Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide

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CONTENTS

Chapter 1

OVERVIEW

About This Manual

This manual is a guide for technical personnel conducting the Affymetrix® Genome-Wide Human SNP Nsp/Sty 5.0/6.0 Assay (Genome-Wide SNP 5.0/6.0 Assay) experiments in the laboratory. It contains:

- Protocols for sample preparation and 48 sample processing
- Instructions for washing, staining, and scanning arrays
- Instructions for generating genotype calls
- Troubleshooting information

A description of each chapter follows.

Chapter 1: Overview

Provides a scientific overview of the concept behind the Genome-Wide SNP 5.0/6.0 Assay, including the biochemical process, data generation, potential applications, and a list of references.

Chapter 2: Laboratory Setup

Describes the appropriate laboratory configuration for running Genome-Wide SNP 5.0/6.0 Assay experiments.

Chapter 3: Genomic DNA Preparation

Describes the requirements for genomic DNA, with recommended sources and methods for purification and quantitation.

Chapter 4: Genome-Wide SNP 6.0 Assay for 48 Samples

Includes a detailed, step-by-step protocol for processing 48 samples of human genomic DNA.

Chapter 5: Genome-Wide SNP 6.0 Assay for 96 Samples

Includes a detailed, step-by-step protocol for processing 96 samples of human genomic DNA.

Chapter 6: Washing, Staining, and Scanning

Includes instructions and protocols for fluidics station and scanner operation.

Chapter 7: Data Analysis

Describes how to analyze data using Affymetrix Genotyping Console™.

Chapter 8: Troubleshooting

Provides additional guidelines for obtaining optimal assay results including troubleshooting tips.

Chapter 9: Instrument Maintenance

Includes maintenance recommendations and procedures for the vacuum manifold and fluidics station.

Appendix A: Alternative Purification Protocol Using a Seahorse Filter Plate

Provides an alternative to the purification stages described in the 48- and 96-sample protocols.

Appendix B: Reagents, Equipment, and Supplies Required for the Genome-Wide SNP 5.0/6.0 Assay

Includes vendor and part number information for the equipment and reagents required.

Appendix C: Thermal Cycler Programs Required for the Genome-Wide SNP 5.0/6.0 Assay

Lists the thermal cycler programs required.

Appendix D: E-Gels

Describes the use of e-gels for the protocol.

About Whole Genome Sampling Analysis

Long before the completion of the human genome sequence, 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 10 million SNPs with a minor allele frequency of at least 5% [7]. The international effort to characterize human haplotypes (HapMap Project) in four major world populations has identified a standard set of common-allele SNPs that have provided 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, 10].

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 [11]. 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 [12-19].

We have developed an assay termed whole-genome sampling analysis (WGSA) for highly multiplexed SNP genotyping of complex DNA [20, 21]. This method reproducibly amplifies a subset of the human genome through a single primer amplification reaction using restriction enzyme digested, adapter-ligated human genomic DNA. 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 [22-41] and association studies [42-47]. The WGSA assay was extended to allow highly accurate SNP genotyping of over 100,000 SNPs using the two array GeneChip® Mapping 100K Set [48]. These arrays have been used for genome-wide LD studies [49] as well as landmark whole-genome association studies in age-related macular degeneration, multiple sclerosis, and cardiac repolarization. [50–52]. The WGSA assay was again extended in 2005 with the fourth-generation product known as the GeneChip® Mapping 500K Assay in which 500, 000 SNPs are queried using a two-array set. These arrays are being used to study a number of gene associations including the identification of genes associated with memory and scizophrenia [53, 54].

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 population and admixture structure [55-56] and DNA copy number changes. The latter include but are not limited to loss of heterozygosity (LOH), deletions, uniparental disomy (UPD) and gene amplifications [59-82]. The integration of DNA copy number changes with gene expression profiles provides a powerful paradigm for elucidating gene function, elegantly illustrated for example by the demonstration that MITF is an oncogene amplified in malignant melanoma [83].

In the last several years there has been an increasing appreciation of the extent of structural variation present among normal individuals [84-90]. Copy number variations (CNVs) can encompass a wide-range of molecular alterations including duplications, losses, and inversions, can span sizes from ~5kb to 50kb (intermediate sized) and 50kb to 3Mb (large scale), and are distinct from the genetic sequence diversity represented by (SNPs). Although there are several clear examples of how CNVs can influence susceptibility to HIV infection [91], modulate drug responses [92], or contribute to genomic micro-deletion and duplication syndromes [93], a comprehensive biological understanding of the roles of CNVs is not yet currently available but will be important in the context of both the normal and disease states. To this end, the GeneChip® Mapping 500K array set (early access version) has recently been used for a comprehensive view of CNVs among 270 HapMap samples. Greater than 1,000 copy number variable regions were found spanning a broad size range from less than 1kb to over 3Mb [94, 95]. Importantly, the genetic correlation between CNVs and SNPs has also been studied. In the case of biallelic CNVs and common deletion polymorphisms, there is evidence of linkage disequilibrium with neighboring SNPs, but this relationship is not nearly as strong in the case of complex CNVs [94, 96-98]. Thus whole genome SNP-based association studies should benefit from the capability to type CNVs directly rather than relying on LD with SNP markers.

The sixth-generation product in the mapping portfolio, the Affymetrix® Genome-Wide Human SNP Array 6.0, also uses the WGSA assay that has been the hallmark characteristic of all previous mapping arrays. This single array interrogates 906,600 SNPs by combining the Nsp I and Sty I PCR fractions prior to the DNA purification step and through a reduction in the absolute number of features associated with each individual SNP on the array. This array also contains 945,826 copy number probes designed to interrogate CNVs in the genome; 115,000 of these probes interrogate previously identified CNVs while the remaining 831,000 are distributed across the genome for improved CNV detection.

In summary, the Genome-Wide Human SNP Array 6.0 leverages the DNA target prep that is successfully used for the GeneChip® Mapping 500K array set such that 906,600 SNPs are genotyped on a single array. The array also contains copy number probes for improved detection of CNVs present in the genome. The Genome-Wide Human SNP Array 6.0 thus provides a robust, 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 SNP-based association studies.

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Chapter 2

LABORATORY SETUP AND RECOMMENDATIONS

General Workflow

Table 2.1 lists the laboratory areas in which the various stages of the Affymetrix® Genome-Wide Human SNP Nsp/Sty 5.0/6.0 Assay (Genome-Wide SNP 5.0/6.0 Assay) should be carried out: Pre-PCR Clean Room, PCR Staging Room, and Main Lab. Guidelines and recommendations for each area are provided in this chapter.

Table 2.1 Overview of the Areas Required to Perform the Genome-Wide SNP 5.0/6.0 Assay

Area	Template (Genomic DNA)	PCR Product
Pre-PCR Clean Room Assay Steps: • Reagent Preparation	⊘	⊘
PCR Staging Room Assay Steps: • Digestion • Ligation • PCR (set up only)	✓	⊘
Main Lab Assay Steps: • PCR thermal cycling • PCR cleanup • Fragmentation • Labeling • Hybridization • Washing and staining • Scanning	✓	✓

Contamination Prevention

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.

NOTE:

- **The most likely potential source of contamination for the Genome-Wide Human SNP Nsp/Sty 5.0/6.0 Assay is previously amplified PCR product.**
- **Each room should contain dedicated equipment such as thermal cyclers, microfuges, pipets, tips, etc.**
- **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.**
- **Maintain an ambient laboratory environment throughout the procedure.**

Precautions that you can take to minimize contaminating pre-PCR steps with amplified PCR product include the following:

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

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.

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 Room. This room should contain dedicated pipets, tips, vortex, etc. Refer to Appendix B, *Reagents, Equipment, and Consumables* for more information.

PCR Staging Room

The PCR Staging Room 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.

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 Room without first showering and changing into freshly laundered clothes.

Safety Precautions

The Affymetrix® Genome-Wide Human SNP Nsp/Sty Assay Kit 5.0/6.0 as well as the Affymetrix® Genome-Wide Human SNP Array 6.0 are for research use only.

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

NOTE: 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

The general requirements for genomic DNA sources and extraction methods are described in this chapter. 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 the Affymetrix® Genome-Wide Human SNP Nsp/Sty Assay Kit 5.0/6.0. 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 described below. However, the reliability of any given result should be assessed in the context of overall experimental design and goals.

General Requirements

- DNA must be double-stranded (not single-stranded).

  This requirement relates to the restriction enzyme digestion step in the protocol.

- 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 *48 Sample Protocol* or Chapter 5 *96 Sample Protocol*.

- 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.

- 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.

- Genomic DNA amplified with the Repli-G® Kit (a 29 whole genome amplification kit; QIAGEN) has been tested successfully with the Affymetrix® Genome-Wide Human SNP Nsp/Sty 5.0/6.0 Assay. The Repli-G Kit was used to amplify 30 ng genomic DNA. The amplified products (without purification) were immediately used in the subsequent protocol steps (using 250 ng amplified DNA for each Nsp I and Sty I restriction digestion).

  This procedure gave Birdseed algorithm call rates averaging 99.5 ± 0.3%, with an average concordance of 99.3 + 0.6%. Other pre-amplification methods or pre-digestion with restriction enzymes other than Nsp I or Sty I have not been tested by Affymetrix. If other methods are desired, we recommend conducting experiments to evaluate their performance with the Genome-Wide SNP 5.0/6.0 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* on page 19.

- 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 under *General Requirements* on page 19.

Whole-genome Amplification

For information on whole-genome amplification, refer to the following technical note which is available on our website:

Linking Whole-genome Amplification to SNP Genotyping, P/N 702722

This technical note contains recommendationsfor whole-genome amplification of small amounts of genomic DNA for analysis using the Affymetrix Genome-Wide Human SNP Arrays 6.0 and 5.0. These recommendations were developed by the systematic assessment of assay performance using different starting genomic DNA amounts and cleanup options.

Genomic DNA Extraction/Purification Methods

Genomic DNA extraction and purification methods that meet the general requirements 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 min.
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 min.
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

48 Sample Protocol

About This Protocol

The Affymetrix® Genome-Wide Human SNP Nsp/Sty 5.0/6.0 Assay (Genome-Wide SNP 5.0/6.0 Assay) is designed for processing 48 samples. The protocol is presented in the following stages:

- *Genomic DNA Plate Preparation*
- *Stage 1: Sty Restriction Enzyme Digestion*
- *Stage 2: Sty Ligation*
- *Stage 3: Sty PCR*
- *Stage 4: Nsp Restriction Enzyme Digestion*
- *Stage 5: Nsp Ligation*
- *Stage 6: Nsp PCR*
- *Stage 7: PCR Product Purification Using a Millipore Filter Plate*
- *Stage 8: Quantitation*
- *Stage 9: Fragmentation*
- *Stage 10: Labeling*
- *Stage 11: Target Hybridization*

Key points regarding the various molecular biology steps that comprise whole-genome sampling analysis (WGSA) are included in the protocol and guidelines.

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 1, genomic DNA is digested with the restriction enzyme Sty I. In stage 2, it is ligated to a common adaptor with T4 DNA ligase. Following ligation, the template undergoes PCR using TITANIUM™ Taq DNA polymerase. Once the product has been purified (stage 7), it is then fragmented in stage 9 with Fragmentation Reagent (DNAse I), and end-labeled using terminal deoxynucleotidyl transferase (stage 10).

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:
  - Ensure that enzymes are kept at –20 °C until needed.
  - Keep all master mixes and working solutions in chilled cooling chambers.
  - 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.
- Keep dedicated equipment in each of the areas used for this protocol (including pipettors, ice buckets, coolers, etc.). To avoid contamination, do not move equipment from one area to another.

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:

- All of the thermal cyclers (PCR Staging Room and Main Lab)
- GeneChip® Hybridization Oven 640
- GeneChip® Fluidics Station 450
- GeneChip® Scanner 3000 7G
- The UV spectrophotometer plate reader
- All multi-channel pipets

About the Cytogenetics Copy Number Assay

! IMPORTANT: The Cytogenetics Copy Number assay protocol is optimized for processing from 4 to 24 samples at a time to obtain copy number results. This protocol is not intended for genome-wide association studies.

The 48 and 96 sample protocols described in this user guide have been optimized for genome-wide association studies.

Workflow Recommendations

Figure 4.1 shows the recommended workflow for one operator processing 48 samples.

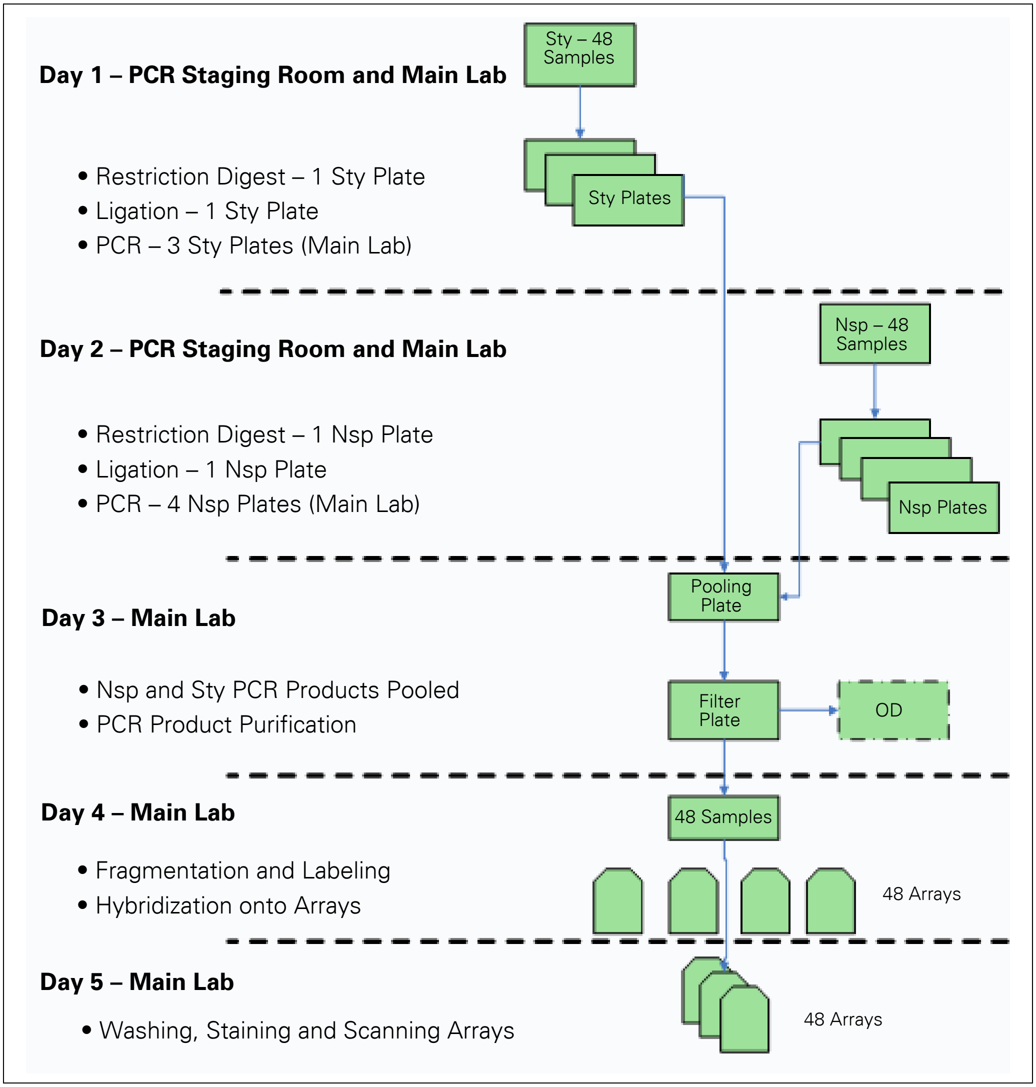


Figure 4.1 Workflow recommended for processing 48 samples

Since WGSA involves a series of ordered stages, the output of one stage directly impacts the performance of the subsequent stage. For example, the quantity and purity of the DNA after purification can affect the kinetics of the Fragmentation Reagent during the subsequent fragmentation stage.

To efficiently process samples in 96-well plates, it is essential that you be proficient with the use of multi-channel pipets. Attempting to use a single channel pipet 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 pipets, we strongly recommend practicing several times before processing actual samples. You can use water to get a feel for aspirating and dispensing solutions to multiple wells simultaneously.

Post-PCR stages 7 through 11 are best performed by the more experienced operators in your laboratory. These operators should be proficient in:

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

When processing multiple full plates, we recommend that the same operator not perform too many stages in a given day. Dedicating small teams to different stages of the protocol has proven to be a highly effective method of managing this workflow.

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

- Team 1: Pre-PCR (digestion and ligation)
- Team 2: PCR (PCR and PCR product purification and quantitation)
- Team 3: Post-PCR (fragmentation and labeling)
- Team 4: Array processing (hybridization, fluidics, and scanning)

Your technical support representative can provide additional guidance on how best to organize lab personnel for this protocol.

Before You Begin

Master Mix Preparation

Carefully follow each master mix recipe. Use pipets that have been calibrated to ± 5%. When molecular biology-grade water is specified, be sure to use the AccuGENE® water listed in Appendix B. Using in-house ddH_2O or other water can negatively affect your results. The enzymatic reaction in *Stage 9: 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 pipets are not accurate. We recommend that you stop and repeat the experiment.

Reagent Handling and Storage

Follow these guidelines for reagent handling and storage.

- Keep dedicated equipment in each of the areas used for this protocol. To avoid contamination, do not move equipment between the Pre-PCR Area, the PCR Staging Room and the Main Lab.
- Unless otherwise indicated, keep all reagents (except enzymes) on ice in a cooling chamber that has been chilled to 4 °C when working on the bench top.
- 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.
- Consult the appropriate MSDS for reagent storage and handling requirements.
- 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.
- When performing the steps for Stages 1 through 10 of the 48-sample protocol:
  - Keep all tubes on ice or in a cooling chamber on ice.
  - Keep all plates in cooling chambers on ice.

Preparing the Work Area for Each Stage

Many of the stages in the Genome-Wide SNP 5.0/6.0 Assay must be performed rapidly and on ice to carefully control enzyme activity and temperature transitions. Therefore, we recommend that you 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 1: Sty Restriction Enzyme Digestion*. Pipets and tips are not shown.

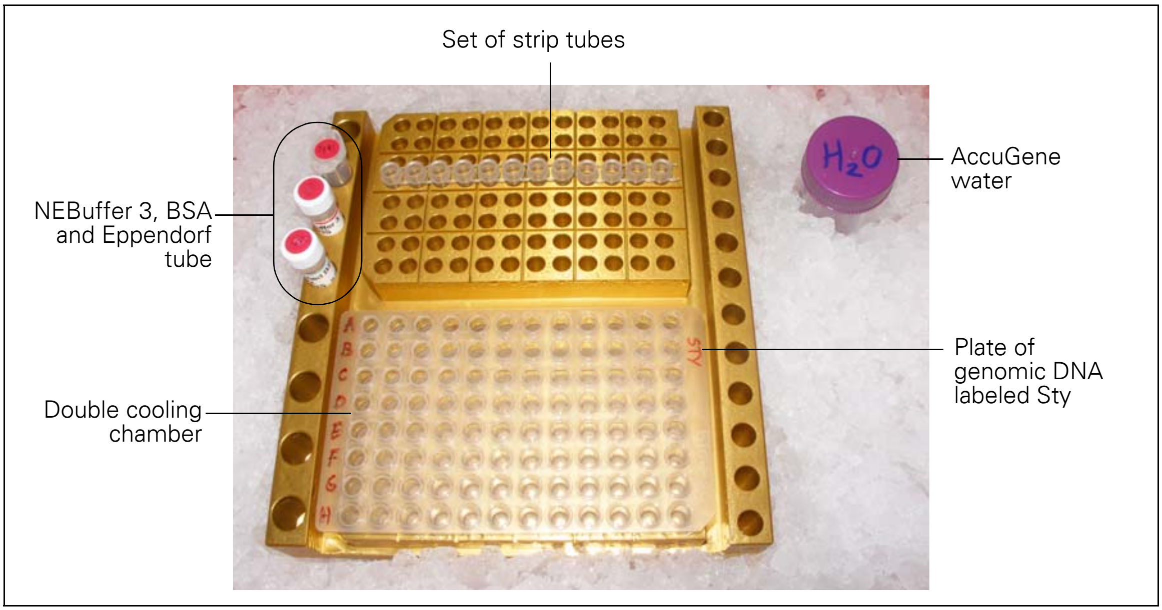


Figure 4.2 Example of Work Area Preparation

Thermal Cyclers, Plates and Plate Seals

The Genome-Wide SNP 5.0/6.0 Assay has been optimized using the following thermal cyclers, reaction plate and adhesive seals.

> **!** **IMPORTANT: Use only the 96-well plate and adhesive seals listed in Table 4.1, and only the thermal cyclers listed in Table 4.2. Using other plates and seals that are incompatible with these thermal cyclers can result in loss of sample or poor results.**

Table 4.1 96-well plate and adhesive seals optimized for use with this protocol

Item	Vendor	Part Number
Multiplate 96-well unskirted PCR plate	Bio-Rad	MLP-9601
Adhesive seals:		
• Microseal 'B' Adhesive Seal	Bio-Rad	MSB1001
• MicroAmp® Clear Adhesive Film	Applied Biosystems	4306311

Table 4.2 Thermal cyclers optimized for use with this protocol

Laboratory	Thermal Cyclers Validated for Use
Pre-PCR Clean Area	Applied Biosystems units: • 2720 Thermal Cycler • GeneAmp® PCR System 9700
	Bio-Rad units: MJ Tetrad PTC-225 DNA Engine Tetrad 2
Post-PCR Area	Applied Biosystems: • GeneAmp® PCR System 9700 (silver block or gold-plated silver block)
	Bio-Rad units: • MJ Tetrad PTC-225 • DNA Engine Tetrad 2

Program Your Thermal Cyclers

The thermal cycler programs listed below are used during this protocol. Before you begin processing samples, enter and store these programs on the appropriate thermal cyclers in the PCR Staging Room and the Main Lab.

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

Table 4.3 Thermal Cycler Programs Required for the 48 Sample Protocol (Figure 4.1 on page 25)

Program Name	# of Thermal Cyclers Required	Laboratory
GW5.0/6.0 Digest	1	PCR Staging Room
GW5.0/6.0 Ligate	1	PCR Staging Room
GW5.0/6.0 PCR	4	Main Lab
GW5.0/6.0 Fragment	1	Main Lab
GW5.0/6.0 Label	1	Main Lab
GW5.0/6.0 Hyb	1	Main Lab

Genomic DNA Plate Preparation

About this Stage

The human genomic DNA you will process using the Genome-Wide SNP 5.0/6.0 Assay 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 the corresponding wells of two 96-well plates.

Location and Duration

- PCR Staging Room
- Hands-on time: time will vary; can be up to 4 hours

Input Required

This protocol is written for processing two replicates of 48 genomic DNA samples including controls.

Table 4.4 Input Required for *Genomic DNA Plate Preparation*

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

Equipment and Consumables Required

The equipment and consumables listed in Table 4.5 are required for this stage.

Table 4.5 Equipment and Consumables Required for *Genomic DNA Plate Preparation*

Quantity	Item
enough for three 96-well plates	Cooling chambers, chilled to 4 °C (do not freeze)
1	Ice bucket, filled with ice
1	Plate centrifuge
1	Pipet, single channel P20
1	Pipet, 12-channel P200
1	Pipet, single channel P200
As needed	Pipet tips
As needed (2 per sample)	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 on page 29.	

Reagents Required

The following reagents are required for this stage.

Table 4.6 Reagents Required for *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 may 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 two 96-well reaction plates.

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

   Two replicates of each sample are required for this protocol: one for Nsp and one for processing Sty.
3. Seal each plate with adhesive film.

What To 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 1: Sty Restriction Enzyme Digestion

About this Stage

During this stage, the genomic DNA is digested by the Sty I restriction enzyme. You will:

1. Prepare a Sty Digestion Master Mix.
2. Add the master mix to one set of 48 samples.
3. Place the samples onto a thermal cycler and run the GW5.0/6.0 Digest program.

Location and Duration

- Pre-PCR Clean Area
- Hands-on time: 30 min
- GW5.0/6.0 Digest thermal cycler program time: 2.5 hours

Input Required From Previous Stage

The input required is shown below.

Quantity	Item
48 samples	Genomic DNA prepared as instructed under *Genomic DNA Plate Preparation* on page 31 (5 µL at 50 ng/µL in each well).

Equipment and Consumables Required

The following equipment and consumables are required for this stage.

Table 4.7 Equipment and Consumables Required for *Stage 1: Sty 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	Pipet, single channel P100
1	Pipet, single channel P200
1	Pipet, single channel P1000
1	Pipet, 12-channel P20
As needed	Pipet tips for pipets listed above; full racks
1	Plate centrifuge
1	Plate seal**
1	Thermal cycler**
1 strip	Tubes, 12-strip, 0.2 mL
1	Tube, Eppendorf 2.0 mL
1	Vortexer
** **IMPORTANT** Use only the PCR plate, adhesive film and thermal cyclers listed on page 29.	

Reagents Required

The following reagents are required for this stage. The amounts listed are sufficient for processing 48 samples.

Table 4.8 Reagents Required for *Stage 1: Sty Restriction Enzyme Digestion*

Quantity	Reagent
1 vial	BSA (100X; 10 mg/mL)
1 vial	NE Buffer 3 (10X)
1 vial	Sty I (10 U/µL; NEB)
2.5 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 I and Sty I digestion reactions on the same day.**

About Using Controls

Positive Controls

We recommend including one positive and one negative control with every set of samples run.

Reference Genomic DNA 103 can be used as a positive control. It is supplied in the Genome-Wide Human SNP Nsp/Sty Assay Kit 5.0/6.0.

A process negative control can be included at the beginning of the assay to assess the presence of contamination. Refer to Chapter 3 and Chapter 8 for more information.

Prepare the Reagents, Equipment and Consumables

Thaw Reagents and Genomic DNA

1. Allow the following reagents to thaw on ice:
   - NE Buffer 3
   - BSA
2. If the genomic DNA is frozen, allow it to thaw in a cooling chamber on ice.

> **IMPORTANT:** **Leave the 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 4.3 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 with genomic DNA 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. Prepare the reagents (except for the enzyme) as follows:
   - **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.

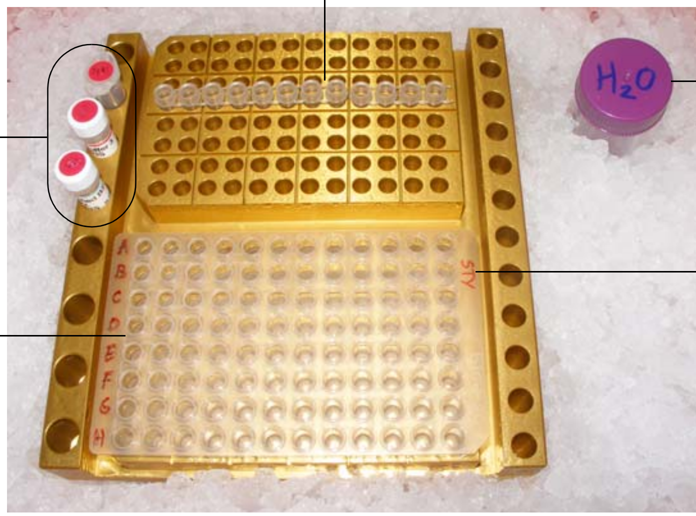


Figure 4.3 Work Area Prepared for Processing Samples with Sty Digest Mix (Sty Enzyme Not Pictured; Still at –20 °C)

Prepare the Sty Digestion Master Mix

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

1. To the 2.0 mL Eppendorf tube, add the volumes of the following reagents as shown in Table 4.9:
   - AccuGENE water
   - NE Buffer 3
   - BSA
2. Remove the Sty I enzyme 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 Sty Digestion Master Mix to Samples* on page 40.

Table 4.9 Sty I Digestion Master Mix

Reagent	1 Sample	48 Samples (15% extra)
AccuGENE® Water	11.55 µL	637.6 µL
NE Buffer 3 (10X)	2 µL	110.4 µL
BSA (100X; 10 mg/mL)	0.2 µL	11 µL
Sty I (10 U/µL)	1 µL	55.2 µL
Total	**14.75 µL**	**814.2 µL**

Add Sty Digestion Master Mix to Samples

To add the Sty Digestion Master Mix to samples:

1. Using a single channel P200 pipet, aliquot 67 µL of Sty Digestion Master Mix to each tube of the strip tubes labeled *Dig*.
2. Using a 12-channel P20 pipet, add 14.75 µL of Sty Digestion Master Mix to each DNA sample in the cooling chamber on ice.

   The total volume in each well is now 19.75 µL.

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 GW5.0/6.0 Digest program.

