhesive film between the used and unused wells. Remove the portion that covers the unused wells you want to use.**

3. Add 8 µL 0.1 M EDTA to each PCR reaction. Seal plate with plate

[1] *Clontech also supports the Millipore MultiScreen®, Promega Vac-Man® 96, and Bio-Rad Aurum vacuum manifolds for use with the Clean-Up Plates.*

cover, vortex at medium speed for 2 seconds, and spin at 2,000 rpm for 1 minute.

4. Consolidate three PCR reactions for each sample into one well of the Clean-Up Plate.

NOTE **For PCR samples prepared in three 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 Clean-Up Plate. With the vacuum on, the three PCR reactions for each sample (300 µL) can be combined into one well of the Clean-Up Plate.**

CAUTION **Be sure to dilute EDTA to 0.1 M. A higher concentration may interfere with downstream steps.**

CAUTION **To avoid piercing the membrane, do not pipet up and down in the Clean-Up Plate.**

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

IMPORTANT **Be sure to maintain the vacuum at 600 mbar.**

IMPORTANT **Three water washes must be done to remove PCR reaction contaminants (e.g., EDTA). Be sure to completely dry the membrane between each wash.**

IMPORTANT **After the final wash, every well must be completely dry before adding elution buffer. This step is critical to prevent the dilution of DNA with water.**

5. Apply a vacuum and maintain at ~600 mbar until the wells are completely dry.

6. Wash the PCR products by adding 50 µL molecular biology grade water and dry the wells completely (approximately 20 minutes). Repeat this step 2 additional times for a total of 3 water washes.
7. Switch off vacuum source and release the vacuum.
8. Carefully remove the Clean-Up Plate from the vacuum manifold and immediately:
   - **A.** Blot the plate on a stack of clean absorbent paper to remove any liquid that might remain on the bottom of the plate
   - **B.** Dry the bottom of each well with an absorbent wipe.

IMPORTANT !

To remove all remaining liquid, blot and wipe the bottom of the Clean-up Plate immediately after taking it off the vacuum manifold. Any water retained underneath the plate may be absorbed back into the wells.

9. Add 45 µL RB buffer to each well. Cover the plate with PCR plate cover film and seal tightly. Moderately shake the Clean-Up Plate on a plate shaker, e.g., Jitterbug (Boekel Scientific, model 130000), for 10 minutes at room temperature.
10. Recover the purified PCR product to a fresh 96-well plate by pipetting the eluate out of each well and transferring it to the corresponding well in the fresh 96-well plate. For easier recovery of the eluates, the plate can be held at a slight angle.

STEP 6: Quantification of Purified PCR Product

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

IMPORTANT **Due to the high PCR yield, the accuracy of the O.D. measurement is critical. Carefully follow the steps below and be sure the O.D. measurement is within the linear range of the instrument.**

TIP **Prepare at least three independent dilutions of each sample for accurate concentration measurement. Average the results before proceeding.**

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 2 µL of the purified PCR product to 198 µL molecular biology grade water (100-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 µg/mL for double-stranded PCR product.
   *This convention assumes a path length of 1 cm. Consult your spectrophotometer handbook for further information.
5. For fragmentation:
   - **A.** Transfer 90 µg of each of the purified DNA samples to the corresponding wells of a new plate.
   - **B.** Bring the total volume of each well up to 45 µL by adding the appropriate volume of RB Buffer.
   - **C.** Cover the plate with PCR plate cover film and seal tightly.
   - **D.** Vortex at medium speed for 2 seconds, and spin down at 2000 rpm for 1 minute.

IMPORTANT !

The amount of DNA in each well will differ. For optimal performance, it is critical that the contents of each well be normalized to 2 µg of DNA/µL before proceeding to the next step.

If necessary, bring the volume of each well to 45 µL by adding RB Buffer; do not add water.

STEP 7: Fragmentation

REAGENTS AND EQUIPMENT

- Fragmentation Reagent (DNase I): Affymetrix, P/N 900131
- 10X Fragmentation Buffer: Affymetrix, P/N 900422 for 30 Rxns and P/N 900695 for 100 Rxns
- Molecular Biology Grade Water: Bio Whittaker Molecular Applications/Cambrex, P/N 51200
- 4% TBE Gel: BMA Reliant precast (4% NuSieve 3:1 Plus Agarose): Cambrex, 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: Bio-Rad, P/N MLP-9601
- 96-well PLT. Clear Adhesive Films: Applied Biosystems, P/N 4306311
- Thermal cycler
  - DNA Engine Tetrad®[1]: MJ Research, or
  - GeneAmp® PCR System 9700 with gold-plated block

[1] *In 2004 MJ GeneWorks, Inc. and its subsidiary MJ Research, Inc. were purchased by Bio-Rad Laboratories, Inc. This model is no longer available. Bio-Rad has indicated that the DNA Engine Tetrad 2 gives similar performance with the same progams. Affymetrix has not tested the newer version.*

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 RB 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 diluted Fragmentation Reagent in excess to avoid errors in taking a small volume of Fragmentation Reagent.**
- **Perform all the dilution, addition and mixing steps ON ICE.**
- **Perform all the steps AS QUICKLY AS POSSIBLE.**
- **Discard remaining Diluted Fragmentation Reagent after use.**

IMPORTANT !