Table 4.10 GW5.0/6.0 Digest Program

GW5.0/6.0 Digest Program	
Temperature	**Time**
37 °C	120 min
65 °C	20 min
4 °C	Hold

8. When the program is finished, remove the plate and spin it down at 2000 rpm for 30 sec.

What To Do Next

Do one of the following:

- If following the recommended workflow (Figure 4.1 on page 25), place the plate in a cooling chamber on ice and proceed immediately to *Stage 2: Sty Ligation* on page 42.
- If not proceeding directly to the next step, store the samples at –20 °C.

Stage 2: Sty Ligation

About this Stage

During this stage, the digested samples are ligated using the Sty Adaptor. You will:

1. Prepare a Sty Ligation Master Mix.
2. Add the master mix to the samples.
3. Place the samples onto a thermal cycler and the GW5.0/6.0 Ligate program is run.
4. Dilute the ligated samples with AccuGENE water.

Location and Duration

- Pre-PCR Clean Area
- Hands-on time: 30 min
- GW5.0/6.0 Ligate thermal cycler program time: 3.3 hours

Input Required From Previous Stage

The input required from *Stage 1: Sty Restriction Enzyme Digestion* is:

Quantity	Item
48 samples	Sty digested samples in a cooling chamber on ice.

Equipment and Consumables Required

The following equipment and consumables are required for this stage.

Table 4.11 Equipment and Consumables Required for *Stage 2: Sty 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	Pipet, single channel P100
1	Pipet, single channel P1000
1	Pipet, 12-channel P20
1	Pipet, 12-channel P200
As needed	Pipet tips for pipets listed above; full racks
1	Plate centrifuge
2	Plate seal**
1	Solution basin, 55 mL
1	Thermal cycler**
1 strip	Tubes, 12-strip, 0.2 mL
1	Tube, Eppendorf 2.0 mL
1	Vortexer
** **IMPORTANT** Use only the PCR plate, adhesive film and thermal cyclers listed on page 29.	

Reagents Required

The following reagents are required for this stage. The amounts listed are sufficient to process 48 samples.

Table 4.12 Reagents Required for *Stage 2: Sty Ligation*

Quantity	Reagent
1 vial	T4 DNA Ligase (400 U/μL; NEB)
1 vial	T4 DNA Ligase Buffer (10X)
1 vial	Adaptor, Sty (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.

Prepare the Reagents, Consumables and Other Components

! **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 Sty adaptor.**

Thaw the Reagents and Sty Digestion Stage Plate

To thaw the reagents and Sty Digestion Stage Plate:

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

   Requires approximately 20 min to thaw.
2. If the Sty digested samples were frozen, allow them 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 4.2 on page 28).
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 digested samples 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 Sty Ligation Master Mix

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

1. To the 2.0 mL Eppendorf tube, add the following reagents based on the volumes shown in Table 4.13:
   - Adaptor Sty I
   - 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 Sty Ligation Master Mix to Reactions*.

Table 4.13 Sty I Ligation Master Mix

Reagent	1 Sample	48 Samples (25% extra)
T4 Ligase Buffer (10X)	2.5 µL	150 µL
Adaptor Sty I (50 µM)	0.75 µL	45 µL
T4 DNA Ligase (400U/µL)	2 µL	120 µL
Total	**5.25 µL**	**315 µL**

Add Sty Ligation Master Mix to Reactions

To add Sty Ligation Master Mix to samples:

1. Using a single channel P100 pipet, aliquot 25 µL of Sty Ligation Master Mix to each tube of the strip tubes on ice.
2. Using a 12-channel P20 pipet, aliquot 5.25 µL of Sty Ligation Master Mix to each reaction on the Sty Digestion Stage Plate.

   The total volume in each well is now 25 µL.

Sty Digested DNA	19.75 µL
Sty 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 GW5.0/6.0 Ligate program.

Table 4.14 GW5.0/6.0 Ligate Thermal Cycler Program

GW5.0/6.0 Ligate Program	
Temperature	**Time**
16ºC	180 min
70ºC	20 min
4ºC	Hold

Dilute the Samples

! **IMPORTANT: It is crucial to dilute the ligated DNA with AccuGENE water prior to PCR.**

To dilute the samples:

8. Place the AccuGENE water on ice 20 min prior to use.

1. When the GW5.0/6.0 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 pipet, add 75 µL of the water to each reaction.

   The total volume in each well is 100 µL.

Sty 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.

What To Do Next

Do one of the following:

- If following the recommended workflow (Figure 4.1 on page 25), proceed immediately to *Stage 3: Sty PCR* on page 49.

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

Stage 3: Sty PCR

About this Stage

During this stage, you will:

1. Transfer equal amounts of each Sty ligated sample into three fresh 96-well plates (Figure 4.4 on page 54).
2. Prepare the Sty PCR Master Mix, and add it to each sample.
3. Place each plate on a thermal cycler and run the GW5.0/6.0 PCR program.
4. Confirm the PCR by running 3 µL of each PCR product on a 2% TBE gel or an E-Gel® *48* 2% agarose gel.

Location and Duration

- Pre-PCR Clean Area: Sty PCR Master Mix preparation
- PCR Staging Area: PCR set up
- Main Lab: PCR Plates placed on thermal cyclers
- Hands-on time: 1 hour
- GW5.0/6.0 PCR thermal cycler program time: 1.5 hours; samples can be held overnight at 4 °C.

Input Required from Previous Stage

The input required from *Stage 2: Sty Ligation* is:

Quantity	Item
48	Diluted Sty ligated samples

Equipment and Materials Required

The following equipment and materials are required to perform this stage.

Table 4.15 Equipment and Consumables Required for *Stage 3: Sty PCR*

Quantity	Item
1	Cooler, chilled to –20 °C
Enough for up to five 96-well plates	Cooling chambers, chilled to 4 °C (do not freeze)
1	Ice bucket, filled with ice
1	Marker, fine point, permanent
1	Microcentrifuge
1	Pipet, single channel P20
1	Pipet, single channel P100
1	Pipet, single channel P200
1	Pipet, single channel P1000
1	Pipet, 12-channel P20
1	Pipet, 12-channel P200
As needed	Pipet tips for pipets listed above; full racks
3	Plates, 96-well reaction**
1	Plate centrifuge
As needed	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 on page 29.	

Reagents Required

The following reagents are required for this stage. The amounts listed are sufficient to process 48 samples.

Table 4.16 Reagents Required for *Stage 3: Sty PCR*

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

Gels and Related Materials Required

Verifying the PCR reaction is required for this stage. You can use the following gels and related materials, or E-Gels as described in Appendix D, *E-gels,* on page 327. The amounts listed are sufficient to process 48 Sty samples.

Table 4.17 Gels and Related Materials Required for *Stage 3: Sty PCR*

Quantity	Reagent
190 µL	DNA Marker
19	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 Sty ligated DNA was diluted to 100 µL with AccuGENE water.**
- **Set up the PCRs in PCR Staging Area.**
- **Prepare Sty 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.**
- **To ensure consistent results, take 3 µL aliquots from each PCR to run on gels.**

About Controls

A PCR negative control can be included in the experiment to assess the presence of contamination. Refer to Chapter 3 and Chapter 8 for more information.

Prepare the Reagents, Consumables and Other Components

Thaw Reagents and Ligated Samples

To thaw the reagents and ligated samples:

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 Sty ligated samples are 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 cooling chambers and one cooler on ice.
2. Label the following, then place in a cooling chamber:
   - Three 96-well reaction plates labeled *P1, P2, P3* (see Figure 4.4 on page 54)
   - One 50 mL Falcon tube labeled *PCR MM*
3. Place on ice:
   - AccuGENE water
   - GC-Melt
   - Solution basin
4. Prepare the Sty ligated samples 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 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.

Aliquot Sty Ligated DNA to the PCR Plates

To aliquot Sty ligated DNA to the PCR plates:

1. Working one row at a time and using a 12-channel P20 pipet, transfer 10 µL of each Sty ligated sample to the corresponding well of each PCR plate.

   Example (Figure 4.4): Transfer 10 µL of each sample from Row A of the Sty Ligation Stage Plate to the corresponding wells of row A on the plates labeled P1, P2, and P3.
2. Seal each plate with adhesive film, and leave in cooling chambers on ice.

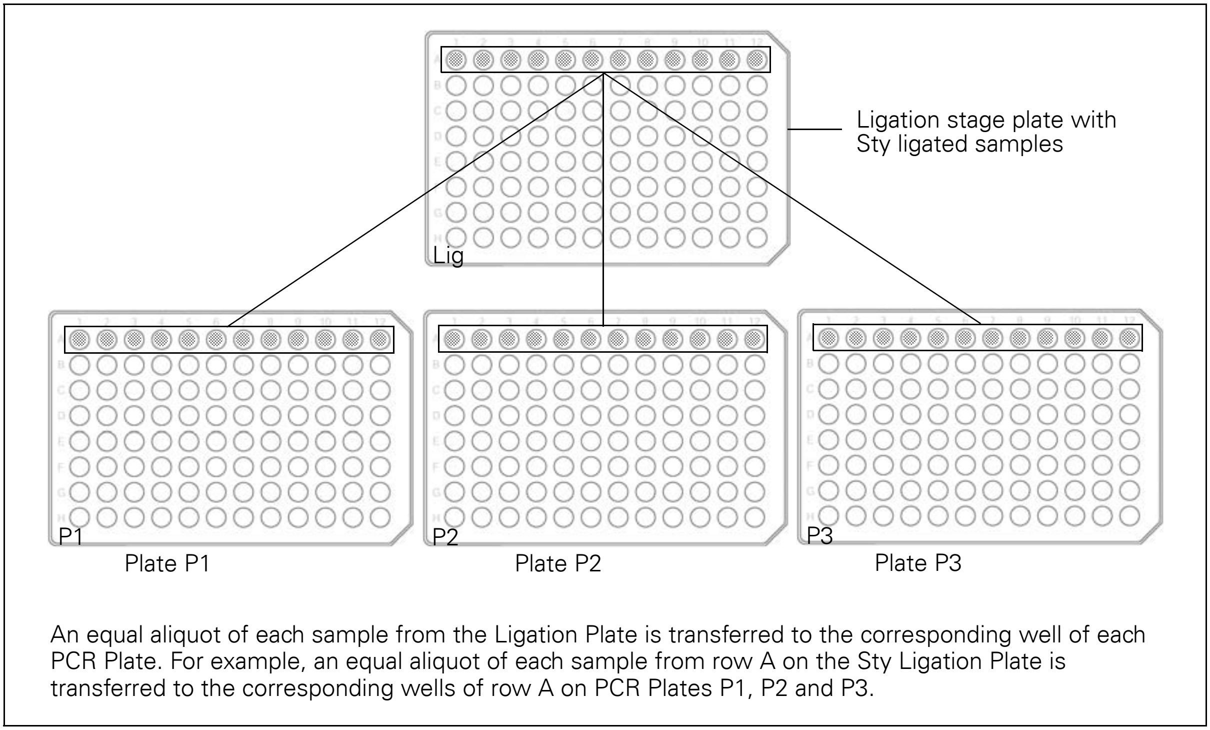


Figure 4.4 Transferring Equal Aliquots of Diluted, Ligated Sty Samples to Three Reaction Plates

Prepare the Sty PCR Master Mix

Location

Pre-PCR Clean Room

Prepare the Sty PCR Master Mix

To prepare the Sty 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.**

1. Keeping the 50 mL Falcon tube in the cooling chamber, add the reagents as shown in Table 4.18 on page 55 (**except for the *Taq* DNA polymerase**).

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 4.18 Sty PCR Master Mix for 48 Samples

Reagent	For 1 Reaction	3 PCR Plates, 48 Samples Each Plate (15% extra)
AccuGENE water	39.5 µL	6.541 mL
TITANIUM *Taq* PCR Buffer (10X)	10 µL	1.656 mL
GC-Melt (5M)	20 µL	3.312 mL
dNTP (2.5 mM each)	14 µL	2.318 mL
PCR Primer 002 (100 µM)	4.5 µL	0.745 mL
TITANIUM *Taq* DNA Polymerase (50X) (do not add until ready to aliquot master mix to ligated samples)	2 µL	0.331 mL
Total	**90 µL**	**14.903 mL**

Add Sty PCR Master Mix to Samples

Location

PCR Staging Area

Procedure

To add Sty PCR Master Mix to samples:

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

   To avoid contamination, change pipet tips after each dispense.

   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 Sty PCR Plates Onto Thermal Cyclers

IMPORTANT: PCR protocols for the MJ Tetrad PTC-225 and Applied Biosystems thermal cyclers are different. See Table 4.19 and Table 4.20 below.

Location

Main Lab

Procedure

To load the plates and run the GW5.0/6.0 PCR program:

1. Transfer the 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 GW5.0/6.0 PCR program.

   The program varies depending upon the thermal cyclers you are using. See Table 4.19 for Applied Biosystems thermal cyclers and Table 4.20 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 4.19 GW5.0/6.0 PCR Thermal Cycler Program for the GeneAmp® PCR System 9700 (silver or gold-plated silver blocks)

GW5.0/6.0 PCR Program for GeneAmp® PCR System 9700		
Temperature	**Time**	**Cycles**
94°C	3 min	1X
94°C	30 sec	30X
60°C	45 sec	
68°C	15 sec	
68°C	7 min	1X
4°C	HOLD (Can be held overnight)	
Volume: 100 µL		
Specify *Maximum* mode.		

Table 4.20 GW5.0/6.0 PCR Thermal Cycler Program for the MJ Tetrad PTC-225

GW5.0/6.0 PCR Program for MJ Tetrad PTC-225		
Temperature	**Time**	**Cycles**
94°C	3 min	1X
94°C	30 sec	30X
60°C	30 sec	
68°C	15 sec	
68°C	7 min	1X
4°C	HOLD (Can be held overnight)	
Volume: 100 µL		
Use *Heated Lid* and *Calculated Temperature*		

Running Gels

The instructions below are for running 2% TBE gels. For information on running E-Gel *48* 2% agarose gels, refer to Appendix D, *E-gels,* on page 327.

Before Running Gels

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

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

Run the Gels

When the GW5.0/6.0 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 in rows A through D of the fresh, labeled P*X*Gel plates.
6. Using a 12-channel P20 pipet, transfer 3 µL of each PCR product from the 3 Sty PCR plates to the corresponding plate, row and wells of the P*X*Gel plates.

   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 the P*X*Gel plates.
8. Vortex the center of each P*X*Gel plate*,* then spin them down at 2000 rpm for 30 sec.
9. Load the total volume from each well of each P*X*Gel plate onto 2% TBE gels.
10. Run the gels at 120V for 40 min to 1 hour.
11. Verify that the PCR product distribution is between ~200 bp to 1100 bp (see Figure 4.5).

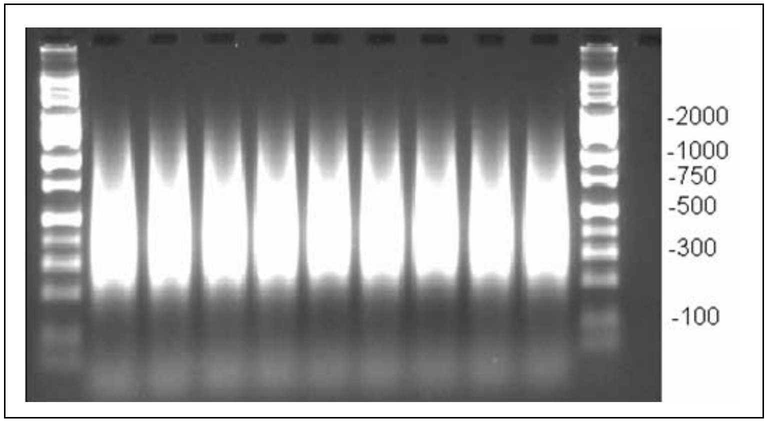


Figure 4.5 Example of PCR products run on 2% TBE agarose gel at 120V for 1 hour. Average product distribution is between ~200 to 1100 bp.

What To Do Next

Do one of the following:

- If following the recommended workflow (Figure 4.1 on page 25), seal the Sty PCR product plates and store them at –20 °C.
- Proceed to the next stage within 60 min.

Stage 4: Nsp Restriction Enzyme Digestion

About this Stage

During this stage, the genomic DNA is digested by the Nsp I enzyme. You will:

1. Prepare a Nsp Digestion Master Mix.
2. Add the master mix to one set of 48 samples.
3. Place the samples onto a thermal cycler and run the GW5.0/6.0 Digest program.

Location and Duration

- Pre-PCR Clean Area
- Hands-on time: 30 min
- GW5.0/6.0 Digest thermal cycler program time: 2.5 hours

Input Required From Previous Stage

The input required is shown below.

Quantity	Item
48 samples	Genomic DNA prepared as instructed under *Genomic DNA Plate Preparation* on page 31 (5 µL at 50 ng/µL in each well).

Equipment and Consumables Required

The following equipment and consumables are required for this stage.

Table 4.21 Equipment and Consumables Required for *Stage 4: Nsp 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	Pipet, single channel P100
1	Pipet, single channel P200
1	Pipet, single channel P1000
1	Pipet, 12-channel P20
As needed	Pipet tips for pipets listed above; full racks
1	Plate centrifuge
1	Plate seal**
1	Thermal cycler**
1 strip	Tubes, 12-strip, 0.2 mL
1	Tube, Eppendorf 2.0 mL
1	Vortexer
** **IMPORTANT** Use only the PCR plate, adhesive film and thermal cyclers listed on page 29.	

Reagents Required

The following reagents are required for this stage. The amounts listed are sufficient for processing 48 samples.

Table 4.22 Reagents Required for *Stage 4: Nsp Restriction Enzyme Digestion*

Quantity	Reagent
1 vial	BSA (100X; 10 mg/mL)
1 vial	NE Buffer 2 (10X)
1 vial	Nsp I (10 U/µL; NEB)
2.5 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

We recommend including one positive and one negative control with every set of samples run.

Reference Genomic DNA 103 can be used as a positive control. It is supplied in the Genome-Wide Human SNP Nsp/Sty Assay Kit 5.0/6.0.

A process negative control can be included at the beginning of the assay to assess the presence of contamination. Refer to Chapter 3 and Chapter 8 for more information.

Prepare the Reagents, Equipment and Consumables

Thaw Reagents and Genomic DNA

To thaw the reagents and genomic DNA:

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

> **IMPORTANT: Leave the NSP 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.
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 with genomic DNA 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. Prepare the reagents (except for the enzyme) as follows:
   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 Nsp Digestion Master Mix

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

1. To the 2.0 mL Eppendorf tube, add the appropriate volumes of the following reagents based on Table 4.23:
   - AccuGENE water
   - NE Buffer 2
   - BSA
2. Remove the Nsp I enzyme 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 Nsp Digestion Master Mix to Samples* on page 65.

Table 4.23 Nsp I Digestion Master Mix

Reagent	1 Sample	48 Samples (15% extra)
AccuGENE® Water	11.55 µL	637.6 µL
NE Buffer 2 (10X)	2 µL	110.4 µL
BSA (100X; 10 mg/mL)	0.2 µL	11 µL
Nsp I (10 U/µL)	1 µL	55.2 µL
Total	**14.75 µL**	**814.2 µL**

Add Nsp Digestion Master Mix to Samples

To add Nsp Digestion Master Mix to samples:

1. Using a single channel P200 pipet, aliquot 67 µL of Nsp Digestion Master Mix to each tube of the strip tubes labeled *Dig*.
2. Using a 12-channel P20 pipet, add 14.75 µL of Nsp Digestion Master Mix to each DNA sample in the cooling chamber on ice.

   The total volume in each well is now 19.75 µL.

Genomic DNA (50 ng/µL)	5 µL
Nsp 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 GW5.0/6.0 Digest program.

Table 4.24 GW5.0/6.0 Digest Program

GW5.0/6.0 Digest Program	
Temperature	**Time**
37 °C	120 min
65 °C	20 min
4 °C	Hold

8. When the program is finished, remove the plate and spin it down at 2000 rpm for 30 sec.

What To Do Next

Do one of the following:

- If following the recommended workflow (Figure 4.1 on page 25), proceed immediately to *Stage 5: Nsp Ligation* on page 66.
- If not proceeding directly to the next step, store the samples at –20 °C.

Stage 5: Nsp Ligation

About this Stage

During this stage, the digested samples are ligated using the Nsp Adaptor. You will:

1. Prepare a Nsp Ligation Master Mix.
2. Add the master mix to the samples.
3. Place the samples onto a thermal cycler and the GW5.0/6.0 Ligate program is run.
4. Dilute the ligated samples with AccuGENE water.

Location and Duration

- Pre-PCR Clean Area
- Hands-on time: 30 min
- GW5.0/6.0 Ligate thermal cycler program time: 3.3 hours

Input Required From Previous Stage

The input required from *Stage 4: Nsp Restriction Enzyme Digestion* is:

Quantity	Item
48 samples	Nsp digested samples in a cooling chamber on ice.

Equipment and Consumables Required

The following equipment and consumables are required for this stage.

Table 4.25 Equipment and Consumables Required for *Stage 5: Nsp 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	Pipet, single channel P100
1	Pipet, single channel P1000
1	Pipet, 12-channel P20
1	Pipet, 12-channel P200
As needed	Pipet tips for pipets listed above; full racks
1	Plate centrifuge
2	Plate seal**
1	Solution basin, 55 mL
1	Thermal cycler**
1 strip	Tubes, 12-strip, 0.2 mL
1	Tube, Eppendorf 2.0 mL
1	Vortexer
** **IMPORTANT** Use only the PCR plate, adhesive film and thermal cyclers listed on page 29.	

Reagents Required

The following reagents are required for this stage. The amounts listed are sufficient to process 48 samples.

Table 4.26 Reagents Required for *Stage 5: Nsp Ligation*

Quantity	Reagent
1 vial	T4 DNA Ligase (400 U/µL; NEB)
1 vial	T4 DNA Ligase Buffer (10X)
1 vial	Adaptor, Nsp (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.

Prepare the Reagents, Consumables and Other Components

! **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 Nsp adaptor.**

Thaw the Reagents and Nsp Digestion Stage Plate

To thaw the reagents and Nsp Digestion Stage Plate:

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

   Takes approximately 20 min to thaw.
2. If the Nsp digested samples were frozen, allow them 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.
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 digested samples 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 Nsp Ligation Master Mix

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

1. To the 2.0 mL Eppendorf tube, add the following reagents based on the volumes shown in Table 4.27:
   - Adaptor Nsp
   - 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 Nsp Ligation Master Mix to Reactions*.

Table 4.27 Nsp I Ligation Master Mix

Reagent	1 Sample	48 Samples (25% extra)
T4 DNA Ligase Buffer (10X)	2.5 µL	150 µL
Adaptor Nsp I (50 µM)	0.75 µL	45 µL
T4 DNA Ligase (400 U/µL)	2 µL	120 µL
Total	**5.25 µL**	**315 µL**

Add Nsp Ligation Master Mix to Reactions

To add Nsp Ligation Master Mix to samples:

1. Using a single channel P100 pipet, aliquot 25 µL of Nsp Ligation Master Mix to each tube of the strip tubes on ice.
2. Using a 12-channel P20 pipet, aliquot 5.25 µL of Nsp Ligation Master Mix to each reaction on the Nsp Digestion Stage Plate.

   The total volume in each well is now 25 µL.

Nsp Digested DNA	19.75 µL
Nsp 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 GW5.0/6.0 Ligate program.

Table 4.28 GW5.0/6.0 Ligate Thermal Cycler Program

GW5.0/6.0 Ligate Program	
Temperature	**Time**
16ºC	180 min
70ºC	20 min
4ºC	Hold

Dilute the Samples

> **IMPORTANT: It is crucial to dilute the ligated DNA with AccuGENE water prior to PCR.**

To dilute the samples:

8. Place the AccuGENE water on ice 20 min prior to use.

1. When the GW5.0/6.0 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 pipet, add 75 µL of the water to each reaction.

   The total volume in each well is 100 µL.

Nsp 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.

What To Do Next

Do one of the following:

- If following the recommended workflow (Figure 4.1 on page 25), proceed immediately to *Stage 6: Nsp PCR* on page 73.

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

Stage 6: Nsp PCR

About this Stage

During this stage, you will:

1. Transfer equal amounts of each Nsp ligated sample into four fresh 96-well plates.
2. Prepare the Nsp PCR Master Mix, and add it to each sample.
3. Place each plate on a thermal cycler and run the GW5.0/6.0 PCR program.
4. Confirm the PCR by running 3 µL of each PCR product on a 2% TBE gel or an E-Gel® *48* 2% agarose gel.

Location and Duration

- Pre-PCR Clean Area: Nsp PCR Master Mix preparation
- PCR Staging Area: PCR set up
- Main Lab: PCR Plates placed on thermal cyclers
- Hands-on time: 1 hour
- GW5.0/6.0 PCR thermal cycler program time: 1.5 hours; samples can be held overnight at 4 °C.

Input Required from Previous Stage

The input required from *Stage 5: Nsp Ligation* is:

Quantity	Item
48	Diluted Nsp ligated samples

Equipment and Materials Required

The following equipment and materials are required to perform this stage.

Table 4.29 Equipment and Consumables Required for *Stage 6: Nsp PCR*

Quantity	Item
1	Cooler, chilled to –20 °C
Enough for five 96-well plates	Cooling chambers, chilled to 4 °C (do not freeze)
1	Ice bucket, filled with ice
1	Marker, fine point, permanent
1	Microcentrifuge
1	Pipet, single channel P20
1	Pipet, single channel P100
1	Pipet, single channel P200
1	Pipet, single channel P1000
1	Pipet, 12-channel P20
1	Pipet, 12-channel P200
As needed	Pipet tips for pipets listed above; full racks
4	Plates, 96-well reaction**
1	Plate centrifuge
As needed	Plate seal**
1	Solution basin, 55 mL
4	Thermal cycler**
1	Tube, Falcon 50 mL
1	Vortexer
** **IMPORTANT** Use only the PCR plate, adhesive film and thermal cyclers listed on page 29.	

Reagents Required

The following reagents are required for this stage. The amounts listed are sufficient to process 48 samples.

Table 4.30 Reagents Required for *Stage 6: Nsp PCR*

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

Gels and Related Materials Required

Verifying the PCR reaction is required for this stage. You can use the following gels and related materials, or E-Gels as described in Appendix D, *E-gels,* on page 327. The amounts listed are sufficient to process 48 Sty samples.

Table 4.31 Gels and Related Materials Required for *Stage 6: Nsp PCR*

Quantity	Reagent
190 µL	DNA Marker
19	Gels, 2% TBE
As needed	Gel loading solution
4	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 Nsp ligated DNA was diluted to 100 µL with AccuGENE water.**
- **Set up the PCRs in PCR Staging Area.**
- **Prepare Nsp 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.**
- **To ensure consistent results, take 3 µL aliquots from each PCR to run on gels.**

About Controls

A PCR negative control can be included in the experiment to assess the presence of contamination. Refer to Chapter 3 and Chapter 8 for more information.

Prepare the Reagents, Consumables and Other Components

Thaw Reagents and Nsp Ligated Samples

To thaw the reagents and Nsp ligated samples:

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 Nsp ligated samples are 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 enough cooling chambers for 5 plates and one cooler on ice.
2. Label the following, then place in a cooling chamber:
   - Four 96-well reaction plates labeled *P1, P2, P3, P4*
   - One 50 mL Falcon tube labeled *PCR MM*
3. Place on ice:
   - AccuGENE water
   - GC-Melt
   - Solution basin
4. Prepare the Nsp ligated samples 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 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.

Aliquot Nsp Ligated DNA to the PCR Plates

To aliquot Nsp ligated DNA to the PCR plates:

1. Working one row at a time and using a 12-channel P20 pipet, transfer 10 µL of each Nsp ligated sample to the corresponding well of each PCR plate (P1, P2, P3 and P4).
2. Seal each plate with adhesive film, and leave in cooling chambers on ice.

Prepare the Nsp PCR Master Mix

Location

Pre-PCR Clean Room

Prepare the Nsp PCR Master Mix

To prepare the Nsp 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 Nsp 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.**

1. Keeping the 50 mL Falcon tube in the cooling chamber, add the reagents as shown in Table 4.32 on page 79 (**except for the *Taq* DNA polymerase**).
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 4.32 Nsp PCR Master Mix for 48 Samples

Reagent	For 1 Reaction	4 PCR Plates (15% extra)
AccuGENE water	39.5 µL	8.722 mL
TITANIUM *Taq* PCR Buffer (10X)	10 µL	2.208 mL
GC-Melt (5M)	20 µL	4.416 mL
dNTP (2.5 mM each)	14 µL	3.091 mL
PCR Primer 002 (100 µM)	4.5 µL	0.994 mL
TITANIUM *Taq* DNA Polymerase (50X) (do not add until ready to aliquot master mix to ligated samples)	2 µL	0.442 mL
Total	**90 µL**	**19.873 mL**

Add Nsp PCR Master Mix to Samples

Location

PCR Staging Area

Procedure

To add Nsp PCR Master Mix to samples:

1. Using a 12-channel P200 pipet, add 90 µL Nsp PCR Master Mix to each sample.
   To avoid contamination, change pipet tips after each dispense.
   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 Nsp PCR Plates Onto Thermal Cyclers

! IMPORTANT: PCR protocols for the MJ Tetrad PTC-225 and Applied Biosystems thermal cyclers are different. Thermal cycler program parameters are on page 81.