Tips on handling the Fragmentation Reagent:

- **Store Fragmentation Reagent stock at –20°C until ready for use.**
- **Transfer Fragmentation Reagent in a –20°C cooler and keep in the cooler until used. Return to the cooler immediately after use.**
- **Work efficiently while preparing mixes for the fragementation reaction.**
- **Briefly spin down Fragmentation Reagent tube. Do not vortex.**
- **Avoid excess enzyme on the outside of the pipette tip while preparing mixes.**

FRAGMENTATION PROCEDURE

IMPORTANT !

Program the thermal cycler in advance. Switch on the thermal cycler 10 minutes before reactions are ready so that the lid is heated.

1. Pre-heat thermal cycler to 37°C.
2. Add 5 µL 10X Fragmentation Buffer to each sample in the fragmentation plate ON ICE.

Table C.9
Fragmentation Mix

Reagent	Volume/Sample
Purified PCR product (90 µg in RB buffer)	45 µL
10X Fragmentation Buffer	5 µL
Total	50 µL

3. 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)

   0.05 U/µL = final concentration of diluted Fragmentation Reagent

   120 µL = final volume of diluted Fragmentation Reagent*

$$Y = \frac{0.05\ \text{U/µL} * 120\ \text{µL}}{X\ \text{U/µL}}$$

   * enough for 20 reactions

IMPORTANT **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. Do calculations prior to diluting sample and Fragmentation Reagent.**

IMPORTANT **To ensure uniform and reproducible fragmentation, completely pipet the viscous reagent from the stock vial into the diluted enzyme mix. Thorougly mix the diluted enzyme before aliquoting to the DNA samples.**

IMPORTANT **The Fragmentation Reagent must be added to the fragmentation mix *quickly* and *ON ICE* to minimize enzyme activity prior to placing the samples on the thermal cycler.**

4. Dilute the stock of Fragmentation Reagent to 0.05 U/µL as follows:

   A. Place the water (Molecular Biology Grade), Fragmentation Buffer, and Fragementation Reagent on ice.

   B. Combine the reagents ON ICE in the order shown in Table C.10.

   C. Vortex at medium speed for 2 seconds.

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

Table C.10
Diluting the Fragmentation Reagent – Combine Reagents in the Order Shown

Reagent	2 units/µL	3 units/µL
H_2O, Molecular Biology Grade	105 µL	106 µL
10X Fragmentation Buffer	12 µL	12 µL
Fragmentation Reagent*	3 µL	2 µL
Total	120 µL	120 µL
* Add the Fragmentation Reagent last, after allowing the water and buffer to cool on ice.		

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

5. Quickly and ON ICE, divide the diluted Fragmentation Reagent into 8 or 12 microtube strips.
6. Add 5 µL of diluted Fragmentation Reagent (0.05 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. Be sure to change tips between samples. The total volume for each sample is listed below.

Table C.11

Reagent	Volume/Sample
Fragmentation Mix	50 µL
Diluted Fragmentation Reagent (0.05 U/µL)	5 µL
Total	55 µL

IMPORTANT !

For 90 µg of purified PCR product, a total of 0.25 U of Fragmentation Reagent is needed in a final reaction volume of 55 µL.

7. 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 at 4°C.
9. Place the fragmentation plate in pre-heated thermal cycler (37°C) as quickly as possible.

IMPORTANT !

Make sure the reaction tubes/plates 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).

Seal the tubes/plates 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 or PCR tubes that do not fit with the thermal cycler.

10. Run the *500K Fragment* program:

500K Fragment Program	
Temperature	**Time**
37°C	35 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 C.3.)
13. Proceed immediately to Labeling step, if your gel matches the example below.

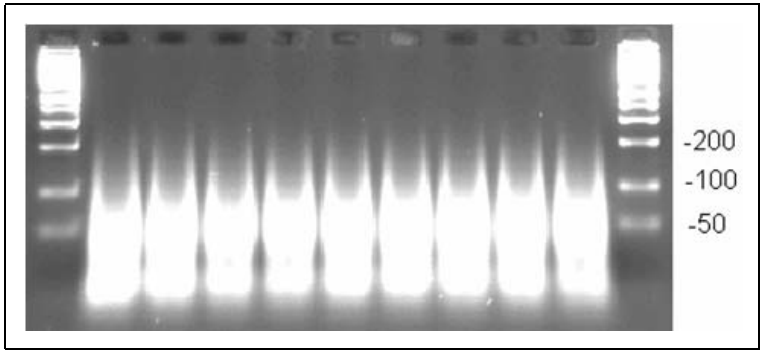

Figure C.3
Typical example of fragmented PCR products run on 4% TBE agarose gel at 120V for 30 minutes to 1 hour, with average size < 180 bp