Location

Main Lab

Procedure

To load the plates and run the GW5.0/6.0 PCR program:

1. Transfer the 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 GW5.0/6.0 PCR program.

   The program varies depending upon the thermal cyclers you are using. See Table 4.33 and Table 4.34 on page 81 program parameters.

! 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 4.33 GW5.0/6.0 PCR Thermal Cycler Program for the GeneAmp® PCR System 9700 (silver or gold-plated silver blocks)

GW5.0/6.0 PCR Program for GeneAmp® PCR System 9700		
Temperature	**Time**	**Cycles**
94°C	3 min	1X
94°C	30 sec	30X
60°C	45 sec	
68°C	15 sec	
68°C	7 min	1X
4°C	HOLD (Can be held overnight)	
Volume: 100 µL		
Specify *Maximum* mode.		

Table 4.34 GW5.0/6.0 PCR Thermal Cycler Program for the MJ Tetrad PTC-225

GW5.0/6.0 PCR Program for MJ Tetrad PTC-225		
Temperature	**Time**	**Cycles**
94°C	3 min	1X
94°C	30 sec	30X
60°C	30 sec	
68°C	15 sec	
68°C	7 min	1X
4°C	HOLD (Can be held overnight)	
Volume: 100 µL		
Use *Heated Lid* and *Calculated Temperature*		

Running Gels

The instructions below are for running 2% TBE gels. For information on running E-Gel *48* 2% agarose gels, refer to Appendix D, *E-gels,* on page 327.

Before Running Gels

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

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

Run the Gels

When the GW5.0/6.0 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 four fresh 96-well reaction plates *P1Gel*, *P2Gel*, *P3Gel,* and *P4Gel.*
5. Aliquot 3 µL of 2X Gel Loading Dye to each well in rows A through D of the fresh, labeled P*X*Gel plates.
6. Using a 12-channel P20 pipet, transfer 3 µL of each PCR product from the 4 Nsp PCR plates to the corresponding plate, row and wells of the P*X*Gel plates.

   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 the P*X*Gel plates.
8. Vortex the center of each P*X*Gel plate*,* then spin them down at 2000 rpm for 30 sec.
9. Load the total volume from each well of each P*X*Gel plate onto 2% TBE gels.
10. Run the gels at 120V for 40 min to 1 hour.
11. Verify that the PCR product distribution is between ~200 bp to 1100 bp (see Figure 4.6 on page 83).

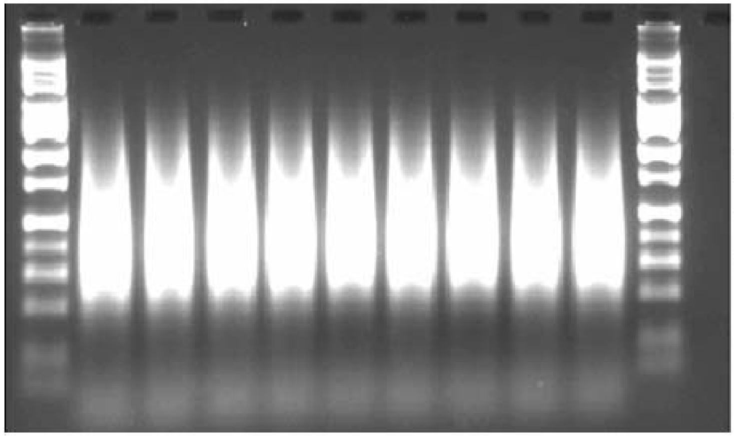


Figure 4.6 Example of PCR products run on 2% TBE agarose gel at 120V for 1 hour. Average product distribution is between ~200 to 1100 bp.

What To Do Next

Do one of the following:

- If following the recommended workflow (Figure 4.1 on page 25), do one of the following:
  - If the Nsp PCR plates are still on the thermal cyclers, remove them now and run gels to confirm the PCR (*Running Gels* on page 82). Then proceed to *Stage 7: PCR Product Purification Using a Millipore Filter Plate*.
  - If the PCR has been confirmed, proceed to *Stage 7: PCR Product Purification Using a Millipore Filter Plate*.
- If not proceeding directly to the next stage, seal the plates with PCR product and store at –20 °C.

Stage 7: PCR Product Purification Using a Millipore Filter Plate

Millipore vs Seahorse Filter Plate

! IMPORTANT: Two different filter plates can be used for the purification stage: Millipore or Seahorse. The instructions in this chapter are based on using a Millipore filter plate. To use a Seahorse filter plate, follow the instructions in **Appendix A, *Alternative Purification Protocol Using a Seahorse Filter Plate,* on page 295.**

About this Stage

During this stage, you will:

- Pool the Sty and Nsp PCR reactions to a single deep well pooling plate
- Add beads to each pool and incubate
- Transfer each pool to a Millipore filter plate and filter on a vacuum manifold
- Wash the PCR products with EtOH and filter
- Elute the PCR products using Buffer EB
- Vacuum and spin transfer the PCR products to a new 96-well plate

Location and Duration

- Main Lab
- Hands-on time: . . . . .approximately 1 hour
- Sample/magnetic bead incubation: . . . . .10 min
- Initial vacuum step: . . . . .approximately 40 to 50 min
- First EtOH vacuum step:. . . . . .approximately 10 to 15 min
- Final EtOH vacuum step: . . . . .10 min
- Resuspend beads in Buffer EB on Jitterbug . . . . .10 min
- Elution on vacuum manifold: . . . . .approximately 5 to 15 min
- Final elution on centrifuge:. . . . . .5 min
- Total time for this stage: . . . . .approximately 2.5 to 3 hr

Input Required from Previous Stage

The input required is:

Quantity	Item
3 plates	Sty PCR product
4 plates	Nsp PCR product

Equipment and Consumables Required

The following equipment and materials are required to perform this stage.

Table 4.35 Equipment and Consumables Required for *Stage 7: PCR Product Purification Using a Millipore Filter Plate*

Quantity	Item
1	Jitterbug
As needed	Kimwipes
1	Pipet, 12-channel P20
1	Pipet, 12-channel P200
1	Pipet, 12-channel P1200
1	Pipet, serological
As needed	Pipet tips for pipets listed above; full racks
1	Plate, 96-well PCR
1	Plate centrifuge with deep-well capacity (54mm H x 160g)
1	Plate, storage, 2.4 mL deep well (referred to as the *pooling plate*)
1	Plate, elution catch, 96-well V-bottom

Table 4.35 Equipment and Consumables Required for *Stage 7: PCR Product Purification Using a Millipore Filter Plate*

Quantity	Item
1	Plate, Multiscreen Deep Well (Millipore, P/N MDRLN0410)
7	Plate holders
As required	Plate seal**
1	Solution basin, 55 mL or larger
1 roll	Tape, lab
1	Vacuum Manifold, Millipore
1	Vortexer
** **IMPORTANT** Use only the PCR plate, adhesive film and thermal cyclers listed on page 29.	

Reagents Required

The following reagents are required for this stage.

Table 4.36 Reagents Required for *Stage 7: PCR Product Purification Using a Millipore Filter Plate*

Volume Required for 48 Samples	Reagent
3 mL	Elution Buffer (Buffer EB)
100 mL	75% EtOH (ACS-grade ethanol diluted to 75% using AccuGENE water)
50 mL	Magnetic Beads (AMPure or SNPClean)

Important Information About This Stage

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

CAUTION: Do not overdry the magnetic beads during the vacuum steps. Overdrying may inhibit elution of the purified DNA.

After adding EtOH to the wells (Step 5 on page 93), the first vacuum step should not exceed approximately 20 min.

The final EtOH vacuum step is 10 min only (Step 8 on page 94). Do not exceed 10 min.

All of the liquid in each well should be pulled through the filter. Although the beads may still be moist, there should be no standing liquid on top of the beads. The wells will appear dull (matte) – not shiny.

If any wells are clogged, do not continue filtering. Proceed with the protocol for the samples that have been successfully purified and eluted. Repeat the experiment for the samples in the clogged wells.

IMPORTANT:

- **Bring the Buffer EB and 75% EtOH to room temperature prior to use.**
- **The storage temperature for the magnetic beads is 4° C (refrigerator).**
- **To avoid cross-contamination, pipet very carefully when pooling the PCR reactions into the deep-well plate.**
- **Maintain the vacuum between 20—24 in Hg (do not exceed 24 in Hg).**
- **Inspect the vacuum manifold for salt buildup after each use, and clean regularly. Refer to Chapter 9 for cleaning instructions.**

Prepare the 75% EtOH

Dilute ACS-grade or equivalent ethanol to 75% using AccuGENE water.

Recipe for 75% EtOH

In a 1 L measuring cylinder:

1. Pour 750 mL 100% EtOH
2. Add 250 mL AccuGENE molecular biology grade water.
3. Transfer to a 1 L bottle and mix well.
4. Seal tightly and store at room temperature.

Prepare the Reagents

Allow the Buffer EB to warm to room temperature prior to use.

Prepare the Vacuum Manifold

To prepare the manifold:

1. Connect the manifold and regulator to a suitable vacuum source able to maintain 20 to 24 in Hg.

   Leave the vacuum turned off at this time.
2. Inspect the manifold for salt and other contaminants and clean if necessary.
3. Place the vacuum flask trap below the level of the manifold.
4. Place the standard collar on the manifold.

! IMPORTANT: Inspect the vacuum manifold for salt buildup before each use. Clean the manifold regularly. Refer to Chapter 9 for cleaning instructions.

If the flask trap is not placed below the level of the manifold, some solution may splash back and adhere to the bottom of the filter plate.

Pool the PCR Products

CAUTION: Be very careful when pooling PCR products. Avoid cross-contaminating neighboring wells with small droplets.

To pool the PCR products:

1. If PCR products are:
   - Frozen, thaw to room temperature on the bench top in plate holders.
   - On thermal cyclers, remove them now.
2. Vortex the center of each plate at high speed for 3 sec.
3. Spin down each plate at 2000 rpm for 30 sec.
4. Place each PCR plate in a plate holder on the bench top.
5. Place a deep well pooling plate on the bench top.
6. On each PCR plate, cut the seal between each row so that it can be removed one row at a time.
7. Using a 12-channel P200 pipet set to 110 µL:
   - **A.** Remove the seal to expose row A only on each PCR plate.
   - **B.** Transfer the reactions from row A of each PCR plate to the corresponding wells of row A on the pooling plate (Table 4.37 below and Figure 4.7 on page 90).
   - **C.** Change your pipet tips.

     Change pipet tips after the PCR product from the same row of each PCR plate has been pooled on the pooling plate.
   - **D.** Remove the seal from each PCR plate to expose the next row.
   - **E.** Transfer each reaction from the same row of each PCR plate to the corresponding row and wells of the pooling plate.
   - **F.** Repeat steps C., D. and E. until all of the reactions from each PCR plate are pooled.
8. When finished, examine the wells of each PCR plate to ensure that all of the product has been transferred and pooled.

Table 4.37 Pooled Sty and Nsp PCR Products

Sty PCR plates (3):	100 µL from each well	= 300 µL/well
Nsp PCR Plate (4):	100 µL from each well	= 400 µL/well
Total Volume Each Well of Pooling Plate		**= 700 µL/well**

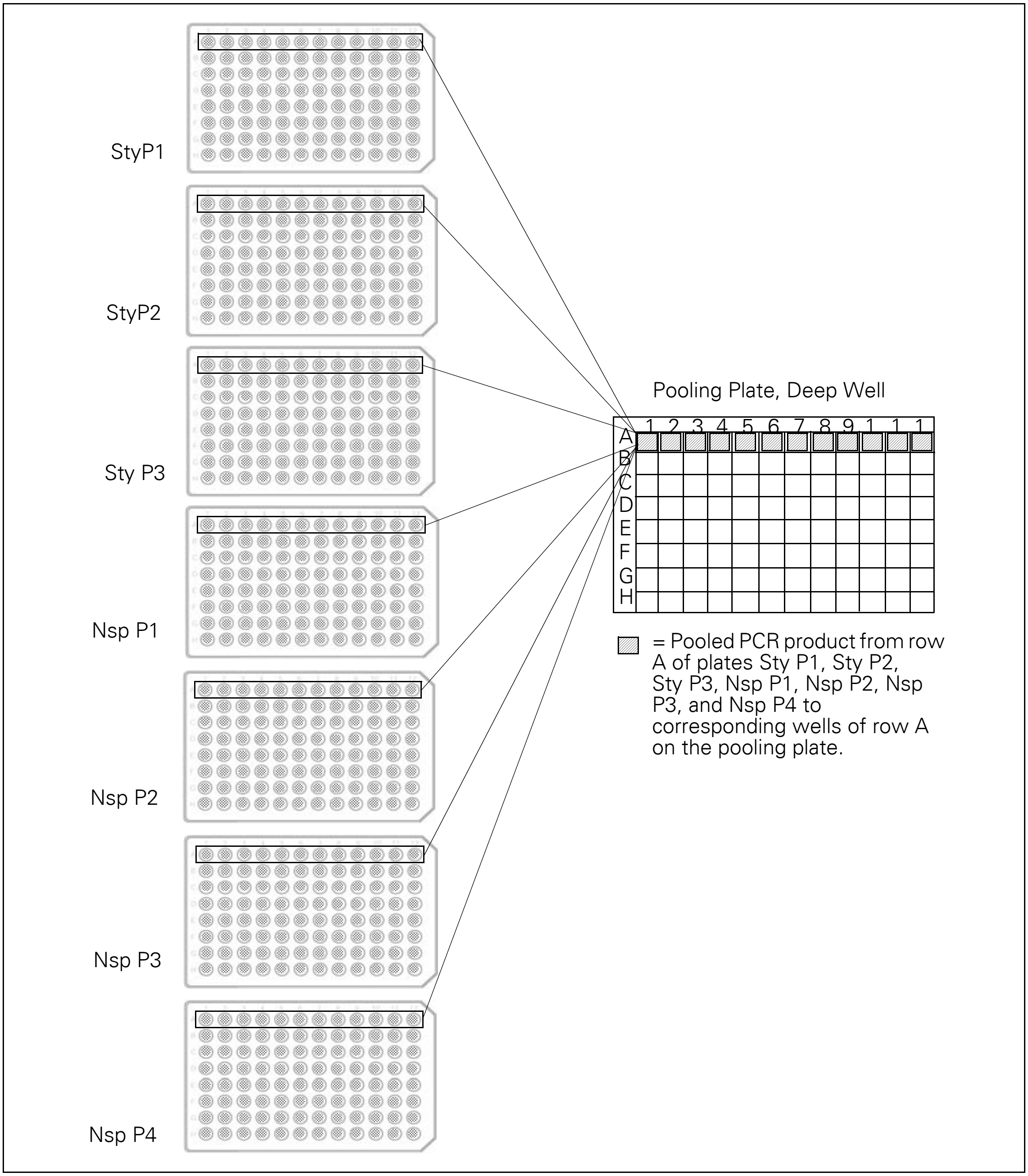


Figure 4.7 Pooling Sty and Nsp PCR Products on a Deep Well Pooling Plate

Purify the Pooled PCR products

Add Magnetic Beads and Incubate

During incubation, the DNA binds to the magnetic beads.

To add magnetic beads and incubate:

1. Mix the magnetic bead stock very well by vigorously shaking the bottle.

   Beads will settle overnight. Examine the bottom of the bottle and ensure that the solution appears homogenous.

2. Pour or pipet 50 mL of magnetic beads to a solution basin.

   1 mL of magnetic beads is required for each reaction. You can add in multiple batches if the solution basin is not large enough.

3. Using a manual (not electronic) 12-channel P1200 pipet:

   A. Slowly add 1.0 mL of magnetic beads to each well of pooled PCR product.

   B. Mix well by pipetting up and down 5 times using the following technique:

      Mixing Technique:

      1) Depress the plunger and place the pipet tips into the top of the solution.
      2) Move the pipet tips down – aspirating at the same time – until the tips are near the bottom of each well.
      3) Raise the tips out of the solution.
      4) Place the pipet tips against the wall of each well just above each reaction, and carefully dispense the solution.

      ! **IMPORTANT: The solution is viscous and sticky. Pipet carefully to ensure that you aspirate and dispense 1 mL. Do not use an electronic pipet.**

      Thorough mixing is critical to ensure that the PCR products bind to the beads.

      5) Change pipet tips for each row.

4. Cover the plate to protect the samples from environmental contaminants and allow to incubate at room temperature for 10 min.

   You can use the lid from a pipet tip box to cover the wells.

Transfer Reactions to a Filter Plate

To transfer the reactions to a filter plate:

1. Place the filter plate on the standard collar on the vacuum manifold (Figure 4.8).
2. Using a 12-channel P1200 pipet, transfer each reaction from the pooling plate to the corresponding row and well of the filter plate.

> **IMPORTANT: You will need to pipet twice to transfer all of the solution from each well to the filter plate. The solution is viscous and sticky, so check to ensure that all of it has been transferred.**

3. Tightly seal the unused wells with a MicroAmp Clear Adhesive Film (Figure 4.9).

   To ensure a tight seal, cover 1/2 to 1/3 of the wells in row D as well. Unused wells ***must be sealed*** to ensure proper vacuum pressure.

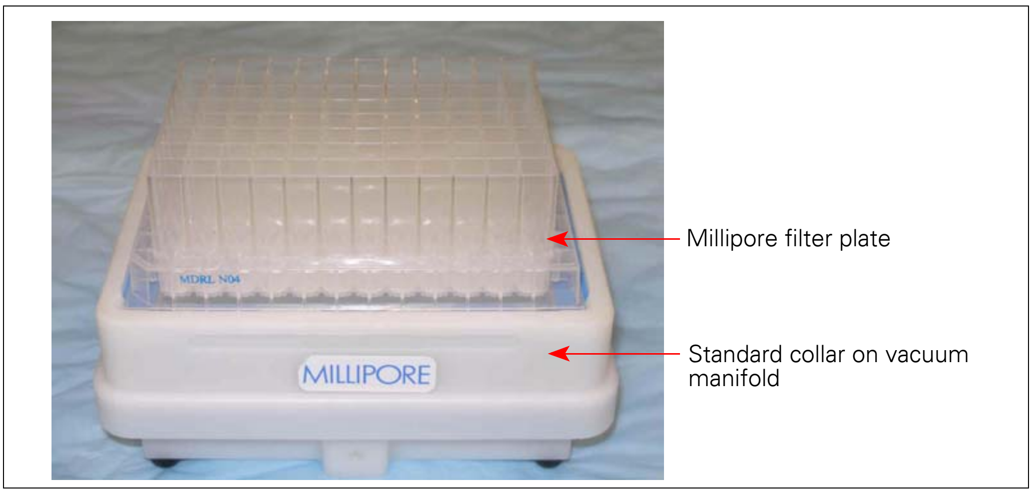


Figure 4.8 Millipore filter plate on standard collar

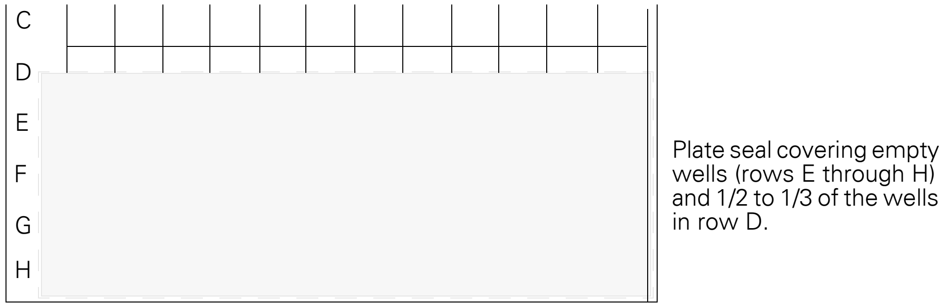


Figure 4.9 Sealing Empty Wells on the Filter Plate

Purify the Reactions

To purify the reactions:

1. Turn on the vacuum to 20 to 24 in Hg and check the seals.

   Do not exceed 24 in Hg. Adjust the leak valve if necessary.
2. Ensure that the unused wells are completely sealed, and cover the plate to protect it from environmental contaminants.
3. Run the vacuum until all of the liquid has been pulled through the filter (approximately 40 to 50 min), then turn off the vacuum.
4. Examine each well.

   There should be no standing liquid in any well, and the wells should appear dull (matte). Wet wells will look shiny.

   If any of the wells are still wet, put the plate back on the vacuum and continue filtering for up to 10 min (total ≤ 60 min).
5. Using a 12-channel P1200 set to 900 µL:

   A. Add 900 µL of 75% EtOH to each reaction.

   B. Turn the vacuum on to 20 to 24 in Hg.

   C. Run the vacuum for approximately 1–2 min (or until the volume in the wells begins to decrease).

   D. Add another 900 µL of 75% EtOH to each reaction (for a total of 1.8 mL EtOH).

   E. Cover the plate.

   F. Run the vacuum until all of the liquid has been pulled through the filter (approximately 10 to 15 min), then turn off the vacuum.

6. Examine each well.

   Again, there should be no standing liquid in any well, and the wells should appear dull (matte). Wet wells will look shiny.

   If any of the wells are still wet, put the plate back on the vacuum and continue filtering for up to 5 min (total ≤20 min; see the Caution on page 87).

7. Remove any excess EtOH as follows:

   A. Blot the bottom of the plate on Kimwipes.

   B. Wipe the bottom of the plate with a clean Kimwipe.

8. Return the filter plate to the manifold and turn on the vacuum for an additional 10 min ONLY.

   Do not exceed 10 min. Less than 10 min is OK if no excess ethanol is present in the wells or on the underside of the filter plate.

NOTE: The purpose of this step is to remove excess EtOH so that it is not carried over into the eluate. Ten minutes is sufficient for this purpose. Leaving the vacuum on for more than 10 minutes may over-dry the beads which may inhibit elution of the purified DNA.

9. Turn off the vacuum, and blot the bottom of the plate on Kimwipes to remove any remaining EtOH.

Elute the Purified Reactions

To elute the purified reactions:

1. Attach the elution catch plate to the bottom of the filter plate using 2 strips of lab tape.

   The filter and elution plate assembly is now referred to as the *plate stack* (Figure 4.10).

IMPORTANT: Do not completely seal with tape. Product will not elute if sealed.

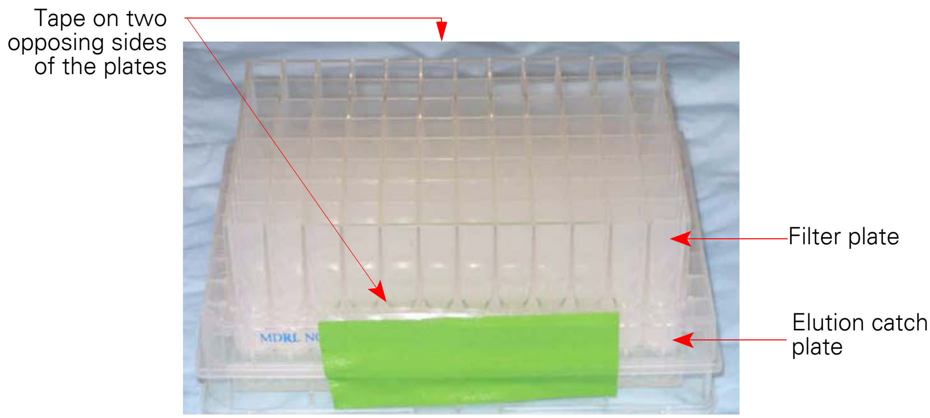


Figure 4.10 Attaching the Elution Catch Plate to the Filter Plate

2. Pour or pipet 3 mL of Buffer EB to a solution basin.
3. Using a 12-channel P200 pipet, add 55 µL of Buffer EB to each well.

   For accurate pipetting, pre-wet pipet tips with EB before dispensing. Dispense as close to the beads as possible without touching them. Buffer EB should be applied directly on top of the beads (see Figure 4.11 and Figure 4.12 on page 96).

> **NOTE: If the volume of eluate in Step 13 on page 97 is < 47 µL, increase the amount of Buffer EB used in this step the next time you perform the protocol. You can increase from 55 to 60 µL (total not to exceed 60 µL).**

4. Tap the plate stack to move all Buffer EB onto the filter at the bottom of the wells.
5. Using an adhesive film, tightly seal the filter plate.
6. Place the plate stack on a Jitterbug for 10 min at *setting 5*.

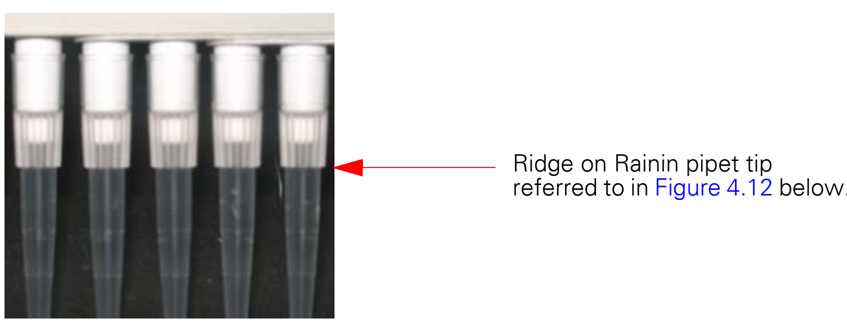


Figure 4.11 Ridge on Rainin pipet tips

If using Rainin pipet tips, rest the ridge of the pipet tip on the lip of the plate when pipetting Buffer EB.

This technique will help ensure that Buffer EB is dispensed as close to the beads as possible without touching them.

Figure 4.12 Adding Buffer EB to Reactions on the Filter Plate

7. Inspect each well to verify that the beads are thoroughly resuspended.

   The beads must be thoroughly resuspended in Buffer EB so that the DNA can come off the beads.

8. Remove the plate stack from the Jitterbug and remove the adhesive seal.

9. Continue elution on the vacuum manifold as follows:

   A. Remove the standard collar from the manifold.

   B. Place the plate stack inside the manifold.

   C. Place the standard collar around the plate stack (Figure 4.13 on page 97).

   D. Seal the empty wells with adhesive film.

   E. Turn the vacuum on to 20 to 24 in Hg and ensure that a seal has been formed between the collar and the base of the manifold.

   F. Ensure that the unused wells are completely sealed.

   G. Cover the plate stack to protect it from environmental contaminants.

   H. Run the vacuum until all of the liquid has been pulled through the filter (approximately 5 to 15 min).

   I. Turn off the vacuum.

Figure 4.13 Plate stack on vacuum manifold with standard collar

10. Examine each well.

Again, there should be no standing liquid in any well, and the wells should appear dull (matte). Wet wells will look shiny.

If any of the wells are still wet, continue filtering for up to 15 additional min.

11. Seal the plate stack with an adhesive film, and spin it down at room temperature for 5 min at 1400 rcf.

Use the following formula to convert relative centrifugal force (rcf) to revolutions per minute (rpm):

rpm = 1000 × square root(rcf/1.12r)

The radius, *r*, is equal to the distance in millimeters between the axis of rotation of the centrifuge and the bottom of the plate bucket.

For example, on the Eppendorf 5804R, spinning at 3100 rpm gives an rcf of 1400 (assuming r = 133 mm).

12. Remove the elution catch plate from the filter plate.

13. Using a 12-channel P200 pipet, transfer 45 µL of eluate to a new PCR plate for fragmentation.

NOTE: If the volume of eluate is < 47 µL, increase the amount of Buffer EB used for elution the next time you perform the protocol. You can increase from 55 to 50 µL (total not to exceed 60 µL).

See also the Caution on page 87, and page 279 of Chapter 8, *Troubleshooting* for more information.

What To Do Next

Take an OD measurement using 2 µL from the remaining eluate as described below.

Do one of the following:

- If following the recommended workflow (Figure 4.1 on page 25) seal the plate containing the eluate and store it overnight at –20 °C.
- Proceed directly to *Stage 9: Fragmentation* on page 107.

Stage 8: Quantitation

About this Stage

During this stage, you will prepare one dilution of each PCR product in optical plates. You will then quantitate the diluted PCR products.

Location and Duration

- Main Lab
- Hands-on time: 20 min

Input Required from Previous Stage

Input required from *Stage 7: PCR Product Purification Using a Millipore Filter Plate* is:

Quantity	Item
1	Plate of purified PCR product

Equipment and Consumables Required

The following equipment and consumables are required for this stage.

Table 4.38 Equipment and Consumables Required for *Stage 8: Quantitation*

Quantity	Item
1	Marker, fine point, permanent
1	Pipet, single channel P20
1	Pipet, single channel P200
1	Pipet, 12-channel P20 (accurate to within ± 5%)
1	Pipet, 12-channel P200
As needed	Pipet tips for pipets listed above; full racks

Table 4.38 Equipment and Consumables Required for *Stage 8: Quantitation*

Quantity	Item
1	Plate, optical For example, the Greiner 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
** **IMPORTANT** Use only the PCR plate, adhesive film and thermal cyclers listed on page 29.	