STEP 8: Labeling

REAGENTS

- GeneChip® DNA Labeling Reagent (30 mM): Affymetrix, P/N 900778 for 30 Rxns and P/N 900699 for 100 Rxns. Available in the Nsp and Sty GeneChip® Mapping 250K Assay Kits:
  - Nsp 30 Rxn Kit P/N 900766
  - Nsp 100 Rxn Kit P/N 900753
  - Sty 30 Rxn Kit P/N 900765
  - Sty 100 Rxn Kit P/N 900754
- Terminal Deoxynucleotidyl Transferase (30 U/µL): Affymetrix, P/N 900508 for 30 Rxns and P/N 900703 for 100 Rxns. Available in the Nsp and Sty GeneChip® Mapping 250K Assay Kits:
  - Nsp 30 Rxn Kit P/N 900766
  - Nsp 100 Rxn Kit P/N 900753
  - Sty 30 Rxn Kit P/N 900765
  - Sty 100 Rxn Kit P/N 900754
- 5X Terminal Deoxynucleotidyl Transferase Buffer: Affymetrix, P/N 900425 for 30 Rxns and P/N 900696 for 100 Rxns. Available in the Nsp and Sty GeneChip® Mapping 250K Assay Kits:
  - Nsp 30 Rxn Kit P/N 900766
  - Nsp 100 Rxn Kit P/N 900753
  - Sty 30 Rxn Kit P/N 900765
  - Sty 100 Rxn Kit P/N 900754

LABELING PROCEDURE

IMPORTANT ! **Program the thermal cycler in advance. Switch on the thermal cycler 10 minutes before reactions are ready so that the lid is heated.**

MAIN LAB

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

Table C.12

Reagent	1X	Final Conc. in Sample
5X TdT Buffer	14 µL	1X
GeneChip® DNA Labeling Reagent (30 mM)	2 µL	0.857 mM
TdT (30 U/µL)	3.5 µL	1.5 U/µL
Total	19.5 µL	

2. Aliquot 19.5 µL of Labeling Master Mix into the fragmentation plate containing 50.5 µL of fragmented DNA samples as follows:

Table C.13

Reagent	Volume/Rx
Fragmented DNA (from Fragmentation step)	50.5 µL
Labeling Mix	19.5 µL
Total	70 µL

Remaining fragmented DNA is used for gel analysis

TIP **To expedite the aliquoting, the Labeling Master Mix can be first divided into 8 or 12 microtube 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. Be sure to change tips between samples.**

3. Seal the plate tightly with a plate cover.
4. Vortex the plate at medium speed for 2 seconds, and spin the plate at 2,000 rpm for 1 minute.
5. Run the *500K Label* program:

500K Label Program	
Temperature	**Time**
37°C	4 hours
95°C	15 minutes
4°C	Hold

IMPORTANT ! **Make sure the plates/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. Spin the plate at 2,000 rpm for 1 minute after the labeling reaction.

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

STEP 9: Target Hybridization

REAGENTS

- 5 M TMACL (Tetramethyl Ammonium Chloride): Sigma, P/N T3411
- 10% Tween-20: Pierce, P/N 28320 (Surfact-Amps®); diluted to 3% in molecular biology grade water
- MES hydrate SigmaUltra: 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 DNA®: Invitrogen, P/N 15279-011
- Oligo Control Reagent, 0100 (OCR, 0100): Affymetrix, P/N 900541 for 30 Rxns and 900701 for 100 Rxns. Available in the Nsp and Sty GeneChip® Mapping 250K Assay Kits:
  - Nsp 30 Rxn Kit P/N 900766
  - Nsp 100 Rxn Kit P/N 900753
  - Sty 30 Rxn Kit P/N 900765
  - Sty 100 Rxn Kit P/N 900754

IMPORTANT !

We have noticed increased Mapping 500K performance variability in the GeneChip® Hybridization Oven 640 models (P/N 800138 or 800189) manufactured prior to 2001. We have not seen this increased variability in later Hybridization Oven 640 models with a serial number of 11215 or higher. Please check the serial number of your hybridization oven(s). If you have one of the older models with a number of 11214 or lower, please contact Affymetrix for an upgrade.

REAGENT PREPARATION

12 X MES Stock

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

IMPORTANT !

Do not autoclave. Store between 2ºC and 8ºC, 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.**

IMPORTANT ! **DMSO is light sensitive and must be stored in a dark glass bottle. Do not store in a plastic container.**

1. Prepare the Hybridization Cocktail Master Mix in the order shown in Table C.14. For multiple samples, prepare 5% excess.

Table C.14
Hybridization Cocktail Master Mix

Reagent	1X	Final Conc. in Sample
MES (12X; 1.22 M)	12 µL	0.056 M
DMSO (100%)	13 µL	5.0%
Denhardt's Solution (50X)	13 µL	2.50X
EDTA (0.5 M)	3 µL	5.77 mM
HSDNA (10 mg/mL)	3 µL	0.115 mg/mL
OCR, 0100	2 µL	1X
Human Cot-1 DNA® (1 mg/mL)	3 µL	11.5 µg/mL
Tween-20 (3%)	1 µL	0.0115%
TMACL (5 M)	140 µL	2.69 M
Total	190 µL	

2. Mix well.

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

3. Transfer each of the labeled samples from the plate to a 1.5 mL Eppendorf tube. Aliquot 190 µL of the Hybridization Cocktail Master Mix into the 70 µL of labeled DNA samples as follows:

Table C.15

Reagent	Volume/Rx
Labeled DNA	70 µL
Hybridization Mix	190 µL
Total	260 µL

4. Heat the 260 µL of hybridization mix and labeled DNA at 99°C in a heat block for exactly 10 minutes to denature.

IMPORTANT **Denaturation of the labeled DNA sample is important to maximize binding to the oligonucleotides on the array surface. The sample must be heated to at least 95°C for 10 minutes prior to adding the DNA to the array. If a precipitate forms during this step, resuspend it before hybridization. Hybridize the arrays for at least 16 hours at 49°C. This temperature has been optimized for this product, and should be stringently followed.**

5. Cool on crushed ice for 10 seconds.

IMPORTANT **To avoid the formation of aggregates, do not leave on ice for longer than 10 seconds.**

6. Spin briefly at 2,000 rpm in a microfuge to collect any condensate.
7. Place the tubes at 49°C for 1 minute.

8. Inject 200 µL denatured hybridization cocktail into the array.

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

IMPORTANT **When processing multiple samples, leave samples at 49°C until ready to load onto the array. After injecting sample into an array, immediately place the array in the hybridization oven.**

9. Hybridize at 49°C for 16 to 18 hours at 60 rpm.

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

IMPORTANT **The hybridization temperature is 49°C. This is different from the GeneChip® expression assay, and the Mapping 10K and 100K assays.**

Appendix D

Reagents, Instruments, and Supplies Required for Low Throughput Protocol

Appendix D

Introduction

This section describes the reagents, supplies and equipment required to implement the GeneChip® Mapping 500K Low Throughput Protocol.

The first section describes the Affymetrix GeneChip® Mapping 250K Assay Kits for Nsp and Sty (Nsp 30 Rxn Kit P/N 900766, Nsp 100 Rxn Kit P/N 900753, Sty 30 Rxn Kit P/N 900765, Sty 100 Rxn Kit P/N 900754), which contains critical reagents and reagent components specifically developed and/or optimized for the GeneChip Mapping 500K Assay, and controls that are integrated for use with the GeneChip® Mapping 500K Array Set. This section also indicates the Affymetrix equipment – scanner, fluidics station, hybridization oven - and GeneChip Mapping 250K arrays 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.

Reagents and Instruments Required

The following reagents and equipment are required to process the GeneChip Mapping 500K Array Set.

Table D.1
Reagents Supplied by Affymetrix - 30 Reaction Kit

Affymetrix GeneChip® Mapping 250K Nsp Assay Kit P/N 900766 This kit includes sufficient reagent for 30 arrays.				
Component	**Volume**	**Concentration**	**Description**	**Box #**
Adaptor, Nsp I	25 µL	50 µM	Two annealed oligonucleotides, specific for ligation to Nsp restriction site.	1
PCR Primer, 002	450 µL	100 µM	PCR primer, to amplify ligated genomic DNA	1
Reference Genomic DNA, 103	30 µL	50 ng/µL	Human genomic DNA (single source)	2
GeneChip® Fragmentation Reagent	25 µL	See label on tube	DNase I enzyme, formulated to fragment purified PCR amplicons	3
10X Fragmentation Buffer	250 µL	10X	Buffer for fragmentation reaction	3
GeneChip® DNA Labeling Reagent (30 mM)	60 µL	30 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 (30 mM)	3
5X Terminal Deoxynucleotidyl Transferase Buffer	420 µL	5X	Buffer for labeling reactio	3
Oligo Control Reagent, 0100 (OCR, 0100)	60 µL	See protocol Chapter 4 (high throughput) or Appendix C (low throughput)	Mixture of five biotin-labeled oligonucleotides, which hybridize to control regions (gridding controls and array controls) on the GeneChip® Mapping 250K Arrays.	3