Reagents Required

The following reagents are required for this stage. The amounts listed are sufficient to process 48 reactions.

Table 4.39 Reagents Required for *Stage 8: Quantitation*

Quantity	Reagent
15 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 accuracy of the OD measurement is critical. Carefully follow this procedure and be sure the OD measurement is within the quantitative linear range of the instrument (0.2 to 2.0 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.**
>
>   **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 min before use.

Prepare Your Work Area

To prepare the work area:

1. Place the following on the bench top:
   - Optical plate
   - Solution basin
   - AccuGENE water
2. Label the optical plate *OP*.
3. 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.** Spin down the plate at 2000 rpm for 30 sec.
   - **C.** Place the plate on the bench top.

Prepare Diluted Aliquots of Purified Sample

IMPORTANT: One row of wells on the optical plate are used as blanks and contain AccuGENE water only.

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

To prepare diluted aliquots of the purified samples:

1. Pour 15 mL of room temperature AccuGENE water into the solution basin.
2. Using a 12-channel P200 pipet aliquot 198 µL of water to each well in rows A through E of the optical plate.
3. Using a 12-channel P20 pipet:
   - A. Transfer 2 µL of each purified PCR product from rows A through D of the purified sample plate to the corresponding rows and wells of the optical plate (see Figure 4.14 on page 103).

     Row E remains water only and will serve as a blank.

     NOTE: If a particular well(s) contain less than 2 µL of purified PCR product, see page 279 of Chapter 8, *Troubleshooting* for instructions.
   - B. Pipet up and down 2 times after each transfer to ensure that all of the product is dispensed.
   - C. Examine the pipet tips and aliquots before and after each dispense to ensure that exactly 2 µL has been transferred.

     The result is a 100-fold dilution.
4. Set a 12-channel P200 pipet to 180 µL.
5. Mix each sample by pipetting up and down 3 times.

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

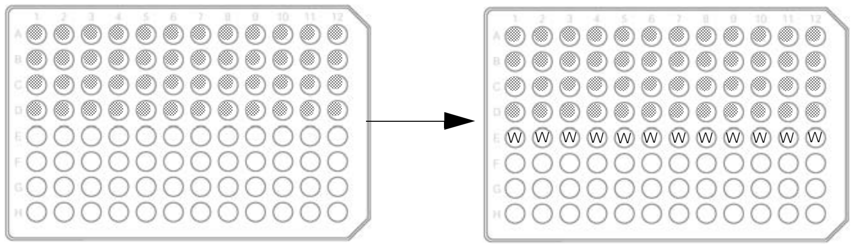


Figure 4.14 Loading the Optical Plate with Purified Sample and Water Blanks

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* on page 104.

2. Determine the OD260 measurement for the water blank and average.

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

   A. Take 1 OD reading for every sample.

      OD = (sample OD) – (average water blank OD)

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

      Sample concentration in µg/µL = OD X 0.05 ug/uL X 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.

Sample OD

A typical sample OD is 0.9 to 1.2. This OD range is equivalent to a final PCR product concentration of 4.5 to 6.0 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 4.40 PROBLEM: Sample OD is greater than 1.2 (6 µg/µL)

If the sample OD is greater than 1.2 (calculated concentration greater than 6 µg/µL), a problem exists with either the elution of PCR products or the OD reading. The limit on PCR yield is approximately 6 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 55 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. • Pipets 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.

Table 4.41 PROBLEM: Sample OD is Less Than 0.9 (4.5 µg/µL)

If the sample OD is less than 0.9 (calculated concentration less than 4.5 µg/µL), a problem may exist 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.

  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.
- Pipet 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 or Tetrad 2).
- 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 Genome-Wide SNP 5.0/6.0 Assay reaction amplifies a size range of fragments that represents 30% of the genome. The Genome-Wide Human SNP Array 6.0 is 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.

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

- The purified PCR product was eluted in a volume greater than 55 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.

Table 4.41 (Continued) PROBLEM: Sample OD is Less Than 0.9 (4.5 µg/µL)

- The water blank reading was not subtracted from each sample OD reading.
- The spectrophotometer plate reader may require calibration.
- Pipets 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.

Table 4.42 PROBLEM: OD260/OD280 ratio is not between 1.8 and 2.0

Possible causes include:

- The PCR product may be not be sufficiently purified. Ensure the vacuum manifold is working properly.
- An error may have been made while taking the OD readings.
- The PCR product may not have been adequately washed. Check the 75% EtOH wash solution.

Table 4.43 PROBLEM: The OD320 measurement is significantly larger than zero (0 ± 0.005)

Possible causes include:

- Magnetic beads may have been carried over into purified sample.
- Precipitate may be present in the eluted samples.
- There may be defects in the OD plate.
- Air bubbles in the OD plate or in solutions.

What To 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 9: Fragmentation

About this Stage

During this stage the purified 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 GW5.0/6.0 Fragment program.

Once the program is finished, you will check the results of this stage by running 1.5 µL of each reaction on a 4% TBE gel or an E-Gel *48* 4% agarose gel.

Location and Duration

- Main Lab
- Hands-on time: 30 min
- GW5.0/6.0 Fragment thermal cycler program time: 1 hour

Input Required from Previous Stage

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

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

Equipment and Consumables Required

The following equipment and consumables are required for this stage.

Table 4.44 Equipment and Consumables Required for *Stage 9: 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	Pipet, single channel P20
1	Pipet, single channel P100
1	Pipet, single channel P1000
1	Pipet, 12-channel P20 (accurate to within ± 5%)
As needed	Pipet tips for pipets listed above; full racks
1	Plate centrifuge
1	Plate seal**
1	Thermal cycler**
2	Tube, Eppendorf 2.0 mL
2	Tubes, 12-strip, 0.2 mL
1	Vortexer
** **IMPORTANT** Use only the PCR plate, adhesive film and thermal cyclers listed on page 29.	

Reagents Required

The following reagents are required for this stage. The amounts listed are sufficient to process 48 samples.

Table 4.45 Reagents Required for *Stage 9: Fragmentation*

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

Gels and Related Materials Required

Verifying the PCR reaction is required for this stage. You can use the following gels and related materials, or E-Gels as described in Appendix D, *E-gels,* on page 327. The amounts listed are sufficient to process 48 Sty samples.

Table 4.46 Gels and Related Materials Required

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:

- **The degree of fragmentation is critical. Perform this stage carefully to ensure uniform, reproducible fragmentation.**
- **Use only the AccuGENE water listed in Appendix B. Using in-house ddH_2O or other water can negatively affect your results. The reaction in *Stage 9: Fragmentation* is particularly sensitive to pH and metal ion contamination.**
- **All additions, dilutions and mixing must be performed on ice. Be sure to allow all reagents to reach equilibrium before adding new fluid.**

About the Fragmentation Reagent

- This reagent is **extremely temperature sensitive** and rapidly loses activity at higher temperatures. To avoid loss of activity:
  - Handle the tube by the cap only. Do not touch the sides of the tube as the heat from your fingers will raise the reagent temperature.
  - Dilute 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.
  - Spin down so that the contents of the tube are uniform.
  - Perform these steps rapidly and without interruption.
- This reagent is **sticky**, and may adhere to the walls of some microfuge tubes and 96-well plates.
- This reagent is **viscous** and requires extra care when pipetting. Follow these guidelines:
  - Pipet slowly to allow enough time for the correct volume of solution to enter the pipet tip.
  - Avoid excess solution on the outside of the pipet tip.

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:

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. Label and place the following in the cooling chamber on ice:
   - Two strips of 12 tubes each: one labeled *Buffer* and one labeled *FR*.
   - One 2.0 mL Eppendorf tube labeled *Frag MM*.
   - Plate of purified PCR product 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:

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

Prepare the Samples for Fragmentation

Add Fragmentation Buffer to Samples

> **IMPORTANT: All additions in this procedure must be performed on ice.**

To prepare the samples for Fragmentation:

1. Aliquot 28 µL of 10X Fragmentation Buffer to each tube of the strip tubes labeled Buffer.
2. Using a 12-channel P20 pipet, add 5 µL of Fragmentation Buffer to each sample in the 96-well reaction plate.

   Check your pipet 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.**

To dilute the Fragmentation Reagent:

1. Read the Fragmentation Reagent tube label and record the concentration.
2. Dilute the Fragmentation Reagent to 0.1 U/µL as described below using the appropriate recipe from Table 4.47:

Table 4.47 Dilution Recipes for the Fragmentation Reagent

Reagent	Fragmentation Reagent Concentration				
	2 U/µL	**2.25 U/µL**	**2.5 U/µL**	**2.75 U/µL**	**3 U/µL**
AccuGENE water	306 µL	308 µL	309.6 µL	310.9 µL	312 µL
10X Fragmentation Buffer	36 µL	36 µL	36 µL	36 µL	36 µL
Fragmentation Reagent	18 µL	16 µL	14.4 µL	13.1 µL	12 µL
Total (enough for 48 samples)	**360 µL**	**360 µL**	**360 µL**	**360 µL**	**360 µL**

A. To the 2.0 mL Eppendorf tube on ice:

1) Add the AccuGENE water and Fragmentation Buffer.

2) Allow to cool on ice for 5 min.

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.

3. 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 28 µL of diluted Fragmentation Reagent to each tube of the strip tubes labeled *FR*.

Avoid introducing air bubbles at the bottom of the strip tubes to ensure the accurate transfer of 5 µL diluted DNA to each sample.

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

Do not pipet up and down.

Sample with Fragmentation Buffer	50 µL
Diluted Fragmentation Reagent (0.1 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 GW5.0/6.0 Fragment program.

Table 4.48 GW5.0/6.0 Fragment Thermal Cycler Program

GW5.0/6.0 Fragment Program	
Temperature	**Time**
37°C	35 min
95°C	15 min
4°C	Hold

7. Discard any remaining diluted Fragmentation Reagent.

   Diluted Fragmentation Reagent should never be reused.

What To Do Next

Proceed directly to the next stage. Concurrently, check the fragmentation reaction by running gels as described under *Check the Fragmentation Reaction* on page 115.

Check the Fragmentation Reaction

The instructions below are for running 4% TBE gels. For information on running E-Gel *48* 4% agarose gels, refer to Appendix D, *E-gels,* on page 327.

To ensure that fragmentation was successful:

1. When the GW5.0/6.0 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 1.5 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 min to 1 hour.
4. Inspect the gel and compare it against the example shown in Figure 4.15 below.

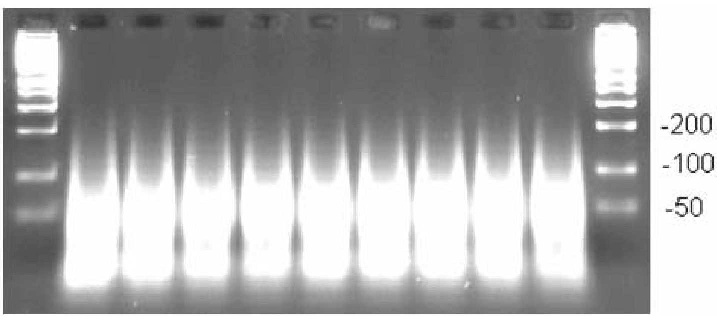


Figure 4.15 Typical example of fragmented PCR products run on 4% TBE agarose gel at 120V for 30 min to 1 hour. Average fragment size is < 180 bp.

Stage 10: Labeling

About this Stage

During this stage, you will:

- Label the fragmented samples using the DNA Labeling Reagent.
- Prepare the Labeling Master Mix.
- Add the mix to each sample.
- Place the samples onto a thermal cycler and run the GW5.0/6.0 Label program.

Location and Duration

- Main Lab
- Hands-on time: 30 min
- GW5.0/6.0 Label thermal cycler program time: 4.25 hours

Input Required from Previous Stage

The input required from *Stage 9: Fragmentation* is:

Quantity	Item
1	Plate of fragmented DNA

Equipment and Consumables Required

The following equipment and consumables are required for this stage.

Table 4.49 Equipment and Consumables Required for *Stage 10: 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	Pipet, single channel P200
1	Pipet, single channel P1000
1	Pipet, 12-channel P20 (accurate to within ± 5%)
As needed	Pipet tips for pipets listed above; full racks
1	Plate centrifuge
1	Plate seal**
1	Thermal cycler**
1	Tube, centrifuge 15 mL
1	Tubes, 12-strip, 0.2 mL
1	Vortexer
** **IMPORTANT** Use only the PCR plate, adhesive film and thermal cyclers listed on page 29.	

Reagents Required

The following reagents are required for this stage. The amounts listed are sufficient to process 48 samples.

Table 4.50 Reagents Required for *Stage 10: Labeling*

Quantity	Reagent
1 vial	DNA Labeling Reagent (30 mM)
1 vial	Terminal Deoxynucleotidyl Transferase (TdT; 30 U/μL)
1 vial	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 GW5.0/6.0 Label thermal cycler program.

Prepare the Reagents, Consumables and Other Components

Thaw Reagents

Thaw the following reagents on ice:

- 5X TdT Buffer
- 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; then place in the cooling chamber.

3. Label one 15 mL centrifuge tube *MM*, and place on ice.
4. Label and place the following in the cooling chamber:
   - One strip of 12 tubes 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 min 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 4.51 on page 120:
   - 5X TdT Buffer
   - 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 4.51 Labeling Master Mix

Reagent	1 Sample	48 Samples (15% extra)
TdT Buffer (5X)	14 µL	772.8 µL
DNA Labeling Reagent (30 mM)	2 µL	110.4 µL
TdT enzyme (30 U/µL)	3.5 µL	193.2 µL
Total	**19.5 µL**	**1076.4 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 89 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 pipet, aliquot 19.5 µL of Labeling Master Mix to each sample.
   B. Pipet 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 73 µL.

Fragmented DNA (less 1.5 µL for gel analysis)	53.5 µL
Labeling Mix	19.5 µL
Total	**73 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 GW5.0/6.0 Label program.

Table 4.52 GW5.0/6.0 Label Thermal Cycler Program

GW5.0/6.0 Label Program	
Temperature	**Time**
37°C	4 hours
95°C	15 min
4°C	Hold

7. When the GW5.0/6.0 Label program is finished:

A. Remove the plate from the thermal cycler.

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

What To 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 11: Target Hybridization

About this Stage

During this stage, each reaction is loaded onto a Genome-Wide Human SNP Array 6.0. Two 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 129.

- **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, MJ Tetrad PTC-225, or MJ Tetrad 2 Thermal Cycler* on page 132.

First, you will prepare a Hybridization Master Mix and add the mix to each sample. Then, you will denature the samples on a thermal cycler.

After denaturation, you will load each sample onto a Genome-Wide Human SNP Array 6.0 – one sample per array. The arrays are then placed into a hybridization oven that has been preheated to 50 °C. Samples are left to hybridize for 16 to 18 hours.

NOTE: Two operators are required for all of the methods.

Location and Duration

- Main Lab
- Hands-on time: 45 min
- Hybridization time: 16 to 18 hours

Input Required from Previous Stage

The input required from *Stage 10: Labeling* is:

Quantity	Item
1	Plate of labeled DNA

Equipment and Consumables Required

The following equipment and consumables are required for this stage.

! **IMPORTANT: Increased variability in Genome-Wide SNP 5.0/6.0 Assay 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.**

The following table lists the equipment and consumables required.

Table 4.53 Equipment and Consumables Required for *Stage 11: Target Hybridization*

Quantity	Item
1	Cooling chamber, chilled to 4 °C (do not freeze)
48	Genome-Wide Human SNP Array 6.0 (one array per sample)
1	GeneChip® Hybridization Oven 640
1	Ice bucket, filled with ice
1	Pipet, single channel P200
1	Pipet, single channel P1000
As needed	Pipet tips for pipets 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 122.
2 per array	Tough-Spots®
1	Tube, centrifuge 50 mL
1	Vortexer
** **IMPORTANT** Use only the PCR plate, adhesive film and thermal cyclers listed on page 29.	

Reagents Required

The following reagents are required for this stage. The amounts listed are sufficient to process 48 samples.

Table 4.54 Reagents Required for *Stage 11: Target Hybridization*

Quantity	Reagent
5 mL	Denhardt's Solution (50X)
1.5 mL	DMSO (100%)
0.5 mL	EDTA (0.5 M)
1 mL	Herring Sperm DNA (HSDNA; 10 mg/mL)
500 µL	Human Cot-1 DNA® (1 mg/mL)
80 g	MES Hydrate SigmaUltra
200 g	MES Sodium Salt
16 mL	Tetramethyl Ammonium Chloride (TMACL; 5M)
10 mL	Tween-20, 10%
250 µL	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.**
- **It is critical that the samples remain on a thermal cycler 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 (see *About this Stage* on page 122).**
- **About DMSO:**

  When adding to the Hybridization Master Mix, pipet 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 equilibrate the arrays 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 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 950 mL.
3. Test the pH.
   The pH should be between 6.5 and 6.7.
4. Adjust the pH so it falls between 6.5 and 6.7.
5. Adjust the volume to 1000 mL.
6. Filter the solution through a 0.2 µm filter.
7. 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 50 °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 the manufacturer's 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 used wells on the plate are cut into 2 strips of 24 wells each.

Preheat the Thermal Cycler Lid

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

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 min.
4. Insert a 200 µL pipet tip into the upper right septum of each array.

> **!** **IMPORTANT: 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 4.55.

   DMSO addition: pipet 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 11 mL, and store the remainder at –20 °C for up to one week.

Table 4.55 Hybridization Master Mix

Reagent	1 Array	48 Arrays (15% extra)
MES (12X; 1.25 M)	12 µL	660 µL
Denhardt's Solution (50X)	13 µL	715 µL
EDTA (0.5 M)	3 µL	165 µL
HSDNA (10 mg/mL)	3 µL	165 µL
OCR, 0100	2 µL	110 µL
Human Cot-1 DNA® (1 mg/mL)	3 µL	165 µL
Tween-20 (3%)	1 µL	55 µL
DMSO (100%)	13 µL	715 µL
TMACL (5 M)	140 µL	7.7 mL
Total	**190 µL**	**10.45 mL**

Using Premixed Hybridization Master Mix

Hybridization Master Mix can be made ahead of time, aliquoted and stored for 1 week at –20 °C.

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 min).
3. Pulse spin for 3 sec.

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

   Total volume in each well is 263 µL.
3. Seal the plate tightly with adhesive film.

   > **!** **IMPORTANT:** **It is critical to seal the plate tightly.**

4. Vortex the center of the plate for 30 sec.
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 GW5.0/6.0 Hyb program.

Table 4.56 GW5.0/6.0 Hyb Thermal Cycler Program

GW5.0/6.0 Hyb Program	
Temperature	**Time**
95 °C	10 min
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 pipet, 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 4.16).
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 48 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 50 °C, 60 rpm for 16 to 18 hours.

! **IMPORTANT: Allow the arrays to rotate in the hybridization ovens for 16 to 18 hours at 50 °C and 60 rpm. This temperature is optimized for this product, and should be stringently followed.**

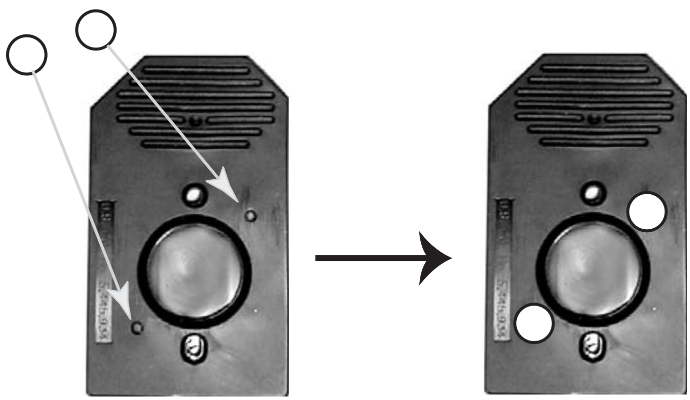


Figure 4.16 Applying Tough-Spots® to the array cartridge

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

For this method, you can use an:

- Applied Biosystems 2720 Thermal Cycler
- MJ Tetrad PTC-225 Thermal Cycler
- MJ Tetrad 2

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

   Total volume in each well is 263 µL.
3. Seal the plate tightly with adhesive film.

   IMPORTANT: It is critical to seal the plate tightly.

4. Vortex the center of the plate for 30 sec.
5. Cut the used wells into 2 strips of two rows each.
6. Put each strip of 24 samples into a plate 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 GW5.0/6.0 Hyb program.

Table 4.57 GW5.0/6.0 Hyb Thermal Cycler Program

GW5.0/6.0 Hyb Program	
Temperature	**Time**
95 °C	10 min
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 pipet, 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 GW5.0/6.0 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 4.16).
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 48 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 50 °C, 60 rpm for 16 to 18 hours.

IMPORTANT: Allow the arrays to rotate in the hybridization ovens for 16 to 18 hours at 50 °C and 60 rpm. This temperature is optimized for this product, and should be stringently followed.

Chapter 5

96 Sample Protocol

About This Protocol

The Genome-Wide Human SNP Nsp/Sty 5.0/6.0 Assay (Genome-Wide SNP 5.0/6.0 Assay) protocol described in this chapter is designed for processing 96 samples. This advanced protocol is intended for experienced users who have:

- Been trained to run the standard 48 sample Genome-Wide SNP 5.0/6.0 Assay protocol
- Demonstrated a consistent pattern of success running the standard 48 sample protocol

The 96 sample protocol is presented in the following stages:

Key points regarding the various molecular biology steps that comprise whole-genome sampling analysis (WGSA) are included in the protocol and guidelines.

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 1, genomic DNA is digested with the restriction enzyme Sty I. In stage 2, it is ligated to a common adaptor with T4 DNA ligase. Following ligation, the template undergoes PCR using TITANIUM™ Taq DNA polymerase. Once the product has been purified (stage 7), it is then fragmented in stage 9 with Fragmentation Reagent (DNAse I), and end-labeled using terminal deoxynucleotidyl transferase (stage 10).

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:
  - Ensure that enzymes are kept at –20 °C until needed.
  - Keep all master mixes and working solutions in chilled cooling chambers.
  - 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.
- Keep dedicated equipment in each of the areas used for this protocol (including pipettors, ice buckets, coolers, etc.). To avoid contamination, do not move equipment from one area to another.

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:

- All of the thermal cyclers (PCR Staging Room and Main Lab)
- GeneChip® Hybridization Oven 640
- GeneChip® Fluidics Station 450
- GeneChip® Scanner 3000 7G
- The UV spectrophotometer plate reader
- All multi-channel pipets

About the Cytogenetics Copy Number Assay

! **IMPORTANT: The Cytogenetics Copy Number assay protocol is optimized for processing from 4 to 24 samples at a time to obtain copy number results. This protocol is not intended for genome-wide association studies.**

The 48 and 96 sample protocols described in this user guide have been optimized for genome-wide association studies.

Workflow Recommendations

The workflow recommended for target preparation for 96 samples is shown below in Figure 5.1. This workflow assumes one full time equivalent (FTE) for target preparation and 2 FTEs for array processing.

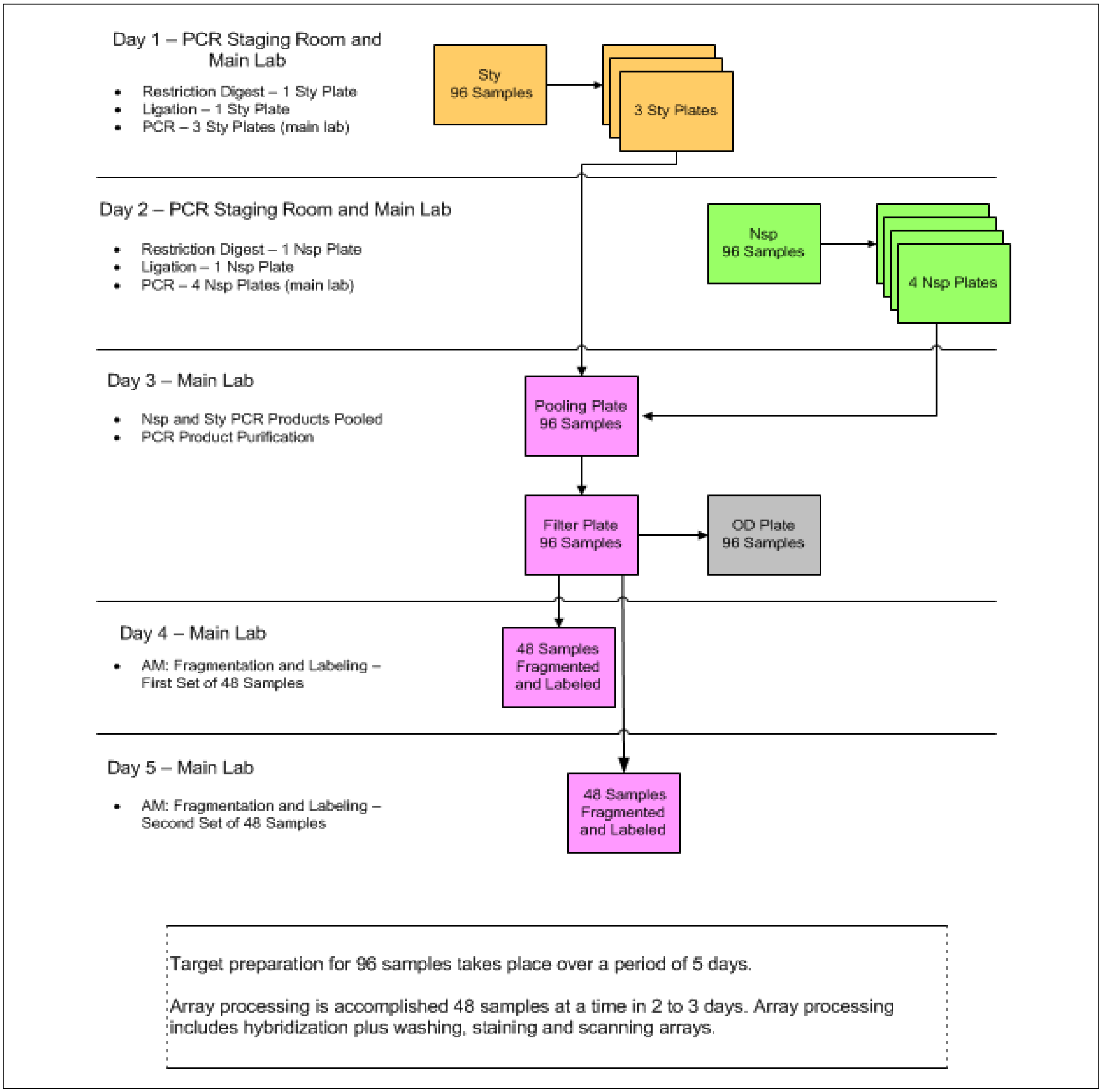


Figure 5.1 Recommended Workflow for Processing 96 Samples

Dedicating small teams to different stages of the protocol has proven to be a highly effective method of managing a high throughput workflow. For example, the full process can be sub-divided into four teams, with each team being responsible for the following stages:

- Team 1: Pre-PCR (digestion and ligation)
- Team 2: PCR (PCR and PCR product purification and quantitation)
- Team 3: Post-PCR (fragmentation and labeling)
- Team 4: Array processing (hybridization, washing, staining and scanning)

When processing multiple full plates, we recommend that the same operator not perform too many stages in a given day. Your technical support representative can provide additional guidance on how best to organize lab personnel for this protocol.

Since WGSA involves a series of ordered stages, the output of one stage directly impacts the performance of the subsequent stage. For example, the quantity and purity of the DNA after purification can affect the kinetics of the Fragmentation Reagent during the subsequent fragmentation stage.

To efficiently process samples in 96-well plates, it is essential that you be proficient with the use of multi-channel pipets. Attempting to use a single channel pipet 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 pipets, we strongly recommend practicing several times before processing actual samples. You can use water to get a feel for aspirating and dispensing solutions to multiple wells simultaneously.

Post-PCR stages 7 through 11 are best performed by the more experienced operators in your laboratory. These operators should be proficient in:

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

Before You Begin

Master Mix Preparation

Carefully follow each master mix recipe. Use pipets that have been calibrated to ± 5%. When molecular biology-grade water is specified, be sure to use the AccuGENE® water listed in Appendix A of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide*. Using in-house ddH_2O or other water can negatively affect your results. The enzymatic reaction in *Stage 9: 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 pipets are not accurate. We recommend that you stop and repeat the experiment.

Reagent Handling and Storage

Follow these guidelines for reagent handling and storage.

- Keep dedicated equipment in each of the areas used for this protocol. To avoid contamination, do not move equipment between the Pre-PCR Clean Area, the PCR Staging Room and the Main Lab.
- Unless otherwise indicated, keep all reagents (except enzymes) on ice in a cooling chamber that has been chilled to 4 °C when working on the bench top.
- 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.
- Consult the appropriate MSDS for reagent storage and handling requirements.
- 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.
- When performing the steps for Stages 1 through 10 of the 96-sample protocol:
  - Keep all tubes on ice or in a cooling chamber on ice.
  - Keep all plates in cooling chambers on ice.