Table D.2
Reagents Supplied by Affymetrix - 100 Reaction Kit

Affymetrix GeneChip® Mapping 250K Nsp Assay Kit P/N 900753 This kit includes sufficient reagent for 100 arrays.				
Component	**Volume**	**Concentration**	**Description**	**Box #**
Adaptor, Nsp I	75 µL	50 µM	Two annealed oligonucleotides, specific for ligation to Nsp restriction site.	1
PCR Primer, 002	2 vials, 750 µL each	100 µM	PCR primer, to amplify ligated genomic DNA	1
Reference Genomic DNA, 103	30 µL	50 ng/µL	Human genomic DNA (single source)	2
GeneChip® Fragmentation Reagent	25 µL	See label on tube	DNase I enzyme, formulated to fragment purified PCR amplicons	3
10X Fragmentation Buffer	835 µL	10X	Buffer for fragmentation reaction	3
GeneChip® DNA Labeling Reagent (30 mM)	200 µL	30 mM	Biotin-labeled reagent for end-labeling fragmented PCR amplicons	3
Terminal Deoxynucleotidyl Transferase	350 µL	30 U/µL	Enzyme used to end-label fragmented PCR amplicons with the GeneChip® DNA Labeling Reagent (30 mM)	3
5X Terminal Deoxynucleotidyl Transferase Buffer	2 vials, 700 µL each	5X	Buffer for labeling reaction	3
Oligo Control Reagent, 0100 (OCR, 0100)	200 µL	See protocol Chapter 4 (high throughput) or Appendix C (low throughput)	Mixture of five biotin-labeled oligonucleotides, which hybridize to control regions (gridding controls and array controls) on the GeneChip® Mapping 250K Arrays.	3

* Adaptor, Nsp

5' ATTATGAGCACGACAGACGCCTGATCTCATG 3'
3' AATACTCGTGCTGTCTGCGGACTAGAp 5'

PCR Primer, 002

5' ATTATGAGCACGACAGACGCCTGATCT 3'

Table D.3
Reagents Supplied by Affymetrix - 30 reaction Kit

Affymetrix GeneChip® Mapping 250K Sty Assay Kit P/N 900765 This kit includes sufficient reagent for 30 arrays.				
Component	**Volume**	**Concentration**	**Description**	**Box #**
Adaptor, Sty I	25 µL	50 µM	Two annealed oligonucleotides, specific for ligation to Sty restriction site.	1
PCR Primer, 002	450 µL	100 µM	PCR primer, to amplify ligated genomic DNA	1
Reference Genomic DNA, 103	30 µL	50 ng/µL	Human genomic DNA (single source)	2
GeneChip® Fragmentation Reagent	25 µL	See label on tube	DNase I enzyme, formulated to fragment purified PCR amplicons	3
10X Fragmentation Buffer	250 µL	10X	Buffer for fragmentation reaction	3
GeneChip® DNA Labeling Reagent (30 mM)	60 µL	30 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 (30 mM)	3
5X Terminal Deoxynucleotidyl Transferase Buffer	420 µL	5X	Buffer for labeling reaction	3
Oligo Control Reagent, 0100 (OCR, 0100)	60 µL	See protocol Chapter 4 (high throughput) or Appendix C (low throughput)	Mixture of five biotin-labeled oligonucleotides, which hybridize to control regions (gridding controls and array controls) on the GeneChip® Mapping 250K Arrays.	3

Table D.4
Reagents Supplied by Affymetrix - 100 reaction Kit

Affymetrix GeneChip® Mapping 250K Sty Assay Kit P/N 900754 This kit includes sufficient reagent for 100 arrays.				
Component	**Volume**	**Concentration**	**Description**	**Box #**
Adaptor, Sty I	75 µL	50 µM	Two annealed oligonucleotides, specific for ligation to Sty restriction site.	1
PCR Primer, 002	2 vials, 750 µL each	100 µM	PCR primer, to amplify ligated genomic DNA	1
Reference Genomic DNA, 103	30 µL	50 ng/µL	Human genomic DNA (single source)	2
GeneChip® Fragmentation Reagent	25 µL	See label on tube	DNase I enzyme, formulated to fragment purified PCR amplicons	3
10X Fragmentation Buffer	835 µL	10X	Buffer for fragmentation reaction	3
GeneChip® DNA Labeling Reagent (30 mM)	200 µL	30 mM	Biotin-labeled reagent for end-labeling fragmented PCR amplicons	3
Terminal Deoxynucleotidyl Transferase	350 µL	30 U/µL	Enzyme used to end-label fragmented PCR amplicons with the GeneChip® DNA Labeling Reagent (30 mM)	3
5X Terminal Deoxynucleotidyl Transferase Buffer	2 vials, 700 µL each	5X	Buffer for labeling reaction	3
Oligo Control Reagent, 0100 (OCR, 0100)	200 µL	See protocol Chapter 4 (high throughput) or Appendix C (low throughput)	Mixture of five biotin-labeled oligonucleotides, which hybridize to control regions (gridding controls and array controls) on the GeneChip® Mapping 250K Arrays.	3

* Adaptor, Sty

5' ATTATGAGCACGACAGACGCCTGATCT 3'
3' AATACTCGTGCTGTCTGCGGACTAGAGWWCp 5'

PCR Primer, 002

5' ATTATGAGCACGACAGACGCCTGATCT 3'

Table D.5
Equipment and Software Supplied by Affymetrix

Item	Part Number
GeneChip® Fluidics Station 450*	00-0079
GeneChip® Hybridization Oven 640*	800139
GeneChip® Scanner 3000 7G*	00-0205
Affymetrix GeneChip® Operating Software version 1.4*	690031
Affymetrix GeneChip® Genotyping Analysis Software 4.0*	690051
GeneChip® Human Mapping 250K Nsp Array*	520330
GeneChip® Human Mapping 250K Sty Array*	520331

** 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.*

Table D.6
Reagents, Equipment and Supplies Not Supplied by Affymetrix

Assay Step	Reagents	Equipment

Genomic DNA Preparation	• Reduced EDTA TE Buffer (10 mM Tris HCL, 0.1 mM EDTA, pH 8.0): TEKnova, P/N T0223
	• **Reference Genomic DNA, 103 (50 ng/µL): Affymetrix*, P/N 900421, available in Box 2 of the GeneChip® Mapping 250K Assay Kits:**
	  • **Nsp 30 Rxn Kit P/N 900766**
	  • **Nsp 100 Rxn Kit P/N 900753**
	  • **Sty 30 Rxn Kit P/N 900765**
	  • **Sty 100 Rxn Kit P/N 900754**	

Restriction Enzyme Digestion	• 250 ng Genomic DNA 50 ng/µL working stock/array
	• **Sty I (10,000 U/mL): New England Biolabs (NEB), P/N R0500S (if running Sty Array) containing:**
	  • **NE Buffer 3: New England Biolabs (NEB), (P/N B7003S to order separately)**
	  • **BSA (Bovine Serum Albumin): New England Biolabs (NEB), (P/N B9001S to order separately)**
	• **Nsp I (10,000 U/mL): New England Biolabs (NEB), P/N R0602L (if running Nsp Array) containing:**
	  • **NE Buffer 2: New England Biolabs (NEB), (P/N B7002S to order separately)**
	• H_2O (Molecular Biology Grade Water): BioWhittaker Molecular Applications/Cambrex, P/N 51200
	• 96-well plate: Bio-Rad, P/N MLP-9601; or Applied Biosystems, P/N 403083
	• 96-well Plate Clear Adhesive Films: Applied Biosystems, P/N 4306311
	• 8-Tube Strips, thin-wall (0.2 mL): Bio-Rad, P/N TBS-0201; Strip of 8 caps: Bio-Rad, P/N TCS-0801
		• Thermal Cycler

Table D.6
Reagents, Equipment and Supplies Not Supplied by Affymetrix

Assay Step	Reagents	Equipment

Ligation	• **T4 DNA Ligase: New England Biolabs (NEB), P/N M0202L containing:**
	  • **T4 DNA Ligase Buffer: New England Biolabs (NEB), P/N B0202S**
	• Molecular Biology Grade Water: BioWhittaker Molecular Applications/Cambrex, P/N 51200
	• Adaptor Nsp (50 µM): Affymetrix, P/N 900596 for 30 Rxnx and P/N 900697 for 100 Rxns (if running Nsp Array) available in the GeneChip® Mapping 250K Nsp Assay Kit:
	  • 30 Rxn Kit P/N 900766, 100 Rxn Kit P/N 900753
	• Adaptor Sty (50 µM): Affymetrix, P/N 900597 for 30 Rxns and P/N 900698 for 100 Rxns (if running Sty Array) available in the GeneChip® Mapping 250K Sty Assay Kit:
	  • 30 Rxn Kit P/N 900765, 100 Rxn Kit P/N 900754
		• Thermal Cycler