Preparing the Work Area for Each Stage

Many of the stages in the Genome-Wide Human SNP Nsp/Sty 5.0/6.0 Assay must be performed rapidly and on ice to carefully control enzyme activity and temperature transitions. Therefore, we recommend that you 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 1: Sty Restriction Enzyme Digestion*. Pipets and tips are not shown.

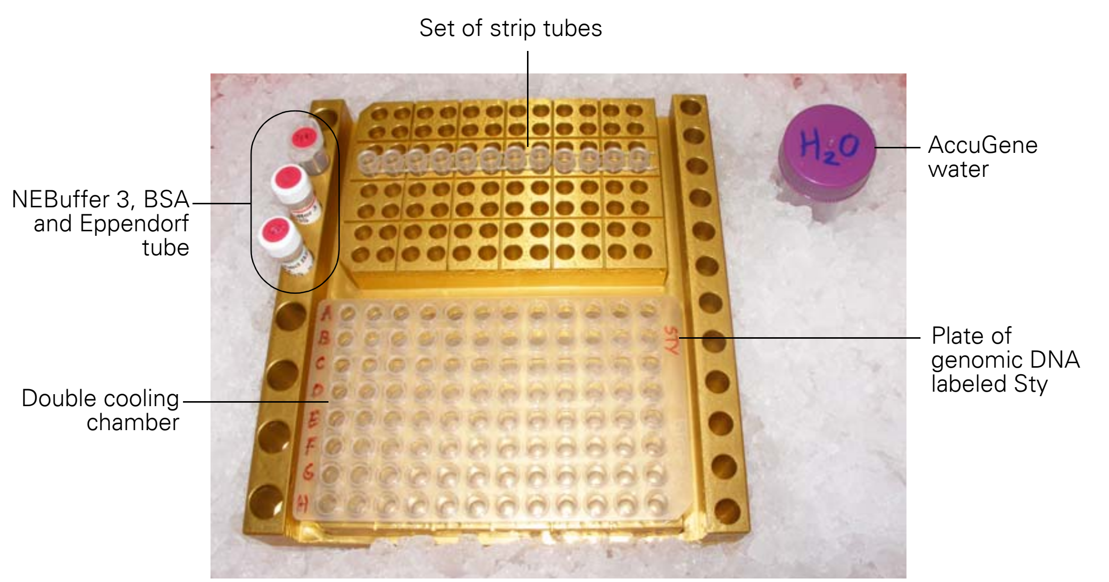


Figure 5.2 Example of Work Area Preparation

Thermal Cyclers, Plates and Plate Seals

The Genome-Wide SNP 5.0/6.0 Assay has been optimized using the following thermal cyclers, reaction plates and adhesive film.

IMPORTANT: Use only the 96-well plate and adhesive seals listed in Table 5.1, and only the thermal cyclers listed in Table 5.2. Using other plates and seals that are incompatible with these thermal cyclers can result in loss of sample or poor results.

Table 5.1 96-well plate and adhesive seals optimized for use with this protocol

Item	Vendor	Part Number
Multiplate 96-well unskirted PCR plate	Bio-Rad	MLP-9601
Adhesive seals:		
• Microseal 'B' Adhesive Seal	Bio-Rad	MSB1001
• MicroAmp® Clear Adhesive Film	Applied Biosystems	4306311

Table 5.2 Thermal cyclers optimized for use with this protocol

Laboratory	Thermal Cyclers Validated for Use
Pre-PCR Clean Area	Applied Biosystems units: • 2720 Thermal Cycler • GeneAmp® PCR System 9700
	Bio-Rad units: • MJ Tetrad PTC-225 • DNA Engine Tetrad 2
Post-PCR Area	Applied Biosystems unit: • GeneAmp® PCR System 9700 (silver block or gold-plated silver block)
	Bio-Rad units: • MJ Tetrad PTC-225 DNA Engine Tetrad 2

Program Your Thermal Cyclers

The thermal cycler programs listed below are used during this protocol. Before you begin processing samples, enter and store these programs on the appropriate thermal cyclers in the PCR Staging Room and the Main Lab.

Thermal cycler program details are listed in Appendix B of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide*.

Table 5.3 Thermal Cycler Programs Required for the 96 Sample Protocol (Figure 5.1 on page 137)

Program Name	# of Thermal Cyclers Required	Laboratory
GW5.0/6.0 Digest	1	PCR Staging Room
GW5.0/6.0 Ligate	1	PCR Staging Room
GW5.0/6.0 PCR	4	Main Lab
GW5.0/6.0 Fragment	1	Main Lab
GW5.0/6.0 Label	1	Main Lab
GW5.0/6.0 Hyb	1	Main Lab

Genomic DNA Plate Preparation

About this Stage

The human genomic DNA you will process using the Genome-Wide SNP 5.0/6.0 Assay should meet the general requirements listed in Chapter 3 of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide*. 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 the corresponding wells of two 96-well plates.

Location and Duration

- PCR Staging Room
- Hands-on time: time will vary; can be up to 4 hours

Input Required

This protocol is written for processing two replicates of 96 genomic DNA samples including controls.

Table 5.4 Input Required for *Genomic DNA Plate Preparation*

Quantity	Item
	Genomic DNA samples that meet the general requirements listed in Chapter 3 of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide*.

Equipment and Consumables Required

The following equipment and consumables are required for this stage. Refer to Appendix A of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide* for vendor and part number information.

Table 5.5 Equipment and Consumables Required for *Genomic DNA Plate Preparation*

Quantity	Item
enough for three 96-well plates	Cooling chambers, chilled to 4 °C (do not freeze)
1	Ice bucket, filled with ice
1	Plate centrifuge
1	Pipet, single channel P20
1	Pipet, 12-channel P200
1	Pipet, single channel P200
As needed	Pipet tips
As needed (2 per sample)	Reaction plates, 96-well**
As needed	Plate seals**
1	Spectrophotometer plate reader
1	Vortexer

! **IMPORTANT: ** Use only the 96-well plate, adhesive seals and thermal cyclers listed in Table 5.1 and Table 5.2 on page 141.**

Reagents Required

The following reagents are required for this stage. Refer to Appendix A of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide* for vendor and part number information.

Table 5.6 Reagents Required for *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 may 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 two 96-well reaction plates.

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

   Two replicates of each sample are required for this protocol: one for Nsp and one for processing Sty.
3. 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 1: Sty Restriction Enzyme Digestion

About this Stage

During this stage, the genomic DNA is digested by the Sty I restriction enzyme. You will:

1. Prepare a Sty Digestion Master Mix.
2. Add the master mix to one set of 96 samples.
3. Place the samples onto a thermal cycler and run the GW5.0/6.0 Digest program.

Location and Duration

- Pre-PCR Clean Area
- Hands-on time: 30 minutes
- GW5.0/6.0 Digest thermal cycler program time: 2.5 hours

Input Required From Previous Stage

The input required is shown below.

Quantity	Item
96 samples	Genomic DNA prepared as instructed under *Genomic DNA Plate Preparation* on page 143 (5 µL at 50 ng/µL in each well).

Equipment and Consumables Required

The following equipment and consumables are required for this stage. Refer to Appendix A of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide* for vendor and part number information.

! **IMPORTANT: ** Use only the 96-well plate, adhesive seals and thermal cyclers listed in Table 5.1 and Table 5.2 on page 141.**

Table 5.7 Equipment and Consumables Required for *Stage 1: Sty 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	Pipet, single channel P100
1	Pipet, single channel P200
1	Pipet, single channel P1000
1	Pipet, 12-channel P20
As needed	Pipet tips for pipets listed above; full racks
1	Plate centrifuge
1	Plate seal**
1	Thermal cycler**
1 strip	Tubes, 12-strip, 0.2 mL
1	Tube, Eppendorf 2.0 mL
1	Vortexer

REAGENTS REQUIRED

The following reagents are required for this stage. Refer to Appendix A of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide* for vendor and part number information. The amounts listed are sufficient for processing 96 samples.

Table 5.8 Reagents Required for *Stage 1: Sty Restriction Enzyme Digestion*

Quantity	Reagent
1 vial	BSA (100X; 10 mg/mL)
1 vial	NE Buffer 3 (10X)
1 vial	Sty I (10 U/µL; NEB)
2.5 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

We recommend including one positive and one negative control with every set of samples run.

Reference Genomic DNA 103 can be used as a positive control. It is supplied in the Genome-Wide Human SNP Nsp/Sty Assay Kit 5.0/6.0.

A process negative control can be included at the beginning of the assay to assess the presence of contamination. Refer to Chapters 3 and 8 of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide* for more information.

Prepare the Reagents, Equipment and Consumables

Thaw Reagents and Genomic DNA

1. Allow the following reagents to thaw on ice:
   - NE Buffer 3
   - BSA
2. If the genomic DNA is frozen, allow it to thaw in a cooling chamber on ice.

> **!** **IMPORTANT: Leave the 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 5.3 on page 151).
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 with genomic DNA 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. Prepare the reagents (except for the enzyme) as follows:
   - **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.

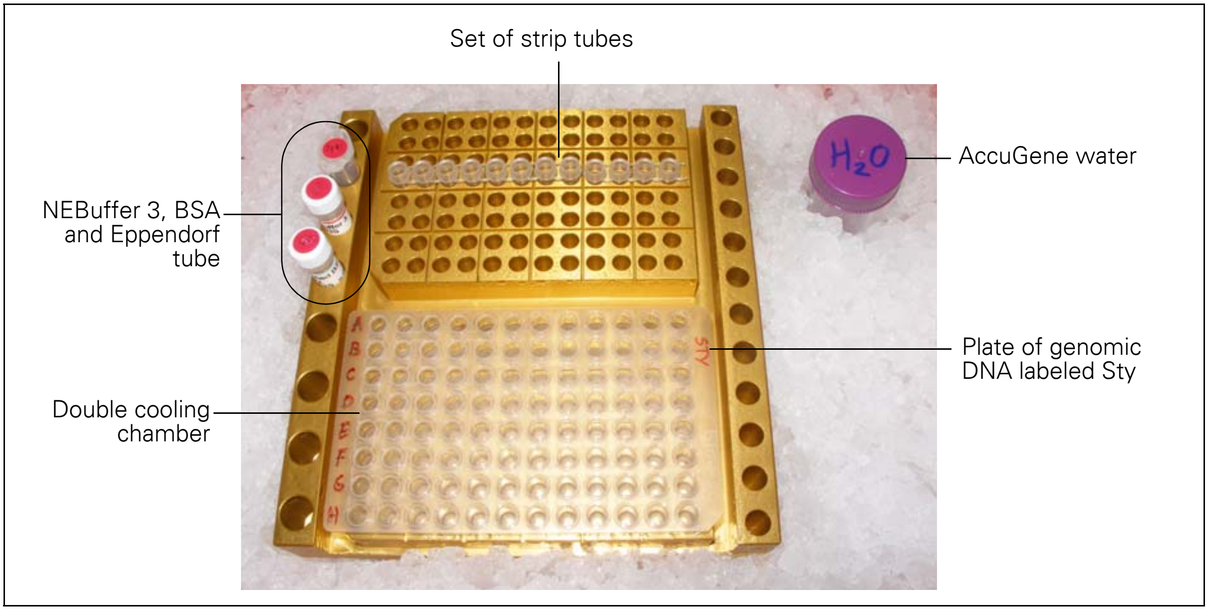


Figure 5.3 Work Area Prepared for Processing Samples with Sty Digest Mix (Sty Enzyme Not Pictured; Still at –20 °C)

Prepare the Sty Digestion Master Mix

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

1. To the 2.0 mL Eppendorf tube, add the volumes of the following reagents as shown in Table 5.9:
   - AccuGENE water
   - NE Buffer 3
   - BSA
2. Remove the Sty I enzyme 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 Sty Digestion Master Mix to Samples* on page 152.

Table 5.9 Sty I Digestion Master Mix

Reagent	1 Sample	96 Samples (~ 15% extra)
AccuGENE® Water	11.55 µL	1270.5 µL
NE Buffer 3 (10X)	2 µL	220 µL
BSA (100X; 10 mg/mL)	0.2 µL	22 µL
Sty I (10 U/µL)	1 µL	110 µL
Total	**14.75 µL**	**1622.5 µL**

Add Sty Digestion Master Mix to Samples

To add the Sty Digestion Master Mix to samples:

1. Using a single channel P200 pipet, aliquot 134 µL of Sty Digestion Master Mix to each tube of the strip tubes labeled *Dig*.
2. Using a 12-channel P20 pipet, add 14.75 µL of Sty Digestion Master Mix to each DNA sample in the cooling chamber on ice.

   The total volume in each well is now 19.75 µL.

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 GW5.0/6.0 Digest program.

Table 5.10 GW5.0/6.0 Digest Program

GW5.0/6.0 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 following the recommended workflow (Figure 5.1 on page 137), place the plate in a cooling chamber on ice and proceed immediately to *Stage 2: Sty Ligation* on page 154.
- If not proceeding directly to the next step, store the samples at –20 °C.

Stage 2: Sty Ligation

About this Stage

During this stage, the digested samples are ligated using the Sty Adaptor. You will:

1. Prepare a Sty Ligation Master Mix.
2. Add the master mix to the samples.
3. Place the samples onto a thermal cycler and the GW5.0/6.0 Ligate program is run.
4. Dilute the ligated samples with AccuGENE water.

Location and Duration

- Pre-PCR Clean Area
- Hands-on time: 30 minutes
- GW5.0/6.0 Ligate thermal cycler program time: 3.3 hours

Input Required From Previous Stage

The input required from *Stage 1: Sty Restriction Enzyme Digestion* is:

Quantity	Item
96 samples	Sty 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 of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide* for vendor and part number information.

Table 5.11 Equipment and Consumables Required for *Stage 2: Sty 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	Pipet, single channel P100
1	Pipet, single channel P1000
1	Pipet, 12-channel P20
1	Pipet, 12-channel P200
As needed	Pipet tips for pipets listed above; full racks
1	Plate centrifuge
2	Plate seal**
1	Solution basin, 55 mL
1	Thermal cycler**
1 strip	Tubes, 12-strip, 0.2 mL
1	Tube, Eppendorf 2.0 mL
1	Vortexer

! IMPORTANT: ** Use only the 96-well plate, adhesive seals and thermal cyclers listed in Table 5.1 and Table 5.2 on page 141.

Reagents Required

The following reagents are required for this stage. Refer to Appendix A of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide* for vendor and part number information. The amounts listed are sufficient to process 96 samples.

Table 5.12 Reagents Required for *Stage 2: Sty Ligation*

Quantity	Reagent
1 vial	T4 DNA Ligase (400 U/µL; NEB)
1 vial	T4 DNA Ligase Buffer (10X)
1 vial	Adaptor, Sty (50 µM)
15 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.

Prepare the Reagents, Consumables and Other Components

! **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 Sty adaptor.**

Thaw the Reagents and Sty Digestion Stage Plate

To thaw the reagents and Sty Digestion Stage Plate:

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

   Requires approximately 20 minutes to thaw.

2. If the Sty digested samples were frozen, allow them 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 5.2 on page 140).
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 digested samples 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 Sty Ligation Master Mix

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

1. To the 2.0 mL Eppendorf tube, add the following reagents based on the volumes shown in Table 5.13:
   - T4 DNA Ligase Buffer (10X)
   - Adaptor Sty I
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 Sty Ligation Master Mix to Reactions*.

Table 5.13 Sty I Ligation Master Mix

Reagent	1 Sample	96 Samples (~ 21% extra)
T4 Ligase Buffer (10X)	2.5 µL	290 µL
Adaptor Sty I (50 µM)	0.75 µL	87 µL
T4 DNA Ligase (400U/µL)	2 µL	232 µL
Total	**5.25 µL**	**609 µL**

Add Sty Ligation Master Mix to Reactions

To add Sty Ligation Master Mix to samples:

1. Using a single channel P100 pipet, aliquot 49 µL of Sty Ligation Master Mix to each tube of the strip tubes on ice.
2. Using a 12-channel P20 pipet, aliquot 5.25 µL of Sty Ligation Master Mix to each reaction on the Sty Digestion Stage Plate.

   The total volume in each well is now 25 µL.

Sty Digested DNA	19.75 µL
Sty 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 GW5.0/6.0 Ligate program.

Table 5.14 GW5.0/6.0 Ligate Thermal Cycler Program

GW5.0/6.0 Ligate Program	
Temperature	**Time**
16ºC	180 minutes
70ºC	20 minutes
4ºC	Hold

Dilute the Samples

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

To dilute the samples:

1. Place the AccuGENE water on ice 20 minutes prior to use.
2. When the GW5.0/6.0 Ligate program is finished, remove the plate and spin it down at 2000 rpm for 30 sec.
3. Place the plate in a cooling chamber on ice.
4. Dilute each reaction as follows:

   A. Pour 15 mL AccuGENE water into the solution basin.

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

   The total volume in each well is 100 µL.

Sty Ligated DNA	25 µL
AccuGENE water	75 µL
Total	**100 µL**

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

What You Can Do Next

Do one of the following:

- If following the recommended workflow (Figure 5.1 on page 137), proceed immediately to *Stage 3: Sty PCR* on page 161.

  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 3: Sty PCR

About this Stage

During this stage, you will:

1. Transfer equal amounts of each Sty ligated sample into three fresh 96-well plates (Figure 5.4 on page 166).
2. Prepare the Sty PCR Master Mix, and add it to each sample.
3. Place each plate on a thermal cycler and run the GW 5.0 PCR program.
4. Confirm the PCR by running 3 µL of each PCR product on a 2% TBE gel or an E-Gel® *96* 2% agarose gel.

Location and Duration

- Pre-PCR Clean Area: Sty PCR Master Mix preparation
- PCR Staging Area: PCR set up
- Main Lab: PCR Plates placed on thermal cyclers
- Hands-on time: 75 minutes
- GW5.0/6.0 PCR thermal cycler program time: 1.5 hours; samples can be held overnight at 4 °C.

Input Required from Previous Stage

The input required from *Stage 2: Sty Ligation* is:

Quantity	Item
96	Diluted Sty ligated samples

Equipment and Materials Required

The following equipment and materials are required to perform this stage. Refer to Appendix A of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide* for vendor and part number information.

Table 5.15 Equipment and Consumables Required for *Stage 3: Sty PCR*

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

! **IMPORTANT: ** Use only the 96-well plate, adhesive seals and thermal cyclers listed in Table 5.1 and Table 5.2 on page 141.**

Reagents Required

The following reagents are required for this stage. Refer to Appendix A of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide* for vendor and part number information. The amounts listed are sufficient to process 96 samples.

Table 5.16 Reagents Required for *Stage 3: Sty PCR*

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

Gels and Related Materials Required

Verifying the PCR reaction is required for this stage. You can use the following gels and related materials, or E-Gels as described in Appendix C of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide*. The amounts listed are sufficient to process 96 Sty samples.

Refer to Appendix A of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide* for vendor and part number information.

Table 5.17 Gels and Related Materials Required for *Stage 3: Sty PCR*

Quantity	Reagent
190 µL	DNA Marker
19	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 Sty ligated DNA was diluted to 100 µL with AccuGENE water.**
- **Set up the PCRs in PCR Staging Area.**
- **Prepare Sty PCR Master Mix immediately prior to use, and prepare in Pre-PCR Clean Area. 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.**
- **To ensure consistent results, take 3 µL aliquots from each PCR to run on gels.**

About Controls

A PCR negative control can be included in the experiment to assess the presence of contamination. Refer to Chapters 3 and 8 of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide* for more information.

Prepare the Reagents, Consumables and Other Components

Thaw Reagents and Ligated Samples

To thaw the reagents and ligated samples:

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 Sty ligated samples are 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 cooling chambers and one cooler on ice.
2. Label the following, then place in a cooling chamber:
   - Three 96-well reaction plates labeled *P1, P2, P3* (see Figure 5.4 on page 166)
   - One 50 mL Falcon tube labeled *PCR MM*
3. Place on ice:
   - AccuGENE water
   - GC-Melt
   - Solution basin
4. Prepare the Sty ligated samples 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 PCR to preheat the lids. The lids must be preheated before loading samples; leave the blocks at room temperature.

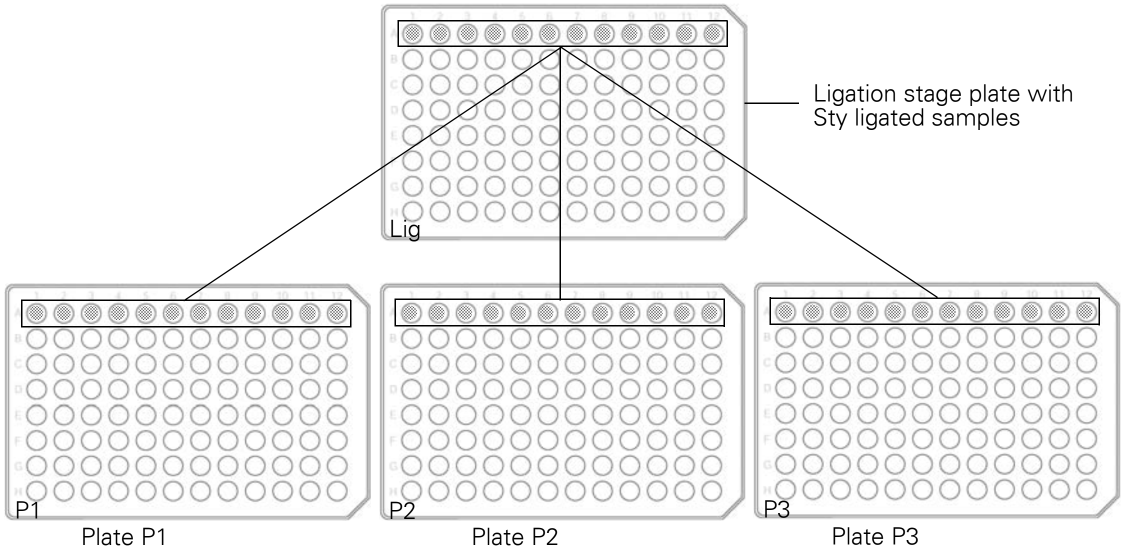
If you are preparing the plates for PCR, it is best not to go from the Pre-PCR Room or Staging Area to the Main Lab and then back again.

Aliquot Sty Ligated DNA to the PCR Plates

To aliquot Sty ligated DNA to the PCR plates:

1. Working one row at a time and using a 12-channel P20 pipet, transfer 10 µL of each Sty ligated sample to the corresponding well of each PCR plate.

   Example (Figure 5.4): Transfer 10 µL of each sample from Row A of the Sty Ligation Stage Plate to the corresponding wells of row A on the plates labeled 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 Plate is transferred to the corresponding well of each PCR Plate. For example, an equal aliquot of each sample from row A on the Sty Ligation Plate is transferred to the corresponding wells of row A on PCR Plates P1, P2 and P3.

Figure 5.4 Transferring Equal Aliquots of Diluted, Ligated Sty Samples to Three Reaction Plates

Prepare the Sty PCR Master Mix

Location

Pre-PCR Clean Area

Prepare the Sty PCR Master Mix

To prepare the Sty 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.**

1. Keeping the 50 mL Falcon tube in the cooling chamber, add the reagents as shown in Table 5.18 on page 167 (**except for the *Taq* DNA polymerase**).

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 5.18 Sty PCR Master Mix for 96 Samples

Reagent	For 1 Reaction	3 PCR Plates, 96 Samples Each Plate (~ 10% extra)
AccuGENE water	39.5 µL	12.482 mL
TITANIUM *Taq* PCR Buffer (10X)	10 µL	3.160 mL
GC-Melt (5M)	20 µL	6.320 mL
dNTP (2.5 mM each)	14 µL	4.424 mL
PCR Primer 002 (100 µM)	4.5 µL	1.422 mL
TITANIUM *Taq* DNA Polymerase (50X) (do not add until ready to aliquot master mix to ligated samples)	2 µL	0.632 mL
Total	**90 µL**	**28.440 mL**

Add Sty PCR Master Mix to Samples

Location

PCR Staging Area

Procedure

To add Sty PCR Master Mix to samples:

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

   To avoid contamination, change pipet tips after each dispense.

   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 Sty PCR Plates Onto Thermal Cyclers

IMPORTANT: PCR protocols for the MJ Tetrad PTC-225 and Applied Biosystems thermal cyclers are different. See Table 5.19 and Table 5.20 below.

Location

Main Lab

Procedure

To load the plates and run the GW5.0/6.0 PCR program:

1. Transfer the 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 GW5.0/6.0 PCR program.

   The program varies depending upon the thermal cyclers you are using. See Table 5.19 for Applied Biosystems thermal cyclers and Table 5.20 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 5.19 GW5.0/6.0 PCR Thermal Cycler Program for the GeneAmp® PCR System 9700 (silver or gold-plated silver blocks)

GW5.0/6.0 PCR Program for GeneAmp® PCR System 9700		
Temperature	**Time**	**Cycles**
94°C	3 minutes	1X
94°C	30 sec	30X
60°C	45 sec	
68°C	15 sec	
68°C	7 minutes	1X
4°C	HOLD (Can be held overnight)	
Volume: 100 µL		
Specify *Maximum* mode.		

Table 5.20 GW5.0/6.0 PCR Thermal Cycler Program for the MJ Tetrad PTC-225

GW5.0/6.0 PCR Program for MJ Tetrad PTC-225		
Temperature	**Time**	**Cycles**
94°C	3 minutes	1X
94°C	30 sec	30X
60°C	30 sec	
68°C	15 sec	
68°C	7 minutes	1X
4°C	HOLD (Can be held overnight)	
Volume: 100 µL		
Use *Heated Lid* and *Calculated Temperature*		

Running Gels

The instructions below are for running 2% TBE gels. For information on running E-Gel *96* 2% agarose gels, refer to Appendix C of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide*.

Before Running Gels

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

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

Run the Gels

When the GW5.0/6.0 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 in rows A through D of the fresh, labeled P*X*Gel plates.
6. Using a 12-channel P20 pipet, transfer 3 µL of each PCR product from the 3 Sty PCR plates to the corresponding plate, row and wells of the P*X*Gel plates.

   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 the P*X*Gel plates.
8. Vortex the center of each P*X*Gel plate*,* then spin them down at 2000 rpm for 30 sec.
9. Load the total volume from each well of each P*X*Gel plate 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 ~200 bp to 1100 bp (see Figure 5.5).

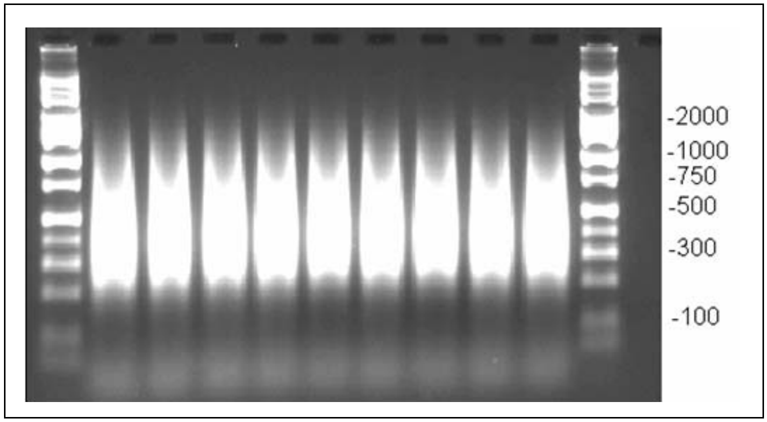


Figure 5.5 Example of PCR products run on 2% TBE agarose gel at 120V for 1 hour. Average product distribution is between ~200 to 1100 bp.

What You Can Do Next

Do one of the following:

- If following the recommended workflow (Figure 5.1 on page 137), seal the Sty PCR product plates and store them at –20 °C.
- Proceed to the next stage within 60 minutes.

Stage 4: Nsp Restriction Enzyme Digestion

About this Stage

During this stage, the genomic DNA is digested by the Nsp I enzyme. You will:

1. Prepare a Nsp Digestion Master Mix.
2. Add the master mix to one set of 96 samples.
3. Place the samples onto a thermal cycler and run the GW5.0/6.0 Digest program.

Location and Duration

- Pre-PCR Clean Area
- Hands-on time: 30 minutes
- GW5.0/6.0 Digest thermal cycler program time: 2.5 hours

Input Required From Previous Stage

The input required is shown below.

Quantity	Item
96 samples	Genomic DNA prepared as instructed under *Genomic DNA Plate Preparation* on page 143 (5 µL at 50 ng/µL in each well).

Equipment and Consumables Required

The following equipment and consumables are required for this stage. Refer to Appendix A of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide* for vendor and part number information.

IMPORTANT: ** Use only the 96-well plate, adhesive seals and thermal cyclers listed in Table 5.1 and Table 5.2 on page 141.

Table 5.21 Equipment and Consumables Required for *Stage 4: Nsp 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	Pipet, single channel P100
1	Pipet, single channel P200
1	Pipet, single channel P1000
1	Pipet, 12-channel P20
As needed	Pipet tips for pipets listed above; full racks
1	Plate centrifuge
1	Plate seal**
1	Thermal cycler**
1 strip	Tubes, 12-strip, 0.2 mL
1	Tube, Eppendorf 2.0 mL
1	Vortexer

Reagents Required

The following reagents are required for this stage. Refer to Appendix A of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide* for vendor and part number information. The amounts listed are sufficient for processing 96 samples.

Table 5.22 Reagents Required for *Stage 4: Nsp Restriction Enzyme Digestion*

Quantity	Reagent
1 vial	BSA (100X; 10 mg/mL)
1 vial	NE Buffer 2 (10X)
1 vial	Nsp I (10 U/µL; NEB)
2.5 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

We recommend including one positive and one negative control with every set of samples run.

Reference Genomic DNA 103 can be used as a positive control. It is supplied in the Genome-Wide Human SNP Nsp/Sty Assay Kit 5.0/6.0.

A process negative control can be included at the beginning of the assay to assess the presence of contamination. Refer to Chapters 3 and 8 of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide* and for more information.

Prepare the Reagents, Equipment and Consumables

Thaw Reagents and Genomic DNA

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

> **!** **IMPORTANT:** **Leave the NSP 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.
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 with genomic DNA 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. Prepare the reagents (except for the enzyme) as follows:
   - **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 Nsp Digestion Master Mix

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

1. To the 2.0 mL Eppendorf tube, add the appropriate volumes of the following reagents based on Table 5.23:
   - AccuGENE water
   - NE Buffer 2
   - BSA
2. Remove the Nsp I enzyme 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 Nsp Digestion Master Mix to Samples* on page 177.

Table 5.23 Nsp I Digestion Master Mix

Reagent	1 Sample	96 Samples (~ 15% extra)
AccuGENE® Water	11.55 µL	1270.5 µL
NE Buffer 2 (10X)	2 µL	220 µL
BSA (100X; 10 mg/mL)	0.2 µL	22 µL
Nsp I (10 U/µL)	1 µL	110 µL
Total	**14.75 µL**	**1622.5 µL**

Add Nsp Digestion Master Mix to Samples

To add Nsp Digestion Master Mix to samples:

1. Using a single channel P200 pipet, aliquot 134 µL of Nsp Digestion Master Mix to each tube of the strip tubes labeled *Dig*.
2. Using a 12-channel P20 pipet, add 14.75 µL of Nsp Digestion Master Mix to each DNA sample in the cooling chamber on ice.

   The total volume in each well is now 19.75 µL.

Genomic DNA (50 ng/µL)	5 µL
Nsp 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 GW5.0/6.0 Digest program.

Table 5.24 GW5.0/6.0 Digest Program

GW5.0/6.0 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 following the recommended workflow (Figure 5.1 on page 137), proceed immediately to *Stage 5: Nsp Ligation* on page 178.
   - If not proceeding directly to the next step, store the samples at –20 °C.

Stage 5: Nsp Ligation

About this Stage

During this stage, the digested samples are ligated using the Nsp Adaptor. You will:

1. Prepare a Nsp Ligation Master Mix.
2. Add the master mix to the samples.
3. Place the samples onto a thermal cycler and the GW5.0/6.0 Ligate program is run.
4. Dilute the ligated samples with AccuGENE water.

Location and Duration

- Pre-PCR Clean Area
- Hands-on time: 30 minutes
- GW5.0/6.0 Ligate thermal cycler program time: 3.3 hours

Input Required From Previous Stage

The input required from *Stage 4: Nsp Restriction Enzyme Digestion* is:

Quantity	Item
96 samples	Nsp 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 of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide* for vendor and part number information.

Table 5.25 Equipment and Consumables Required for *Stage 5: Nsp 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	Pipet, single channel P100
1	Pipet, single channel P1000
1	Pipet, 12-channel P20
1	Pipet, 12-channel P200
As needed	Pipet tips for pipets listed above; full racks
1	Plate centrifuge
2	Plate seal**
1	Solution basin, 55 mL
1	Thermal cycler**
1 strip	Tubes, 12-strip, 0.2 mL
1	Tube, Eppendorf 2.0 mL
1	Vortexer

! **IMPORTANT: ** Use only the 96-well plate, adhesive seals and thermal cyclers listed in Table 5.1 and Table 5.2 on page 141.**

Reagents Required

The following reagents are required for this stage. Refer to Appendix A of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide* for vendor and part number information. The amounts listed are sufficient to process 96 samples.

Table 5.26 Reagents Required for *Stage 5: Nsp Ligation*

Quantity	Reagent
1 vial	T4 DNA Ligase (400 U/μL; NEB)
1 vial	T4 DNA Ligase Buffer (10X)
1 vial	Adaptor, Nsp (50 μM)
15 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.

Prepare the Reagents, Consumables and Other Components

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 Nsp adaptor.**

Thaw the Reagents and Nsp Digestion Stage Plate

To thaw the reagents and Nsp Digestion Stage Plate:

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

   Takes approximately 20 minutes to thaw.
2. If the Nsp digested samples were frozen, allow them 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.
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 digested samples 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 Nsp Ligation Master Mix

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

1. To the 2.0 mL Eppendorf tube, add the following reagents based on the volumes shown in Table 5.27:
   - Adaptor Nsp
   - 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 Nsp Ligation Master Mix to Reactions*.

Table 5.27 Nsp I Ligation Master Mix

Reagent	1 Sample	96 Samples (~ 21% extra)
T4 DNA Ligase Buffer (10X)	2.5 µL	290 µL
Adaptor Nsp I (50 µM)	0.75 µL	87 µL
T4 DNA Ligase (400 U/µL)	2 µL	232 µL
Total	**5.25 µL**	**609 µL**

Add Nsp Ligation Master Mix to Reactions

To add Nsp Ligation Master Mix to samples:

1. Using a single channel P100 pipet, aliquot 49 µL of Nsp Ligation Master Mix to each tube of the strip tubes on ice.
2. Using a 12-channel P20 pipet, aliquot 5.25 µL of Nsp Ligation Master Mix to each reaction on the Nsp Digestion Stage Plate.

The total volume in each well is now 25 µL.

Nsp Digested DNA	19.75 µL
Nsp 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 GW5.0/6.0 Ligate program.

Table 5.28 GW5.0/6.0 Ligate Thermal Cycler Program

GW5.0/6.0 Ligate Program	
Temperature	**Time**
16ºC	180 minutes
70ºC	20 minutes
4ºC	Hold

Dilute the Samples

> **!** **IMPORTANT: It is crucial to dilute the ligated DNA with AccuGENE water prior to PCR.**

To dilute the samples:

1. Place the AccuGENE water on ice 20 minutes prior to use.
2. When the GW5.0/6.0 Ligate program is finished, remove the plate and spin it down at 2000 rpm for 30 sec.
3. Place the plate in a cooling chamber on ice.
4. Dilute each reaction as follows:

   A. Pour 15 mL AccuGENE water into the solution basin.

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

   The total volume in each well is 100 µL.

Nsp Ligated DNA	25 µL
AccuGENE water	75 µL
Total	**100 µL**

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

What You Can Do Next

Do one of the following:

- If following the recommended workflow (*Recommended Workflow for Processing 96 Samples* on page 137), proceed immediately to *Stage 6: Nsp PCR* on page 185.

  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 6: Nsp PCR

About this Stage

During this stage, you will:

1. Transfer equal amounts of each Nsp ligated sample into four fresh 96-well plates.
2. Prepare the Nsp PCR Master Mix, and add it to each sample.
3. Place each plate on a thermal cycler and run the GW 5.0 PCR program.
4. Confirm the PCR by running 3 µL of each PCR product on a 2% TBE gel or an E-Gel® *96* 2% agarose gel.

Location and Duration

- Pre-PCR Clean Area: Nsp PCR Master Mix preparation
- PCR Staging Area: PCR set up
- Main Lab: PCR Plates placed on thermal cyclers
- Hands-on time: 75 minutes
- GW5.0/6.0 PCR thermal cycler program time: 1.5 hours; samples can be held overnight at 4 °C.

Input Required from Previous Stage

The input required from *Stage 5: Nsp Ligation* is:

Quantity	Item
96	Diluted Nsp ligated samples

Equipment and Materials Required

The following equipment and materials are required to perform this stage. Refer to Appendix A of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide* for vendor and part number information.

Table 5.29 Equipment and Consumables Required for *Stage 6: Nsp PCR*

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

! **IMPORTANT: ** Use only the 96-well plate, adhesive seals and thermal cyclers listed in Table 5.1 and Table 5.2 on page 141.**

Reagents Required

The following reagents are required for this stage. Refer to Appendix A of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide* for vendor and part number information. The amounts listed are sufficient to process 96 samples.

Table 5.30 Reagents Required for *Stage 6: Nsp PCR*

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

Gels and Related Materials Required

Verifying the PCR reaction is required for this stage. You can use the following gels and related materials, or E-Gels as described in Appendix C of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide*. The amounts listed are sufficient to process 96 Sty samples.

Refer to Appendix A of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide* for vendor and part number information.

Table 5.31 Gels and Related Materials Required for *Stage 6: Nsp PCR*

Quantity	Reagent
190 µL	DNA Marker
19	Gels, 2% TBE
As needed	Gel loading solution
4	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 Nsp ligated DNA was diluted to 100 µL with AccuGENE water.**
- **Set up the PCRs in PCR Staging Area.**
- **Prepare Nsp PCR Master Mix immediately prior to use, and prepare in Pre-PCR Clean Area. 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.**
- **To ensure consistent results, take 3 µL aliquots from each PCR to run on gels.**

About Controls

A PCR negative control can be included in the experiment to assess the presence of contamination. Refer to Chapters 3 and 8 of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide* for more information.

Prepare the Reagents, Consumables and Other Components

Thaw Reagents and Nsp Ligated Samples

To thaw the reagents and Nsp ligated samples:

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 Nsp ligated samples are 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 enough cooling chambers for 5 plates and one cooler on ice.
2. Label the following, then place in a cooling chamber:
   - Four 96-well reaction plates labeled *P1, P2, P3, P4*
   - One 50 mL Falcon tube labeled *PCR MM*
3. Place on ice:
   - AccuGENE water
   - GC-Melt
   - Solution basin
4. Prepare the Nsp ligated samples 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 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.

Aliquot Nsp Ligated DNA to the PCR Plates

To aliquot Nsp ligated DNA to the PCR plates:

1. Working one row at a time and using a 12-channel P20 pipet, transfer 10 µL of each Nsp ligated sample to the corresponding well of each PCR plate (P1, P2, P3 and P4).
2. Seal each plate with adhesive film, and leave in cooling chambers on ice.

Prepare the Nsp PCR Master Mix

Location

Pre-PCR Clean Area

Prepare the Nsp PCR Master Mix

To prepare the Nsp 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 Nsp 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.

1. Keeping the 50 mL Falcon tube in the cooling chamber, add the reagents as shown in Table 5.32 on page 191 (**except for the *Taq* DNA polymerase**).
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 5.32 Nsp PCR Master Mix for 96 Samples

Reagent	For 1 Reaction	4 PCR Plates 96 Samples Each Plate (~ 10% extra)
AccuGENE water	39.5 µL	16.669 mL
TITANIUM *Taq* PCR Buffer (10X)	10 µL	4.220 mL
GC-Melt (5M)	20 µL	8.440 mL
dNTP (2.5 mM each)	14 µL	5.908 mL
PCR Primer 002 (100 µM)	4.5 µL	1.899 mL
TITANIUM *Taq* DNA Polymerase (50X) do not add until ready to aliquot master mix to ligated samples	2 µL	0.844 mL
Total	**90 µL**	**37.980 mL**

Add Nsp PCR Master Mix to Samples

Location

PCR Staging Area

Procedure

To add Nsp PCR Master Mix to samples:

1. Using a 12-channel P200 pipet, add 90 µL Nsp PCR Master Mix to each sample.
   To avoid contamination, change pipet tips after each dispense.
   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 Nsp PCR Plates Onto Thermal Cyclers

IMPORTANT: PCR protocols for the MJ Tetrad PTC-225 and Applied Biosystems thermal cyclers are different. Thermal cycler program parameters are on page 193.

Location

Main Lab

Procedure

To load the plates and run the GW5.0/6.0 PCR program:

1. Transfer the 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 GW5.0/6.0 PCR program.

   The program varies depending upon the thermal cyclers you are using. See Table 5.33 and Table 5.34 on page 193 program parameters.

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 5.33 GW5.0/6.0 PCR Thermal Cycler Program for the GeneAmp® PCR System 9700 (silver or gold-plated silver blocks)

GW5.0/6.0 PCR Program for GeneAmp® PCR System 9700		
Temperature	**Time**	**Cycles**
94°C	3 minutes	1X
94°C	30 sec	30X
60°C	45 sec	
68°C	15 sec	
68°C	7 minutes	1X
4°C	HOLD (Can be held overnight)	
Volume: 100 µL		
Specify *Maximum* mode.		

Table 5.34 GW5.0/6.0 PCR Thermal Cycler Program for the MJ Tetrad PTC-225

GW5.0/6.0 PCR Program for MJ Tetrad PTC-225		
Temperature	**Time**	**Cycles**
94°C	3 minutes	1X
94°C	30 sec	30X
60°C	30 sec	
68°C	15 sec	
68°C	7 minutes	1X
4°C	HOLD (Can be held overnight)	
Volume: 100 µL		
Use *Heated Lid* and *Calculated Temperature*		

Running Gels

The instructions below are for running 2% TBE gels. For information on running E-Gel *96* 2% agarose gels, refer to Appendix C of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide*.

Before Running Gels

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

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

Run the Gels

When the GW5.0/6.0 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 four fresh 96-well reaction plates *P1Gel*, *P2Gel*, *P3Gel,* and *P4Gel.*
5. Aliquot 3 µL of 2X Gel Loading Dye to each well in rows A through D of the fresh, labeled P*X*Gel plates.
6. Using a 12-channel P20 pipet, transfer 3 µL of each PCR product from the 4 Nsp PCR plates to the corresponding plate, row and wells of the P*X*Gel plates.

   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 the P*X*Gel plates.
8. Vortex the center of each P*X*Gel plate*,* then spin them down at 2000 rpm for 30 sec.
9. Load the total volume from each well of each P*X*Gel plate 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 ~200 bp to 1100 bp (see Figure 5.6 on page 195).

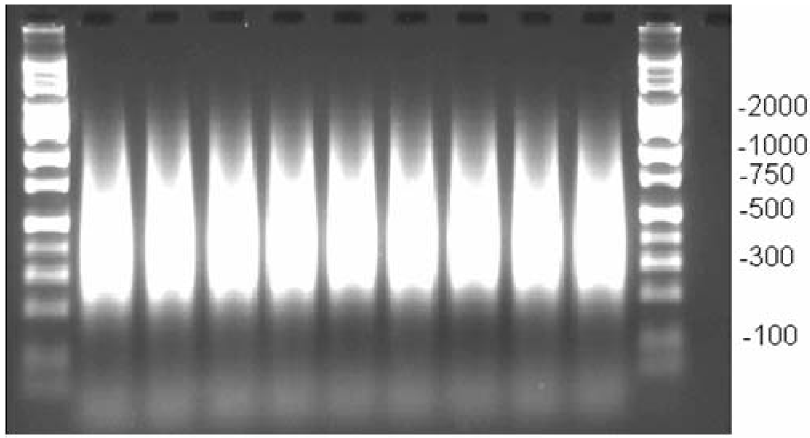


Figure 5.6 Example of PCR products run on 2% TBE agarose gel at 120V for 1 hour. Average product distribution is between ~200 to 1100 bp.

What You Can Do Next

Do one of the following:

- If following the recommended workflow (Figure 5.1 on page 137), do one of the following:
  - If the Nsp PCR plates are still on the thermal cyclers, remove them now and run gels to confirm the PCR (*Running Gels* on page 194). Then proceed to *Stage 7: PCR Product Purification Using a Millipore Filter Plate* on page 196.
  - If the PCR has been confirmed, proceed to *Stage 7: PCR Product Purification Using a Millipore Filter Plate* on page 196.
- If not proceeding directly to the next stage, seal the plates with PCR product and store at –20 °C.

Stage 7: PCR Product Purification Using a Millipore Filter Plate

Millipore vs Seahorse Filter Plate

IMPORTANT: Two different filter plates can be used for the purification stage: Millipore or Seahorse. The instructions in this chapter are based on using a Millipore filter plate. To use a Seahorse filter plate, follow the instructions in **Appendix A, *Alternative Purification Protocol Using a Seahorse Filter Plate,* on page 295.**

About this Stage

During this stage, you will:

- Pool the Sty and Nsp PCR reactions to a single deep well pooling plate
- Add beads to each pool and incubate
- Transfer each pool to a Millipore filter plate and filter on a vacuum manifold
- Wash the PCR products with EtOH and filter
- Elute the PCR products using Buffer EB
- Vacuum and spin transfer the PCR products to a new 96-well plate

Location and Duration

- Main Lab
- Hands-on time: . . . . .approximately 1 hour
- Sample/magnetic bead incubation. . . . . .10 min
- Initial dry-down: . . . . .approximately 40 to 50 min
- First EtOH dry-down:. . . . . .approximately 10 to 15 min
- Final EtOH dry-down: . . . . .10 min
- Resuspend beads in Buffer EB on Jitterbug . . . . .10 min
- Elution on vacuum manifold: . . . . .approximately 5 to 15 min
- Final elution on centrifuge:. . . . . .5 min
- Total time for this stage: . . . . .approximately 2.5 to 3 hr

Input Required from Previous Stage

The input required is:

Quantity	Item
3 plates	Sty PCR product
4 plates	Nsp PCR product

Equipment and Consumables Required

The following equipment and materials are required to perform this stage.

Table 5.35 Equipment and Consumables Required for *Stage 7: PCR Product Purification Using a Millipore Filter Plate*

Quantity	Item
1	Jitterbug
As needed	Kimwipes
1	Pipet, 12-channel P20
1	Pipet, 12-channel P200
1	Pipet, 12-channel P1200
1	Pipet, serological
As needed	Pipet tips for pipets listed above; full racks
1	Plate, 96-well PCR
1	Plate centrifuge with deep-well capacity (54mm H x 160g)
1	Plate, storage, 2.4 mL deep well (referred to as the *pooling plate*)
1	Plate, elution catch, 96-well V-bottom

Table 5.35 Equipment and Consumables Required for *Stage 7: PCR Product Purification Using a Millipore Filter Plate*

Quantity	Item
1	Plate, Multiscreen Deep Well (Millipore, P/N MDRLN0410)
7	Plate holders
As required	Plate seal**
1	Solution basin, 100 mL or larger
1 roll	Tape, lab
1	Vacuum Manifold, Millipore
1	Vortexer
** **IMPORTANT** Use only the PCR plate, adhesive film and thermal cyclers listed on page 141.	

Reagents Required

The following reagents are required for this stage.

Table 5.36 Reagents Required for *Stage 7: PCR Product Purification Using a Millipore Filter Plate*

Volume Required for 96 Samples	Reagent
6 mL	Elution Buffer (Buffer EB)
200 mL	75% EtOH (ACS-grade ethanol diluted to 75% using AccuGENE water)
100 mL	Magnetic Beads (AMPure or SNPClean)

Important Information About This Stage

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

CAUTION: Do not overdry the magnetic beads during the vacuum steps. Overdrying may inhibit elution of the purified DNA.

After adding EtOH to the wells (Step 5 on page 205), the first vacuum step should not exceed approximately 20 min.

The final EtOH vacuum step is 10 min only (Step 8 on page 205). Do not exceed 10 min.

All of the liquid in each well should be pulled through the filter. Although the beads may still be moist, there should be no standing liquid on top of the beads. The wells will appear dull (matte) – not shiny.

If any wells are clogged, do not continue filtering. Proceed with the protocol for the samples that have been successfully purified and eluted. Repeat the experiment for the samples in the clogged wells.

IMPORTANT:

- **Bring the Buffer EB and 75% EtOH to room temperature prior to use.**
- **The storage temperature for the magnetic beads is 4° C (refrigerator).**
- **To avoid cross-contamination, pipet very carefully when pooling the PCR reactions into the deep-well plate.**
- **Maintain the vacuum between 20—24 in Hg (do not exceed 24 in Hg).**
- **Inspect the vacuum manifold for salt buildup after each use, and clean regularly. Refer to Chapter 9 for cleaning instructions.**

Prepare the 75% EtOH

Dilute ACS-grade or equivalent ethanol to 75% using AccuGENE water.

Recipe for 75% EtOH

In a 1 L measuring cylinder:

1. Pour 750 mL 100% EtOH
2. Add 250 mL AccuGENE molecular biology grade water.
3. Transfer to a 1 L bottle and mix well.
4. Seal tightly and store at room temperature.

Prepare the Reagents

Allow the Buffer EB to warm to room temperature prior to use.

Prepare the Vacuum Manifold

To prepare the manifold:

1. Connect the manifold and regulator to a suitable vacuum source able to maintain 20 to 24 in Hg.

   Leave the vacuum turned off at this time.
2. Inspect the manifold for salt and other contaminants and clean if necessary.
3. Place the vacuum flask trap below the level of the manifold.
4. Place the standard collar on the manifold.

IMPORTANT: Inspect the vacuum manifold for salt buildup before each use. Clean the manifold regularly. Refer to Chapter 9 for cleaning instructions.

If the flask trap is not placed below the level of the manifold, some solution may splash back and adhere to the bottom of the filter plate.

Pool the PCR Products

CAUTION: Be very careful when pooling PCR products. Avoid cross-contaminating neighboring wells with small droplets.

To pool the PCR products:

1. If the PCR products are:
   - Frozen, thaw to room temperature on the bench top in plate holders.
   - On thermal cyclers, remove them now.
2. Vortex the center of each plate at high speed for 3 sec.
3. Spin down each plate at 2000 rpm for 30 sec.
4. Place each PCR plate in a plate holder on the bench top.
5. Place a deep well pooling plate on the bench top.
6. On each PCR plate, cut the seal between each row so that it can be removed one row at a time.
7. Using a 12-channel P200 pipet set to 110 µL:

   A. Remove the seal to expose row A only on each PCR plate.

B. Transfer the reactions from row A of each PCR plate to the corresponding wells of row A on the pooling plate (Table 5.37 below and Figure 5.7 on page 202).

C. Change your pipet tips.

Change pipet tips after the PCR product from the same row of each PCR plate has been pooled on the pooling plate.

D. Remove the seal from each PCR plate to expose the next row.

E. Transfer each reaction from the same row of each PCR plate to the corresponding row and wells of the pooling plate.

F. Repeat steps C., D. and E. until all of the reactions from each PCR plate are pooled on the pooling plate.

8. When finished, look at the wells of each PCR plate to ensure that all of the product has been transferred and pooled.

Table 5.37 Pooled Sty and Nsp PCR Products

Sty PCR plates (3):	100 µL from each well	= 300 µL/well
Nsp PCR Plate (4):	100 µL from each well	= 400 µL/well
Total Volume Each Well of Pooling Plate		**= 700 µL/well**

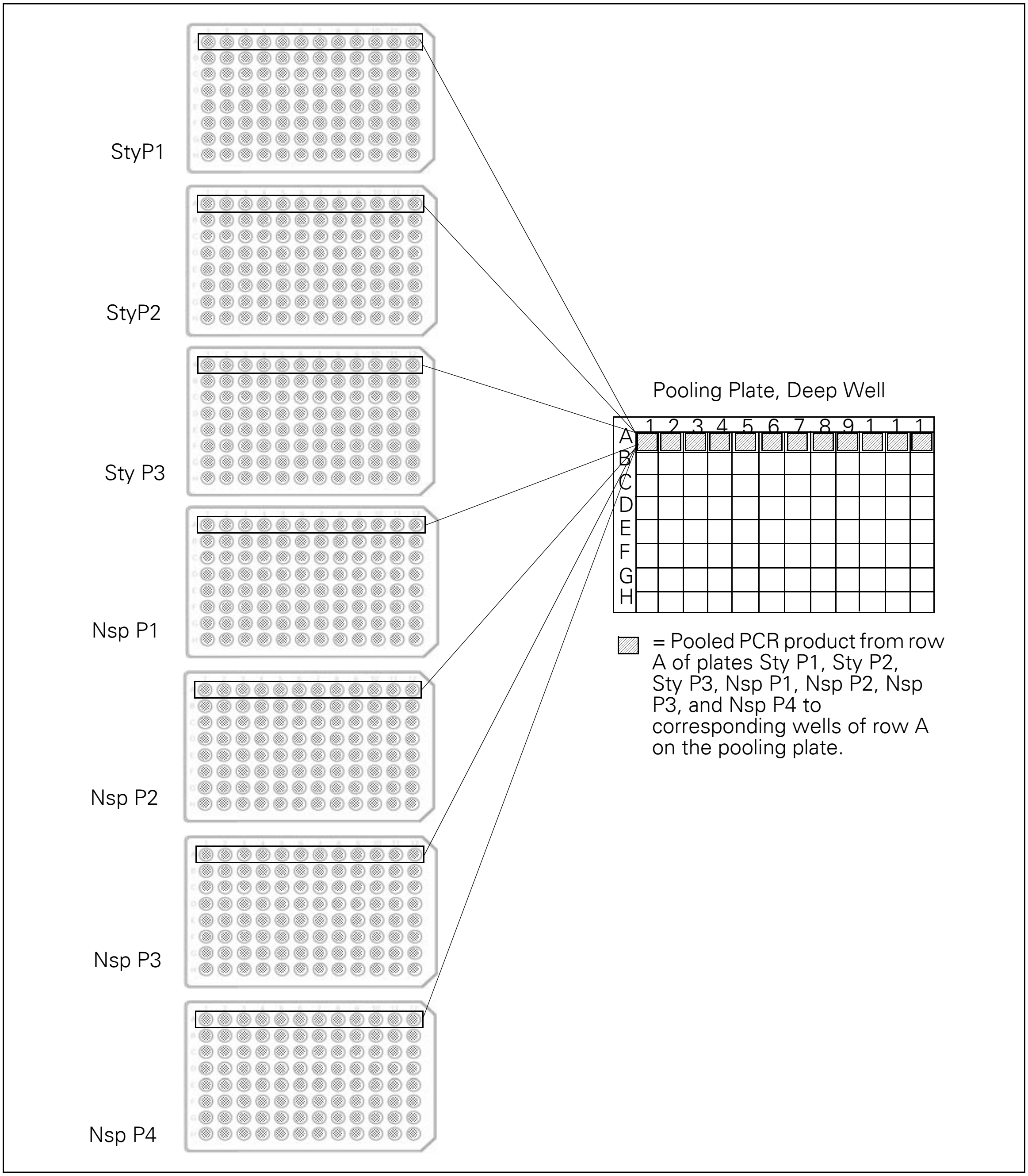


Figure 5.7 Pooling Sty and Nsp PCR Products on a Deep Well Pooling Plate

Purify the Pooled PCR products

Add Magnetic Beads and Incubate

During incubation, the DNA binds to the magnetic beads.

To add magnetic beads and incubate:

1. Mix the magnetic bead stock very well by vigorously shaking the bottle.

   Beads will settle overnight. Examine the bottom of the bottle and ensure that the solution appears homogenous.

2. Pour or pipet 100 mL of magnetic beads to a solution basin.

   1 mL of magnetic beads is required for each reaction. You can add in multiple batches if the solution basin is not large enough.

3. Using a manual (not electronic) 12-channel P1200 pipet:

   A. Add 1.0 mL of magnetic beads to each well of pooled PCR product.

   B. Mix well by pipetting up and down 5 times using the following technique:

   Mixing Technique:

   1) Depress the plunger and place the pipet tips into the top of the solution.
   2) Move the pipet tips down – aspirating at the same time – until the tips are near the bottom of each well.
   3) Raise the tips out of the solution.
   4) Place the pipet tips against the wall of each well just above each reaction, and carefully dispense the solution.

   ! IMPORTANT: The solution is viscous and sticky. Pipet carefully to ensure that you aspirate and dispense 1 mL.

   Thorough mixing is critical to ensure that the PCR products bind to the beads.

   5) Change pipet tips for each row.

4. Cover the plate to protect the samples from environmental contaminants and allow to incubate at room temperature for 10 minutes.

   You can use the lid from a pipet tip box to cover the wells.

Transfer Reactions to a Filter Plate

To transfer the reactions to a filter plate:

1. Place the filter plate on the standard collar on the vacuum manifold (Figure 5.8).
2. Using a 12-channel P1200 pipet, transfer each reaction from the pooling plate to the corresponding row and well of the filter plate.