Table D.6
Reagents, Equipment and Supplies Not Supplied by Affymetrix

Assay Step	Reagents	Equipment

PCR	• G-C Melt (5 M): Clontech, P/N 639238
	• H_2O (Molecular Grade Biology Water): BioWhittaker Molecular Applications/Cambrex, P/N 51200
	• **dNTP (2.5 mM each): Takara, P/N 4030; Fisher Scientific, P/N TAK 4030; or Invitrogen, P/N R72501† (FN1)**
	• **PCR Primer, 002 (100 µM): Affymetrix, P/N 900595 for 30 Rxns and P/N 900702 for 100 Rxns (if running Nsp Array), available in the GeneChip® Mapping 250K Nsp Assay Kits:**
	  • **30 Rxn Kit P/N 900766**
	  • **100 Rxn Kit P/N 900753**
	• **PCR Primer, 002 (100 µM): Affymetrix, P/N 900595 for 30 Rxns and P/N 900702 for 100 Rxns (if running Sty Array), available in the GeneChip® Mapping 250K Sty Assay Kits:**
	  • **30 Rxn Kit P/N 900765**
	  • **100 Rxn Kit P/N 900754**
	• Clontech TITANIUM™ Taq DNA Polymerase (50X): Clontech, P/N 639209
	  • 50X Clontech TITANIUM™ Taq DNA Polymerase
	  • 10X Clontech TITANIUM™ Taq PCR Buffer
	• 2% TBE Gel:BMA Reliant precast (2% SeaKem® Gold): Cambrex Bio Science, P/N 54939
	• All Purpose Hi-Lo DNA Marker: Bionexus, Inc., P/N BN2050, or Direct Load Wide Range DNA Marker: Sigma, P/N D7058
	• Gel Loading Solution: Sigma, P/N G2526
	• **PCR Tubes***: (must be compatible and qualified with MJ DNA Engine Tetrad®**, or ABI GeneAmp® PCR System) For example:
	  • Individual tubes: Bio-Rad, P/N TWI-0201
	  • 8-Tube Strips, thin-wall (0.2 mL): Bio-Rad, P/N TBS-0201; Strip of 8 caps: Bio-Rad, P/N TCS-0801
	• Plate: (must be compatible and qualified with MJ DNA Engine Tetrad®**, or ABI GeneAmp® PCR System)
	  • 96-well plate: Bio-Rad, P/N MLP-9601
	• 96-well PLT. Clear Adhesive Films: Applied Biosystems, P/N 4306311
		• **PCR Thermal Cycler‡*** (Ramp speeds on these models are critical - assay protocols have been optimized for these two thermal cycler models only):
		  • **MJ Research DNA Engine Tetrad®** (PTC-225) 96 well block, or**
		  • **ABI GeneAmp® PCR System 9700 - gold plated 96 well block**
		• gel apparatus
		• **Jitterbug™: Boekel Scientific, model 130000**

Table D.6
Reagents, Equipment and Supplies Not Supplied by Affymetrix

Assay Step	Reagents	Equipment
PCR Purification and Elution	• **DNA Amplification Clean-Up Kit, to be used with Affymetrix products: Clontech P/N 636974 (1 plate), or P/N 636975 (4 plates)** • **Manifold - QIAvac multiwell unit: QIAGEN P/N 9014579**[††] • EDTA (0.5 M, pH 8.0): Ambion, P/N 9260G • Molecular Biology Grade Biology Water: BioWhittaker Molecular Applications/Cambrex, P/N 51200 • Biomek® Seal and Sample Aluminum Foil Lids: Beckman, P/N 538619	• Vacuum Regulator for use during the PCR clean up step. QIAGEN Vacuum Regulator: QIAGEN, P/N 19530[‡‡]

Table D.6
Reagents, Equipment and Supplies Not Supplied by Affymetrix

Assay Step	Reagents	Equipment

Fragmentation and Labeling	• **GeneChip® Fragmentation Reagent (DNase I): Affymetrix, P/N 900131**
	• **10X Fragmentation Buffer: Affymetrix, P/N 900422 for 30 Rxns and P/N 900695 for 100 Rxns**
	• Molecular Biology Grade Water: Bio Whittaker Molecular Applications/Cambrex, P/N 51200
	• 4% TBE Gel: BMA Reliant precast (4% NuSieve 3:1 Plus Agarose): Cambrex, P/N 54929
	• All Purpose Hi-Lo DNA Marker: Bionexus, Inc., 50 - 10000 bp; P/N BN2050
	• Gel Loading Solution: Sigma, P/N G2526
	• 96-well plate: Bio-Rad, P/N MLP-9601
	• 96-well Clear Adhesive Films: Applied Biosystems, P/N 4306311
	• **GeneChip® DNA Labeling Reagent (30 mM): Affymetrix, P/N 900778 for 30 Rxns and P/N 900699 for 100 Rxns, available in the Nsp and Sty GeneChip® Mapping 250K Assay Kits:**
	  • **Nsp 30 Rxn Kit P/N 900766**
	  • **Nsp 100 Rxn Kit P/N 900753**
	  • **Sty 30 Rxn Kit P/N 900765**
	  • **Sty 100 Rxn Kit P/N 900754**
	• **Terminal Deoxynucleotidyl Transferase (30 U/µL): Affymetrix, P/N 900508 for 30 Rxns and P/N 900703 for 100 Rxns, available in the Nsp and Sty GeneChip® Mapping 250K Assay Kits:**
	  • **Nsp 30 Rxn Kit P/N 900766**
	  • **Nsp 100 Rxn Kit P/N 900753**
	  • **Sty 30 Rxn Kit P/N 900765**
	  • **Sty 100 Rxn Kit P/N 900754**
	• **5X Terminal Deoxynucleotidyl Transferase Buffer: Affymetrix, P/N 900425 for 30 Rxns and P/N 900696 for 100 Rxns, available in the Nsp and Sty GeneChip® Mapping 250K Assay Kits:**
	  • **Nsp 30 Rxn Kit P/N 900766**
	  • **Nsp 100 Rxn Kit P/N 900753**
	  • **Sty 30 Rxn Kit P/N 900765**
	  • **Sty 100 Rxn Kit P/N 900754**
		• **PCR Thermal Cycler*** (Ramp speeds on these models are critical - assay protocols have been optimized for these two thermal cycler models only):
		  • **MJ Research DNA Engine Tetrad®** (PTC-225) 96 well block, or**
		  • **ABI GeneAmp® PCR System 9700 - gold-plated 96 well block**