> **!** **IMPORTANT: You will need to pipet twice to transfer all of the solution from each well to the filter plate. The solution is viscous and sticky, so check to ensure that all of it has been transferred.**

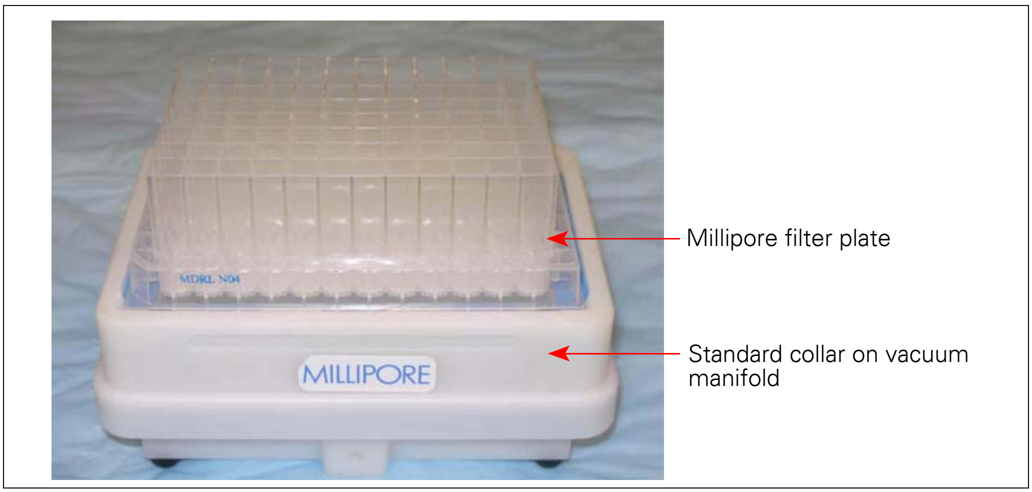


Figure 5.8 Millipore filter plate on standard collar

Purify the Reactions

To purify the reactions:

1. Turn on the vacuum to 20 to 24 in Hg and check the seals.

   Do not exceed 24 in Hg. Adjust the leak valve if necessary.
2. Cover the plate to protect it from environmental contaminants.
3. Run the vacuum until all of the liquid has been pulled through the filter (approximately 40 to 50 min), then turn off the vacuum.
4. Examine each well.

   There should be no standing liquid in any well, and the wells should appear dull (matte). Wet wells will look shiny.

   If any of the wells are still wet, put the plate back on the vacuum and continue filtering for up to 10 min (total ≤ 60 min).

5. Using a 12-channel P1200 set to 900 µL:
   - **A.** Add 900 µL of 75% EtOH to each reaction.
   - **B.** Turn the vacuum on to 20 to 24 in Hg.
   - **C.** Run the vacuum for approximately 1–2 min (or until the volume in the wells begins to decrease).
   - **D.** Add another 900 µL of 75% EtOH to each reaction (for a total of 1.8 mL EtOH).
   - **E.** Cover the plate.
   - **F.** Run the vacuum until all of the liquid has been pulled through the filter (approximately 10 to 15 min), then turn off the vacuum.
6. Examine each well.

   Again, there should be no standing liquid in any well, and the wells should appear dull (matte). Wet wells will look shiny.

   If any of the wells are still wet, put the plate back on the vacuum and continue filtering for up to 5 min (total ≤20 min; see the Caution on page 199).
7. Remove any excess EtOH as follows:
   - **A.** Blot the bottom of the plate on Kimwipes.
   - **B.** Wipe the bottom of the plate with a clean Kimwipe.
8. Return the filter plate to the manifold and turn on the vacuum for an additional 10 min ONLY.

   Do not exceed 10 min. Less than 10 min is OK if no excess ethanol is present in the wells or on the underside of the filter plate.

   NOTE: The purpose of this step is to remove excess EtOH so that it is not carried over into the eluate. Ten minutes is sufficient for this purpose. Leaving the vacuum on for more than 10 minutes may over-dry the beads which may inhibit elution of the purified DNA.
9. Turn off the vacuum, and blot the bottom of the plate on Kimwipes to remove any remaining EtOH.

Elute the Purified Reactions

To elute the purified reactions:

1. Attach the elution catch plate to the bottom of the filter plate using 2 strips of lab tape.

   The filter and elution plate assembly is now referred to as the *plate stack* (Figure 5.9).

IMPORTANT: **Do not completely seal with tape. Product will not elute if sealed.**

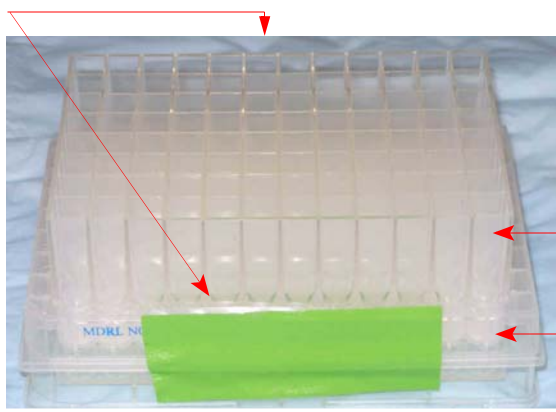


Figure 5.9 Attaching the Elution Catch Plate to the Filter Plate

2. Pour or pipet 6 mL of Buffer EB to a solution basin.
3. Using a 12-channel P200 pipet, add 55 µL of Buffer EB to each well.

   For accurate pipetting, pre-wet pipet tips with EB before dispensing. Dispense as close to the beads as possible without touching them. Buffer EB should be applied directly on top of the beads (Figure 5.11 on page 207).

NOTE: **If the volume of eluate in Step 13 on page 208 is < 47 µL, increase the amount of Buffer EB used in this step the next time you perform the protocol. You can increase from 55 to 60 µL (total not to exceed 60 µL).**

4. Tap the plate stack to move all Buffer EB onto the filter at the bottom of the wells.
5. Using an adhesive film, tightly seal the filter plate.
6. Place the plate stack on a Jitterbug for 10 minutes at *setting 5*.

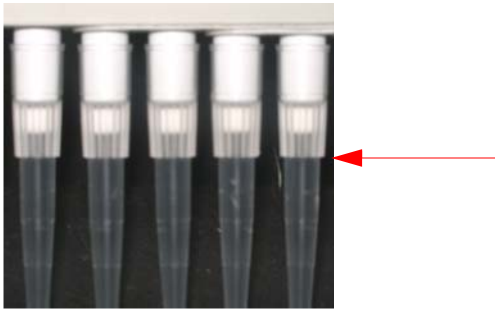


Figure 5.10 Ridge on Rainin pipet tips

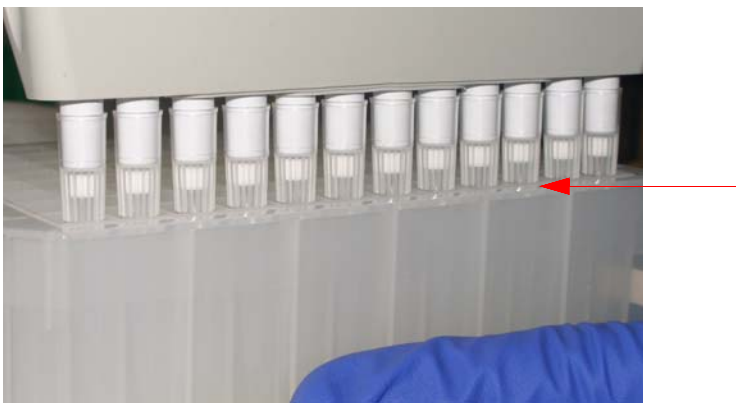


Figure 5.11 Adding Buffer EB to Reactions on the Filter Plate

7. Inspect each well to verify that the beads are thoroughly resuspended.

The beads must be thoroughly resuspended in Buffer EB so that the DNA can come off the beads.

8. Remove the plate stack from the Jitterbug and remove the adhesive seal.

9. Continue elution on the vacuum manifold as follows:

A. Remove the standard collar from the manifold.

B. Place the plate stack inside the manifold.

C. Place the standard collar around the plate stack (Figure 5.12 on page 208).

D. Turn the vacuum on to 20 to 24 in Hg and ensure that a seal has been formed between the collar and the base of the manifold.

E. Cover the plate stack to protect it from environmental contaminants.

F. Run the vacuum until all of the liquid has been pulled through the filter (approximately 5 to 15 min).

G. Turn off the vacuum.

Figure 5.12 Plate stack on vacuum manifold with standard collar

10. Examine each well.

Again, there should be no standing liquid in any well, and the wells should appear dull (matte). Wet wells will look shiny.

If any of the wells are still wet, continue filtering for up to 15 additional min.

11. Seal the plate stack with an adhesive film, and spin it down at room temperature for 5 min at 1400 rcf.

Use the following formula to convert relative centrifugal force (rcf) to revolutions per minute (rpm):

rpm = 1000 × square root(rcf/1.12r)

The radius, *r*, is equal to the distance in millimeters between the axis of rotation of the centrifuge and the bottom of the plate bucket.

For example, on the Eppendorf 5804R, spinning at 3100 rpm gives an rcf of 1400 (assuming r = 133 mm).

12. Remove the elution catch plate from the filter plate.

13. Transfer the samples from the elution catch plate to two fresh PCR plates — 48 samples per plate — using a 12-channel P200 pipet as follows:

A. Label two fresh PCR plate — one FLH 1, the other FLH 2 (Figure 5.13).

(FLH = Fragmentation Label Hyb)

B. Transfer 45 µL of eluate from each well of rows A through D of the elution catch plate to the corresponding rows and wells of plate FLH 1.

C. Transfer 45 µL of eluate from each well of rows E through H of the elution catch plate to the corresponding wells of rows A through D of plate FLH 2.

NOTE: If the volume of eluate is < 47 µL, increase the amount of Buffer EB used for elution the next time you perform the protocol. You can increase from 55 to 50 µL (total not to exceed 60 µL).

See also the Caution on page 199, and page 279 of Chapter 8, *Troubleshooting* for more information.

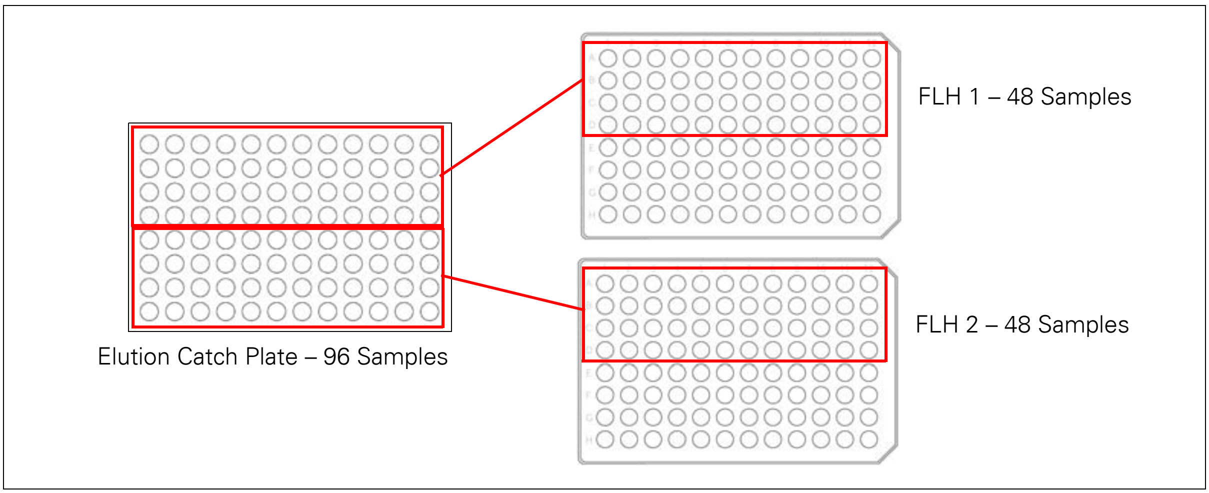


Figure 5.13 Transferring Samples from the Elution Catch Plate to Two Fresh PCR Plates

What To Do Next

Take an OD measurement using 2 µL of the remaining eluate as described under *Stage 8: Quantitation* on page 210.

Then do one of the following:

- If following the recommended workflow (Figure 5.1 on page 137) seal plates FHL 1 and FLH 2 and store them at –20 °C.
- Proceed directly to *Stage 9: Fragmentation* on page 219.

Stage 8: Quantitation

About this Stage

During this stage, you will prepare one dilution of each PCR product in optical plates. You will then quantitate the diluted PCR products.

Location and Duration

- Main Lab
- Hands-on time: 40 minutes

Input Required from Previous Stage

Input required from *Stage 7: PCR Product Purification Using a Millipore Filter Plate* is:

Quantity	Item
1	Plate of remaining purified PCR products

Equipment and Consumables Required

The following equipment and consumables are required for this stage. Refer to Appendix A of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide* for vendor and part number information.

IMPORTANT: **** Use only the 96-well plate, adhesive seals and thermal cyclers listed in Table 5.1 and Table 5.2 on page 141.**

Table 5.38 Equipment and Consumables Required for *Stage 8: Quantitation*

Quantity	Item
1	Marker, fine point, permanent
1	Pipet, single channel P20
1	Pipet, single channel P200
1	Pipet, 12-channel P20 (accurate to within ± 5%)
1	Pipet, 12-channel P200
As needed	Pipet tips for pipets listed above; full racks
2	Plate, optical For example, the Greiner UV Star Transparent, 96-well. Use the optical plate recommended for use with your plate reader.
1	Plate centrifuge
6	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 of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide* for vendor and part number information. The amounts listed are sufficient to process 96 reactions.

Table 5.39 Reagents Required for *Stage 8: Quantitation*

Quantity	Reagent
30 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 accuracy of the OD measurement is critical. Carefully follow this procedure and be sure the OD measurement is within the quantitative linear range of the instrument (0.2 to 2.0 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.**

  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 the following on the bench top:
   - Optical plates
   - Solution basin
   - AccuGENE water
2. Label one plate *OP1*; the other plate *OP2*.
3. If the purified PCR products to be used for quantitation were frozen, allow the plate to thaw in a cooling chamber on ice.
4. Spin down the plate at 2000 rpm for 30 sec, and place on the bench top.

Prepare Diluted Aliquots of Purified Sample

> **IMPORTANT: One row of wells on the optical plate are used as blanks and contain AccuGENE water only.**
>
> **The 12-channel P20 pipet must be accurate to within ± 5%.**

To prepare diluted aliquots of the purified samples:

1. Pour 30 mL of room temperature AccuGENE water into the solution basin.
2. Using a 12-channel P200 pipet aliquot 198 µL of water to each well in rows A through E of each optical plate.
3. Using a 12-channel P20 pipet:

   A. To optical plate OP1:

   1) Transfer 2 µL of each purified PCR product from rows A through D of the purified sample plate to the corresponding rows and wells of optical plate OP1 (see Figure 5.14 on page 214).

      Row E remains water only and will serve as a blank.

      > **NOTE: If a particular well(s) contain less than 2 µL of purified PCR product, see Chapter 8 of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide* for instructions.**

   2) Pipet up and down 2 times after each transfer to ensure that all of the product is dispensed.
   3) Examine the pipet tips and aliquots before and after each dispense to ensure that exactly 2 µL has been transferred.

      The result is a 100-fold dilution.
   4) Set a 12-channel P200 pipet to 180 µL.
   5) Mix each sample by pipetting up and down 3 times.

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

   B. To optical plate OP2:

   1) Transfer 2 µL of each purified PCR product from rows E through H of the purified sample plate to rows A through D of optical plate OP2 (see Figure 5.14 on page 214).

      Row E remains water only and will serve as a blank.

      > **NOTE: If a particular well(s) contain less than 2 µL of purified PCR product, see Chapter 8 of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide* for instructions.**

2) Pipet up and down 2 times after each transfer to ensure that all of the product is dispensed.

3) Examine the pipet tips and aliquots before and after each dispense to ensure that exactly 2 µL has been transferred.

   The result is a 100-fold dilution.

4) Set a 12-channel P200 pipet to 180 µL.

5) Mix each sample by pipetting up and down 3 times.

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

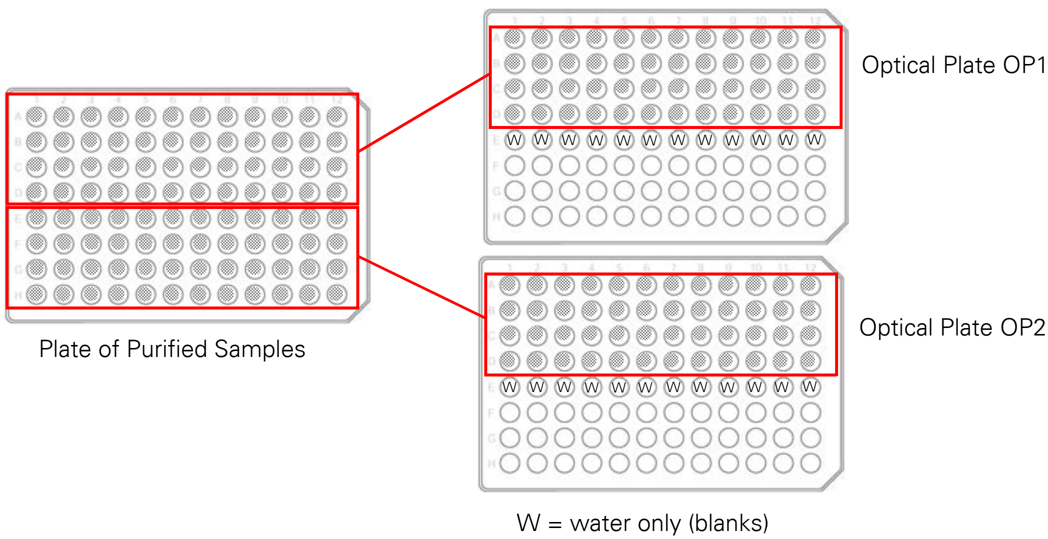


Figure 5.14 Loading the Optical Plate with Purified Sample and Water Blanks

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* on page 215.

2. Determine the OD260 measurement for the water blank and average.

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

   A. Take 1 OD reading for every sample.

      OD = (sample OD) – (average water blank OD)

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

Sample concentration in µg/µL = OD X 0.05 ug/uL X 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.

Sample OD

A typical sample OD is 0.9 to 1.2. This OD range is equivalent to a final PCR product concentration of 4.5 to 6.0 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 5.40 PROBLEM: Sample OD is greater than 1.2 (6 µg/µL)

If the sample OD is greater than 1.2 (calculated concentration greater than 6 µg/µL), a problem exists with either the elution of PCR products or the OD reading. The limit on PCR yield is approximately 6 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 55 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. • Pipets may require calibration.

Table 5.40 PROBLEM: Sample OD is greater than 1.2 (6 µg/µL)

- 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.

Table 5.41 PROBLEM: Sample OD is Less Than 0.9 (4.5 µg/µL)

If the sample OD is less than 0.9 (calculated concentration less than 4.5 µg/µL), a problem may exist 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.

  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.
- Pipet 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 or Tetrad 2).
- 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.

Table 5.41 (Continued) PROBLEM: Sample OD is Less Than 0.9 (4.5 μg/μL)

NOTE: The Genome-Wide SNP 5.0/6.0 Assay reaction amplifies a size range of fragments that represents 30% of the genome. The Genome-Wide Human SNP Array 6.0 is 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.
Troubleshooting Possible Problems with the Elution or OD Readings – possible causes include: • The purified PCR product was eluted in a volume greater than 55 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. • Pipets 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.

Table 5.42 PROBLEM: OD260/OD280 ratio is not between 1.8 and 2.0

Possible causes include: • The PCR product may be not be sufficiently purified. Ensure the vacuum manifold is working properly. • An error may have been made while taking the OD readings. • The PCR product may not have been adequately washed. Check the 75% EtOH wash solution.

Table 5.43 PROBLEM: The OD320 measurement is significantly larger than zero (0 ± 0.005)

Possible causes include: • Magnetic beads may have been carried over into purified sample. • Precipitate may be present in the eluted samples. • There may be defects in the OD plate. • Air bubbles in the OD plate or in solutions.

What To Do Next

Do one of the following:

- Proceed immediately to the next step.
- If following the recommended workflow (Figure 5.1 on page 137), seal plates FHL 1 and FLH 2 and store them at –20 °C.

Stage 9: Fragmentation

About this Stage

The remaining stages of this protocol — fragmentation, labeling, and hybridization — are performed twice over a two day period, 48 samples at a time (see *Workflow Recommendations* on page 137).

During fragmentation, the purified PCR products are 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 GW5.0/6.0 Fragment program.

Once the program is finished, you will verify fragmentation by running 1.5 µL of each reaction on a 4% TBE gel or an E-Gel *48* 4% agarose gel.

Location and Duration

- Main Lab
- Hands-on time: 30 minutes
- GW5.0/6.0 Fragment thermal cycler program time: 1 hour

Input Required from Previous Stage

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

Quantity	Item
1	Plate of 48 quantitated PCR products in a cooling chamber on ice

Equipment and Consumables Required

The following equipment and consumables are required for this stage. Refer to Appendix A of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide* for vendor and part number information.

! **IMPORTANT: ** Use only the 96-well plate, adhesive seals and thermal cyclers listed in Table 5.1 and Table 5.2 on page 141.**

Table 5.44 Equipment and Consumables Required for *Stage 9: 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	Pipet, single channel P20
1	Pipet, single channel P100
1	Pipet, single channel P1000
1	Pipet, 12-channel P20 (accurate to within ± 5%)
As needed	Pipet tips for pipets listed above; full racks
1	Plate, 96-well PCR**
1	Plate centrifuge
2	Plate seal**
1	Thermal cycler**
2	Tube, Eppendorf 1.5 mL
2	Tubes, strip of 12
1	Vortexer

Reagents Required

The following reagents are required for this stage. Refer to Appendix A of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide* for vendor and part number information. The amounts listed are sufficient to process 48 samples.

Table 5.45 Reagents Required for *Stage 9: Fragmentation*

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

Gels and Related Materials Required

Verifying the fragmentation reaction is required for this stage. You can use the following gels and related materials, or E-Gels as described in Appendix C of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide*. The amounts listed are sufficient to process 48 samples.

Refer to Appendix A of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide* for vendor and part number information.

Table 5.46 Gels and Related Materials Required

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:

- **The degree of fragmentation is critical. Perform this stage carefully to ensure uniform, reproducible fragmentation.**
- **Use only the AccuGENE water listed in Appendix A of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide*. Using in-house ddH$_2$O or other water can negatively affect your results. The reaction in *Stage 9: Fragmentation* is particularly sensitive to pH and metal ion contamination.**
- **All additions, dilutions and mixing must be performed on ice. Be sure to allow all reagents to reach equilibrium before adding new fluid.**

About the Fragmentation Reagent

- This reagent is **extremely temperature sensitive** and rapidly loses activity at higher temperatures. To avoid loss of activity:
  - Handle the tube by the cap only. Do not touch the sides of the tube as the heat from your fingers will raise the reagent temperature.
  - Dilute 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.
  - Spin down so that the contents of the tube are uniform.
  - Perform these steps rapidly and without interruption.
- This reagent is **sticky**, and may adhere to the walls of some microfuge tubes and 96-well plates.
- This reagent is **viscous** and requires extra care when pipetting. Follow these guidelines:
  - Pipet slowly to allow enough time for the correct volume of solution to enter the pipet tip.
  - Avoid excess solution on the outside of the pipet tip.

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:

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. Label and place the following in the cooling chamber on ice:
   - Two strips of 12 tubes each: one labeled *Buffer* and one labeled *FR*.
   - One 1.5 mL Eppendorf tube labeled *Frag MM*.
5. Place the plate of 48 purified PCR products in the cooling chamber on ice.

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 additions in this procedure must be performed on ice.

To prepare the samples for Fragmentation:

1. Aliquot 28 µL of 10X Fragmentation Buffer to each tube of the strip tubes labeled Buffer.
2. Using a 12-channel P20 pipet, add 5 µL of Fragmentation Buffer to each sample in the 96-well reaction plate.

   Check your pipet 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.

To dilute the Fragmentation Reagent:

1. Read the Fragmentation Reagent tube label and record the concentration.
2. Dilute the Fragmentation Reagent to 0.1 U/µL as described below using the appropriate recipe from Table 5.47:

Table 5.47 Dilution Recipes for the Fragmentation Reagent

Reagent	Fragmentation Reagent Concentration				
	2 U/µL	2.25 U/µL	2.5 U/µL	2.75 U/µL	3 U/µL
AccuGENE water	306 µL	308 µL	309.6 µL	310.9 µL	312 µL
10X Fragmentation Buffer	36 µL	36 µL	36 µL	36 µL	36 µL
Fragmentation Reagent	18 µL	16 µL	14.4 µL	13.1 µL	12 µL
Total (enough for 48 samples)	**360 µL**	**360 µL**	**360 µL**	**360 µL**	**360 µL**

A. To the 1.5 mL Eppendorf tube on ice:
   1) Add the AccuGENE water and Fragmentation Buffer.
   2) Allow to cool on ice for 5 minutes.

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.

3. 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 28 µL of diluted Fragmentation Reagent to each tube of the strip tubes labeled *FR*.

   Avoid introducing air bubbles at the bottom of the strip tubes to ensure the accurate transfer of 5 µL diluted DNA to each sample.
2. Using a 12-channel P20 pipet, add 5 µL of diluted Fragmentation Reagent to each sample.

   Do not pipet up and down.

Sample with Fragmentation Buffer	50 µL
Diluted Fragmentation Reagent (0.1 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 GW5.0/6.0 Fragment program.

Table 5.48 GW5.0/6.0 Fragment Thermal Cycler Program

GW5.0/6.0 Fragment Program	
Temperature	**Time**
37ºC	35 minutes
95ºC	15 minutes
4ºC	Hold

7. Discard any remaining diluted Fragmentation Reagent.

   Diluted Fragmentation Reagent should never be reused.

What To Do Next

Proceed directly to *Stage 10: Labeling* on page 228.

Concurrently, check the fragmentation reaction by running gels as described under *Check the Fragmentation Reaction* on page 227.

Check the Fragmentation Reaction

The instructions below are for running 4% TBE gels. For information on running E-Gel *48* 4% agarose gels, refer to Appendix C of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide*.

To ensure that fragmentation was successful:

1. When the GW5.0/6.0 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 1.5 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 5.15 below.

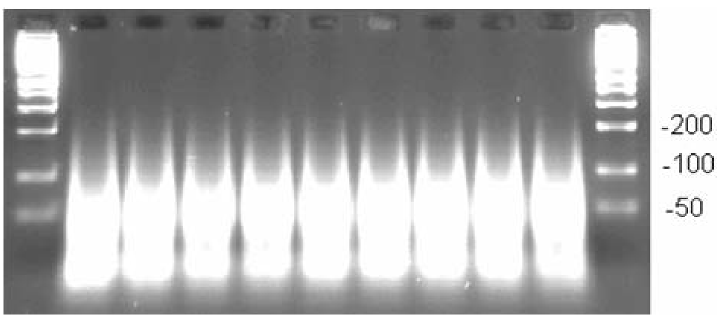


Figure 5.15 Typical example of fragmented PCR products run on 4% TBE agarose gel at 120V for 30 minutes to 1 hour. Average fragment size is < 180 bp.

Stage 10: Labeling

The fragmentation, labeling, and hybridization stages of this protocol are performed twice over a two day period, 48 samples at a time (see *Workflow Recommendations* on page 137).

About this Stage

During this stage, 48 fragmented samples are labeled using the 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 GW5.0/6.0 Label program.

Location and Duration

- Main Lab
- Hands-on time: 30 minutes
- GW5.0/6.0 Label thermal cycler program time: 4.25 hours

Input Required from Previous Stage

The input required from *Stage 9: Fragmentation* is:

Quantity	Item
1	Plate of 48 fragmented samples

Equipment and Consumables Required

The following equipment and consumables are required for this stage. Refer to Appendix A of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide* for vendor and part number information.

! **IMPORTANT:** ** Use only the 96-well plate, adhesive seals and thermal cyclers listed in Table 5.1 and Table 5.2 on page 141.**

Table 5.49 Equipment and Consumables Required for *Stage 10: 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	Pipet, single channel P200
1	Pipet, single channel P1000
1	Pipet, 12-channel P20 (accurate to within ± 5%)
As needed	Pipet tips for pipets listed above; full racks
1	Plate centrifuge
1	Plate seal**
1	Thermal cycler**
1	Tube, centrifuge 15 mL
1	Tubes, 12-strip, 0.2 Ml
1	Vortexer

Reagents Required

The following reagents are required for this stage. Refer to Appendix A of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide* for vendor and part number information. The amounts listed are sufficient to process 48 samples.

Table 5.50 Reagents Required for *Stage 10: Labeling*

Quantity	Reagent
1 vial	DNA Labeling Reagent (30 mM)
1 vial	Terminal Deoxynucleotidyl Transferase (TdT; 30 U/μL)
1 vial	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 GW5.0/6.0 Label thermal cycler program.

Prepare the Reagents, Consumables and Other Components

Thaw Reagents

Thaw the following reagents on ice:

- 5X TdT Buffer
- 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 one 1.5 mL centrifuge tube *MM*, and place on ice.
4. Label one strip of 12 tubes *MM* and place in the cooling chamber.
5. Place the plate of fragmented samples in the cooling chamber.