Table D.6
Reagents, Equipment and Supplies Not Supplied by Affymetrix

Assay Step	Reagents	Equipment

Target Hybridization	• 5 M TMACL (Tetramethyl Ammonium Chloride): Sigma, P/N T3411
	• 10% Tween-20: Pierce, P/N 28320 (Surfact-Amps®); diluted to 3% in molecular biology grade water
	• MES hydrate SigmaUltra: Sigma, P/N M5287
	• MES Sodium Salt: Sigma, P/N M5057
	• DMSO: Sigma, P/N D5879
	• 0.5 M EDTA: Ambion, P/N 9260G
	• Denhardt's Solution: Sigma, P/N D2532
	• HSDNA (Herring Sperm DNA): Promega, P/N D1815
	• Human Cot-1 DNA®: Invitrogen, P/N 15279-011
	• **Oligo Control Reagent, 0100 (OCR, 0100): Affymetrix, P/N 900541 for 30 Rxns and 900701 for 100 Rxns, available in the Nsp and Sty GeneChip® Mapping 250K Assay Kits:**
	  • **Nsp 30 Rxn Kit P/N 900766**
	  • **Nsp 100 Rxn Kit P/N 900753**
	  • **Sty 30 Rxn Kit P/N 900765**
	  • **Sty 100 Rxn Kit P/N 900754**
		• GeneChip® Hybridization Oven 640

Wash and Staining	• 20X SSPE: BioWhittaker Molecular Applications/ Cambrex, P/N 51214 • Denhardt's Solution, 50X concentrate: Sigma, P/N D2532 • 10% Tween-20: Pierce, P/N 28320 (Surfact-Amps®) • Molecular Biology Grade Water: BioWhittaker Molecular Applications/Cambrex, P/N 51200 • **SAPE (Streptavidin, R-phycoerythrin conjugate): Invitrogen, P/N S866, 1 mg/mL*** • **Ab (Biotinylated Anti-Streptividin): Vector Laboratories, P/N BA-0500, 0.5mg;* reconstitute according to product instructions** • Distilled water: Invitrogen Life Technologies, P/N 15230147 • Bleach (5.25% Sodium Hypochlorite): VWR Scientific, P/N 21899-504 (or equivalent)	• GeneChip® Fluidics Station 450
Scanning		• GeneChip® Scanner 3000 7G

*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.

†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.

‡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. You should obtain authorization from Roche or ABI (see PCR licensing information in the MJ Research User Manual), if you are not already licensed. 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.

‡‡In 2004 MJ GeneWorks, Inc. and its subsidiary MJ Research, Inc. were purchased by Bio-Rad Laboratories, Inc. This model is no longer available. Bio-Rad has indicated that the DNA Engine Tetrad 2 gives similar performance with the same progams. Affymetrix has not tested the newer version.

†††Clontech also supports the Millipore MultiScreen®, Promega Vac-Man® 96, and BioRad Aurum vacuum manifolds for use with the Clontech Clean-Up Plates.

‡‡‡The Clontech protocol requires ~600 mb vacuum. If your lab does not have an internally regulated vacuum source, this vacuum regulator is strongly suggested.

Supplier Contact List

Table D.7

Supplier	Web Site
Ambion	www.ambion.com
Applied Biosystems	home.appliedbiosystems.com
Bionexus Inc.	www.bionexus.net
Bio-Rad	www.bio-rad.com
Boekel Scientific	www.boekelsci.com
Cambrex	www.cambrex.com
Clontech	www.clontech.com/clontech
Fisher Scientific	www.fisherscientific.com
Invitrogen Life Technologies	www.invitrogen.com
Molecular Probes	www.probes.com
New England Biolabss	www.neb.com
Operon Technologies	www.operon.com
PerkinElmer Life Sciences	lifesciences.perkinelmer.com
Pierce Chemical	www.piercenet.com
Promega	www.promega.com
QIAGEN	www.qiagen.com
Sigma-Aldrich	www.sigma-aldrich.com
TaKaRa Bio Inc.	www.takara-bio.co.jp/english/index.htm
TEKnova	www.teknova.com
Vector Laboratories	www.vectorlabs.com