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 1.5 mL centrifuge tube on ice using the volumes shown in Table 5.51:
   - 5X TdT Buffer
   - 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 5.51 Labeling Master Mix

Reagent	1 Sample	48 Samples (15% extra)
TdT Buffer (5X)	14 µL	772.8 µL
DNA Labeling Reagent (30 mM)	2 µL	110.4 µL
TdT enzyme (30 U/µL)	3.5 µL	193.2 µL
Total	**19.5 µL**	**1076.4 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 89 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 pipet, aliquot 19.5 µL of Labeling Master Mix to each sample.

B. Pipet 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 73 µL.

Fragmented DNA (less 1.5 µL for gel analysis)	53.5 µL
Labeling Mix	19.5 µL
Total	**73 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 GW5.0/6.0 Label program.

Table 5.52 GW5.0/6.0 Label Thermal Cycler Program

GW5.0/6.0 Label Program	
Temperature	**Time**
37ºC	4 hours
95ºC	15 minutes
4ºC	Hold

7. When the GW5.0/6.0 Label program is finished:

A. Remove the plate from the thermal cycler.

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

What To 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 11: Target Hybridization

The fragmentation, labeling, and hybridization stages of this protocol are performed twice over a two day period, 48 samples at a time (see *Workflow Recommendations* on page 137).

About this Stage

The target hybridization stage is performed twice — 48 samples at time. First, you will prepare a Hybridization Master Mix and add the mix to each sample. Then, you will denature the samples on a thermal cycler. Two approaches to denaturation 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 240.

- **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, MJ Tetrad PTC-225, or MJ Tetrad 2 Thermal Cycler* on page 243.

After denaturation, you will load each sample onto a Genome-Wide Human SNP Array 6.0 – one sample per array. The arrays are then placed into a hybridization oven that has been preheated to 50 °C. Samples are left to hybridize for 16 to 18 hours.

NOTE: **Two operators are required for each method.**

Location and Duration

- Main Lab
- Hands-on time: 45 minutes
- Hybridization time: 16 to 18 hours

Input Required from Previous Stage

The input required from *Stage 10: 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 of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide* for vendor and part number information.

IMPORTANT: Increased variability in Genome-Wide SNP 5.0/6.0 Assay 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.

The following table lists the equipment and consumables required.

Table 5.53 Equipment and Consumables Required for *Stage 11: Target Hybridization*

Quantity	Item
1	Cooling chamber, chilled to 4 °C (do not freeze)
96	Genome-Wide Human SNP Array 6.0 (one array per sample)
2	GeneChip® Hybridization Oven 640
1	Ice bucket, filled with ice
1	Pipet, single channel P200
1	Pipet, single channel P1000
As needed	Pipet tips for pipets 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 233.)
2 per array	Tough-Spots®
1	Tube, centrifuge 50 mL
1	Vortexer

! **IMPORTANT:** ** Use only the 96-well plate, adhesive seals and thermal cyclers listed in Table 5.1 and Table 5.2 on page 141.

Reagents Required

The following reagents are required for this stage. Refer to Appendix A of the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide* for vendor and part number information. Volumes listed are sufficient to process 96 samples.

Table 5.54 Reagents Required for *Stage 11: Target Hybridization*

Quantity	Reagent
10 mL	Denhardt's Solution (50X)
3 mL	DMSO (100%)
1 mL	EDTA (0.5 M)
2 mL	Herring Sperm DNA (HSDNA; 10 mg/mL)
1 mL	Human Cot-1 DNA® (1 mg/mL)
80 g	MES Hydrate SigmaUltra
200 g	MES Sodium Salt
32 mL	Tetramethyl Ammonium Chloride (TMACL; 5M)
20 mL	Tween-20, 10%
500 µL	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.**
- **It is critical that the samples remain on a thermal cycler 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 (see *About this Stage* on page 233).**
- **About DMSO:**

  When adding to the Hybridization Master Mix, pipet 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 equilibrate the arrays 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 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 950 mL.
3. Test the pH.
   The pH should be between 6.5 and 6.7.
4. Adjust the pH so it falls between 6.5 and 6.7.
5. Adjust the volume to 1000 mL.
6. Filter the solution through a 0.2 µm filter.
7. 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 50 °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 the manufacturer's 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 used wells on the plate are cut into 2 strips of 24 wells each.

Preheat the Thermal Cycler Lid

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

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 pipet tip into the upper right septum of each array.

> **!** **IMPORTANT:** **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 5.55.

   DMSO addition: pipet directly into the solution of other reagents. Avoid pipetting along the side of the tube.

2. Mix well.
3. Aliquot out 10.45 mL, and store the remainder at –20 °C for up to one week.

Table 5.55 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	15.4 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 at –20 °C.

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.

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 10.45 mL Hybridization Master Mix into a solution basin.
2. Using a 12-channel P200 pipet, add 190 µL of Hybridization Master Mix to each sample on the Label Plate.
   Total volume in each well is 263 µL.
3. Seal the plate tightly with adhesive film.

   IMPORTANT: It is critical to seal the plate tightly.

4. Vortex the center of the plate for 30 sec.
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 GW5.0/6.0 Hyb program.

Table 5.56 GW5.0/6.0 Hyb Thermal Cycler Program

GW5.0/6.0 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 pipet, 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 5.16).
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 48 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 50 °C, 60 rpm for 16 to 18 hours.

! **IMPORTANT: Allow the arrays to rotate in the hybridization ovens for 16 to 18 hours at 50 °C and 60 rpm. This temperature is optimized for this product, and should be stringently followed.**

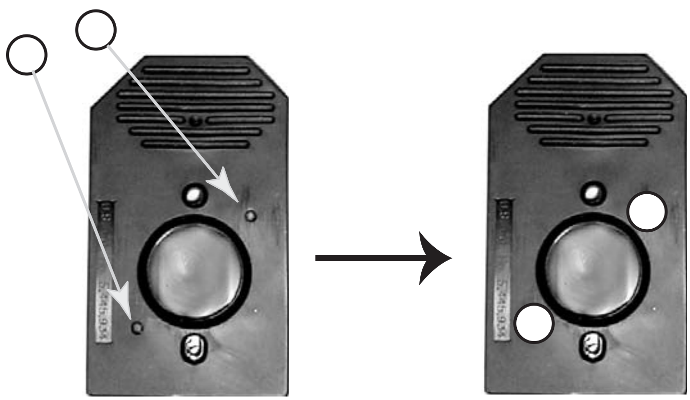


Figure 5.16 Applying Tough-Spots® to the array cartridge

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

For this method, you can use an:

- Applied Biosystems 2720 Thermal Cycler
- MJ Tetrad PTC-225 Thermal Cycler
- MJ Tetrad 2

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

   Total volume in each well is 263 µL.
3. Seal the plate tightly with adhesive film.

   ! IMPORTANT: It is critical to seal the plate tightly.
4. Vortex the center of the plate for 30 sec.
5. Cut the used wells into 2 strips of two rows each.
6. Put each strip of 24 samples into a plate 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 GW5.0/6.0 Hyb program.

Table 5.57 GW5.0/6.0 Hyb Thermal Cycler Program

GW5.0/6.0 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 pipet, 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 GW5.0/6.0 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 5.16).
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 48 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 50 °C, 60 rpm for 16 to 18 hours.

! IMPORTANT: Allow the arrays to rotate in the hybridization ovens for 16 to 18 hours at 50 °C and 60 rpm. This temperature is optimized for this product, and should be stringently followed.

Chapter 6

WASHING, STAINING AND SCANNING ARRAYS

This chapter describes how to wash, stain and scan Affymetrix® Genome-Wide Human SNP Array 6.0. The instrument you will use include the:

- Fluidics Station 450 to wash and stain arrays
- GeneChip® Scanner 3000 7G to scan arrays

Once the arrays are scanned, the array image (.dat file) is ready for analysis.

Equipment and Consumables Required

The following equipment and consumables are required for washing, staining and scanning arrays.

Table 6.1 Equipment and Consumables Required for Washing and Staining Arrays

Item	Vendor	Part Number
GeneChip® Scanner 3000 7G	Affymetrix	—
GeneChip® Fluidics Station 450	Affymetrix	—
One of the following instrument control applications: • Affymetrix GeneChip® Operating Software • Affymetrix GeneChip® Command Console	Affymetrix	—
Sterile, RNase-free, microcentrifuge vials, 1.5 mL	USA Scientific	1415-2600 (or equivalent)
Micropipettors, (P-2, P-20, P-200, P-1000)	Rainin Pipetman® (or equivalent)	—
Sterile-barrier pipet tips and non-barrier pipet tips	—	—
Tygon® Tubing, 0.04” inner diameter	Cole-Parmer	H-06418-04
Tough-Spots®, Label Dots	USA Scientific	9185-0000

Reagents Required

The following reagents are required for washing and staining arrays. These reagents are recommendations, and have been tested and evaluated by Affymetrix scientists. Information and part numbers listed are based on U.S. catalog information.

Table 6.2 Reagents Required for Washing and Staining Arrays

Reagent	Vendor	Part Number
AccuGENE® Molecular Biology-Grade Water, 1 L	Lonza	51200
Distilled water	Invitrogen	15230147
20X SSPE (3 M NaCl, 0.2 M NaH2PO4, 0.02 M EDTA)	Lonza	51214
Anti-streptavidin antibody (goat), biotinylated (reconstitute according to product instructions)	Vector Laboratories	BA-0500
R-Phycoerythrin Streptavidin	Molecular Probes	S-866
10% Surfact-Amps® 20 (Tween-20)	Pierce Chemical	28320
Bleach (5.25% Sodium Hypochlorite)	VWR Scientific	21899-504 (or equivalent)
Denhardt's Solution, 50X concentrate	Sigma-Aldrich	D2532
MES hydrate	Sigma-Aldrich	M5287
MES Sodium Salt	Sigma-Aldrich	M5057
5 M NaCl, RNase-free, DNase-free	Ambion	9760G

Reagent Preparation

Prepare the following buffers and antibody:

- Wash A (Non-Stringent Wash Buffer)
- Wash B (Stringent Wash Buffer)
- Anti-streptavidin Antibody (0.5 mg/mL)
- MES Stock Buffer
- Array Holding Buffer

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. Tightly seal the container 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.

Fluidics Station and Scanner Control Software

You will use one of the instrument control applications listed below to operate the fluidics station and the scanner. For more information on these applications, refer to the appropriate user's guide.

- Affymetrix GeneChip® Operating Software (GCOS)
  Affymetrix *GeneChip® Operating Software User's Guide*
- Affymetrix GeneChip® Command Console (AGCC)
  Affymetrix GeneChip® Command Console™ User's Guide

Register a New Experiment or Sample

To register a new experiment or sample:

- If using GCOS, register a new Experiment
- If using AGCC, register a new Sample

Prime the Fluidics Station

The Fluidics Station 450 is used to wash and stain the arrays; it is operated using either GCOS or AGCC software.

To prime the Fluidics Station:

1. Turn on the Fluidics Station.
2. Prime the Fluidics Station.
   - Select protocol **Prime_450** for each module
   - Intake buffer reservoir: use **Non-Stringent Wash Buffer**
   - Intake buffer reservoir B: use **Stringent Wash Buffer**

About 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

Wash and Stain Arrays

The staining protocol for mapping arrays is a three stage process:

1. A Streptavidin Phycoerythin (SAPE) stain.
2. An antibody amplification step.
3. A final stain with SAPE.

Once stained, each array is filled with Array Holding Buffer prior to scanning.

Prepare Arrays for Washing and Staining

To prepare the arrays for washing and staining:

1. After 16 to 18 hours of hybridization, remove the arrays from the oven.
2. Extract the hybridization cocktail from each array and transfer it to the corresponding well of a 96-well plate.

   Store on ice during the procedure, or at –80 °C for long-term storage.
3. Fill each array completely with 270 µL of Array Holding Buffer.

   See *Array Holding Buffer* on page 254 for buffer recipe.
4. Allow the arrays to equilibrate to room temperature before washing and staining.

NOTE: Arrays can be stored in the Array Holding Buffer at 4°C for up to 3 hours before proceeding with washing and staining. Equilibrate arrays to room temperature before washing and staining.

Prepare Buffers and Solutions

Prepare the following buffers and solutions (recipes follow). Volumes given are sufficient for one array. Mix well.

- Stain Buffer
- SAPE Stain Solution
- Antibody Stain Solution
- Array Holding Buffer

Stain Buffer

Mix well.

Table 6.3 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. Mix well. Do not freeze either concentrated SAPE or diluted SAPE stain solution.

Table 6.4 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	

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

Antibody Stain Solution

Mix well.

Table 6.5 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	

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

Array Holding Buffer

Mix well.

Table 6.6 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 1 mL 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.

Washing and Staining Arrays

Wash and Stain Protocol

The GenomeWideSNP6_450 protocol is an antibody amplification protocol for mapping targets (described in Table 6.7). Use it to wash and stain the Genome-Wide Human SNP Array 6.0.

Table 6.7 GenomeWideSNP6_450 protocol for the Fluidics Station 450

GenomeWideSNP6_450 Protocol for 49 Format (Standard) Arrays	
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 array for 10 min 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 array for 10 min in Antibody Stain Solution at 25°C
3rd Stain	Stain the array for 10 min 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

! **IMPORTANT: The wash and stain buffers are different from the GeneChip® expression buffers.**

Washing and Staining Arrays

To wash and stain the arrays:

1. Select the correct experiment (GCOS) or sample (AGCC) name.

   The Probe Array Type appears automatically.

2. Select the protocol **GenomeWideSNP6_450**.
3. Start the protocol and follow the instructions in the LCD on the fluidics station.

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

4. Insert an array into the designated module of the fluidics station while the cartridge lever is in the Down or Eject position.
5. When finished, verify that the cartridge lever is returned to the Up or Engaged position.
6. Remove any microcentrifuge vials remaining in the sample holders of the fluidics station module(s) being used.
7. When prompted to “Load Vials 1-2-3,” place the three vials into the sample holders 1, 2 and 3 on the fluidics station.
   A. Place one vial containing 600 µL Streptavidin Phycoerythrin (SAPE) stain solution mix in sample holder 1.
   B. Place one vial containing 600 µL anti-streptavidin biotinylated antibody stain solution in sample holder 2.
   C. Place one vial containing 1 mL Array Holding Buffer in sample holder 3.
   D. Press down on the needle lever to snap needles into position and to start the run.

   Once these steps are complete, the fluidics protocol begins. The Fluidics Station dialog box at the workstation terminal and the LCD window displays the status of the washing and staining steps.
8. When staining is finished, remove the microcentrifuge vials containing stain and replace with three empty microcentrifuge vials as prompted.
9. Remove the arrays from the fluidics station by first pressing down the cartridge lever to the eject position.
10. Check the array window for large bubbles or air pockets.

    If bubbles are present, 1) use a pipet to manually fill the array with Array Holding Buffer, 2) remove one-half of the solution, then 3) manually fill the array with Array Holding Buffer.

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

11. If the array has no large bubbles, it is ready for scanning. Pull up on the cartridge lever to engage wash block and proceed to *Scanning Arrays* on page 257.

    If the arrays cannot be scanned promptly, store them at 4°C in the dark until ready for scanning. Scan must be performed within 24 hours.
12. When finished washing and staining, shut down the fluidics station following the procedure listed under *Shutting Down the Fluidics Station* on page 259.

Scanning Arrays

The GeneChip Scanner 3000 7G is controlled by GCOS or AGCC software.

Prepare the Scanner

Turn on the scanner at least 10 min before use.

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 an array. Refer to the *GeneChip® Scanner 3000 Quick Reference Card* (P/N 08-0075).

Prepare Arrays for Scanning

To prepare arrays for scanning:

1. If the arrays were stored at 4°C, allow them to warm to room temperature before scanning.
2. If necessary, clean the glass surface of the array with a non-abrasive towel or tissue before scanning.

   Do not use alcohol to clean the glass.
3. On the back of the array cartridge, clean excess fluid from around the septa.
4. Carefully cover both septa with Tough Spots (See Figure 6.1 on page 258).

   Press to ensure the spots remain flat. If the spots do not apply smoothly (e.g. if you see bumps, bubbles, tears or curled edges) do not attempt to smooth out the spot. Remove the spot and apply a new spot.
5. Insert an array 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.

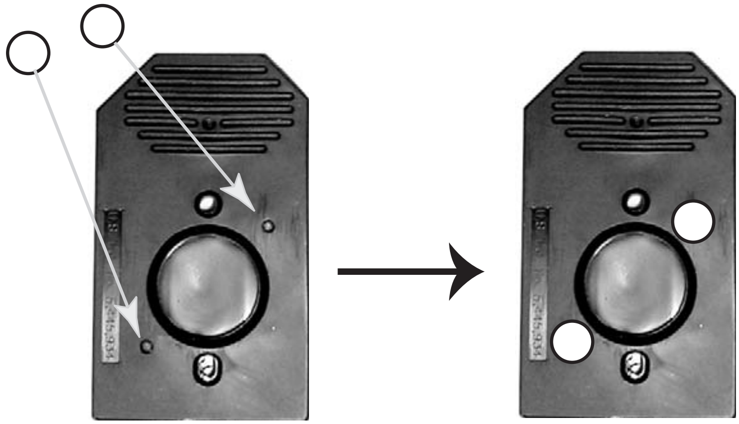


Figure 6.1 Applying Tough-Spots® to Arrays

Scanning the Array

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

To scan arrays:

1. Select the experiment name (GCOS) or sample name (AGCC) that corresponds to the array being scanned.
2. Following the GCOS or AGCC instructions as appropriate, load the array into the scanner and begin the scan.

   Only one scan per array is required. Pixel resolution and wavelength are preset and cannot be changed.

   > **WARNING: The scanner door will open and close automatically. Do not attempt to manually open or close the scanner door as this may damage the instrument.**
   >
   > **Do not force the array into the holder.**

Shutting Down the Fluidics Station

To shut down the Fluidics Station:

1. Gently lift up the cartridge lever to engage (close) the washblock.

   After removing an array from the holder, the LCD window displays the message **ENGAGE WASHBLOCK**. The instrument automatically performs a Cleanout procedure. The LCD window indicates the progress of this procedure.

2. When **REMOVE VIALS is displayed in the LCD**, remove the vials from the sample holders.

   The REMOVE VIALS message indicates the Cleanout procedure is complete.

3. If no other processing is to be performed, place the wash lines into a bottle filled with deionized water.
4. Using GCOS or AGCC, choose the **Shutdown_450** protocol for all modules.
5. Run the protocol for all modules.

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

6. When the protocol is complete, turn the instrument off.
7. Place the wash 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 7

DATA ANALYSIS

The purpose of this chapter is to:

- Describe the workflow used to analyze data from the Affymetrix® Genome-Wide Human SNP Array 6.0.
- Present some guidelines for assessing data quality.

The information in this chapter is intended as a supplement to the documentation listed below.

- *Affymetrix Genotyping Console™ Manual*
- One of the following manuals as appropriate:
  - *GeneChip® Operating Software User's Guide* (GCOS)
  - *Affymetrix GeneChip® Command Console™ User's Guide* (AGCC)

About Genotyping Console™

Genotyping Console 2.1 is a stand-alone application. It can be installed on computers that have GCOS, AGCC, or neither.

Genotyping Console has been verified on the following operating systems:

- Microsoft Windows XP with Service Pack 2.0
- Microsoft Windows Vista

File Requirements

The following files are required for data analysis using Genotyping Console:

- Affymetrix Genome-Wide Human SNP Array 6.0 library files (GenomeWideSNP_6)
- Affymetrix Genome-Wide Human SNP Array 6.0 SNP Annotation files from NetAffx

NOTE: The library and annotation files can be downloaded by Genotyping Console if the computer has access to the internet.

Additional files:

- CEL files (GCOS or AGCC format)
- Optional:
  - XML sample files
  - ARR sample files
  - CHP genotyping files (AGCC format)

NOTE: If using GCOS format CEL files, we recommend using the Affymetrix Data Transfer Tool (DTT v1.1.1) to move the CEL files out of the GCOS directory.

Overview of the QC and Genotyping Analysis Workflow

This section provides an overview of the analysis workflow for the data collected from Genome-Wide Human SNP Arrays 6.0. Raw data acquisition using GCOS or AGCC precedes data analysis by Genotyping Console (see Figure 7.1).

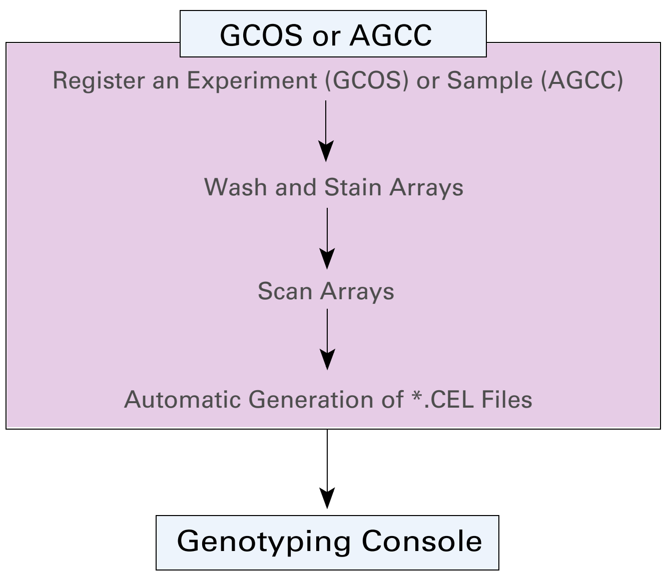


Figure 7.1 Raw data acquisition using GCOS or AGCC

The basic genotyping workflow for Genotyping Console is shown in Figure 7.2.

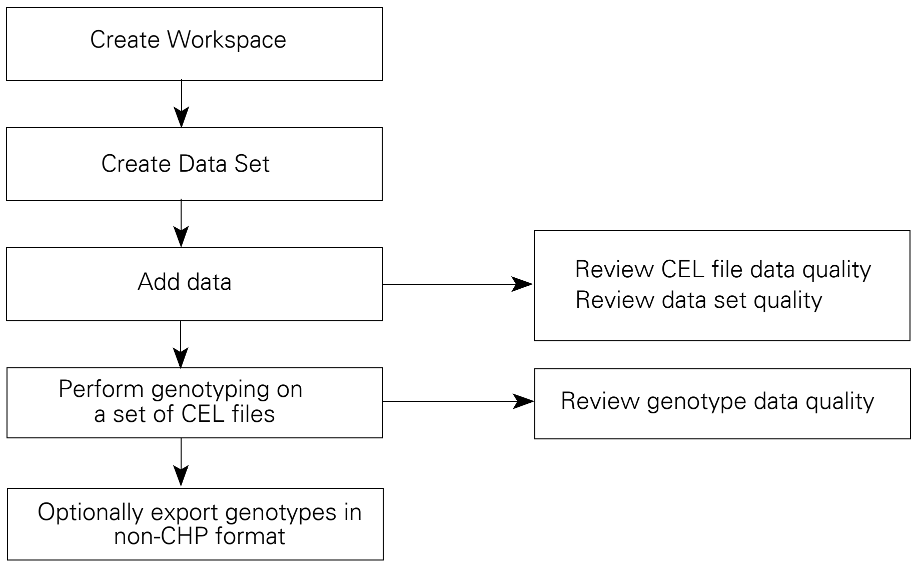


Figure 7.2 Basic genotyping workflow in Genotyping Console

You begin by creating a Workspace. A Workspace contains Data Sets, data files and SNP lists that are available within a single session of the software. Each Workspace should contain related data only (for example, data that belongs to one Principal Investigator or to one research study).

Each Workspace can have multiple Data Sets. A Data Set is a group of ARR/XML, CEL, and CHP files from a single array type. Within a Data Set, information can be displayed in tables and graphs. Examples of information that can be viewed, graphed and exported includes sample attribute information, Contrast QC values, Signature SNP genotypes, CHP and SNP Summary Data, SNP Cluster Graphs, and SNP Lists.

Once a Data Set is created, Quality Control (QC) analysis can be performed on a select set of CEL files or on all CEL files. QC can also be performed automatically upon import of CEL files. After QC, the CEL files are auto-grouped into All, In Bounds, and Out of Bounds groups based on the Contrast QC threshold (see *Assessing Data Quality* on page 265 for more information). Additional custom groupings of CEL files can also be made. The resulting Contrast QC values and other metrics are displayed in tables and graphs that can be exported.

You can initiate genotyping from any group or set of CEL files in a Data Set. Genotyping batch results are grouped together, and additional custom grouping of CHP files can also be made. The following summary results are displayed in tables and graphs that can be exported:

- CHP summary results

  Contains the Call Rate and other metrics for each CHP file
- SNP summary results

  Contains SNP Call Rate, Hardy-Weinberg p-Value, Minor Allele Frequency, and SNP annotations

A SNP list can be generated by filtering on any of these values (see *Downstream Analysis Considerations* on page 270 and *Data Filtering* on page 270). SNP Cluster graphs can be displayed based on a SNP List group of CHP files (see *Downstream Analysis Considerations* on page 270 and *SNP Cluster Visualization* on page 271). Genotypes can be exported in tab-delimited text format for all SNPs or a subset based on a SNP list.

The Workspace and Data Sets in Genotyping Console are organized into a tree structure (Figure 7.3). This structure is designed to guide you through the genotyping workflow. Refer to the Affymetrix *Genotyping Console™ Manual* for more information.

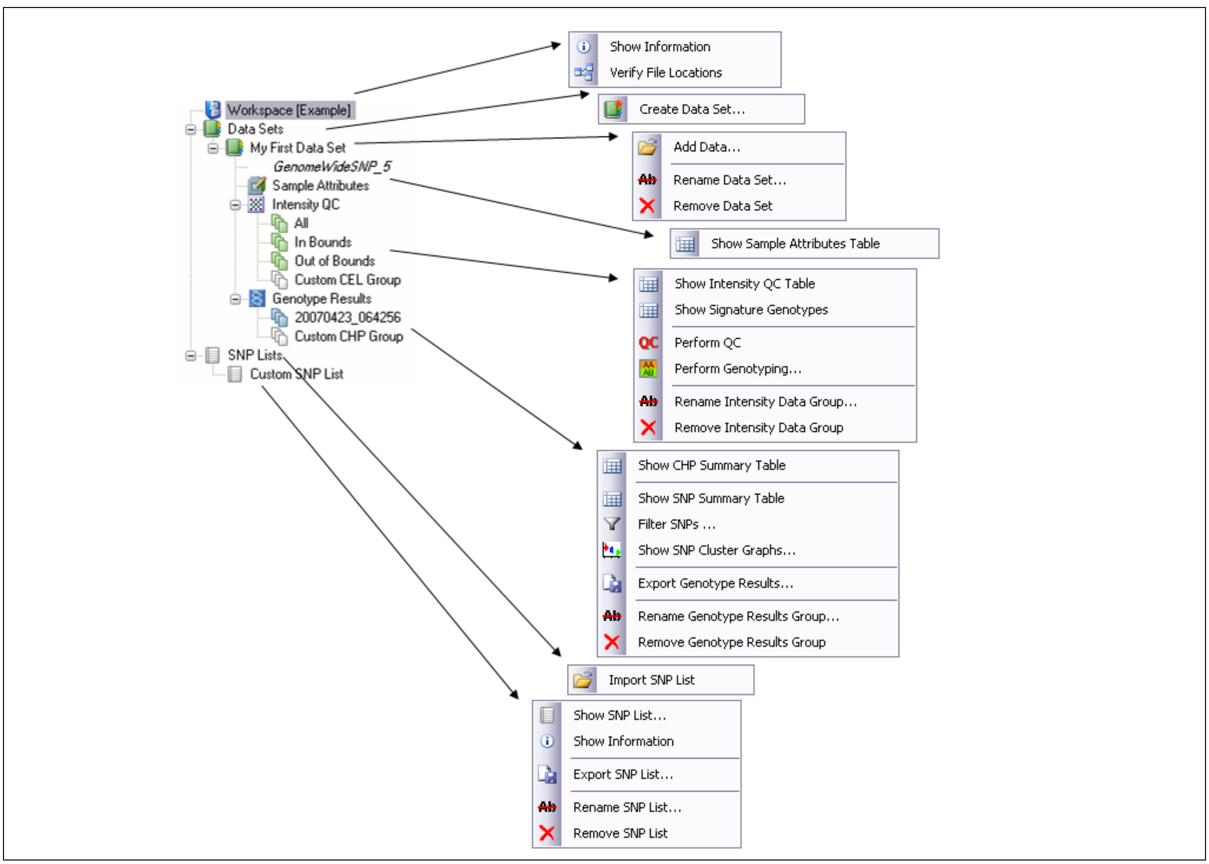


Figure 7.3 Genotyping Console tree structure

Assessing Data Quality

The following information is provided to assist you with establishing guidelines for evaluating the results generated from genotyping experiments. To assess data quality and to identify outlier samples, the Genotyping Console Intensity QC Table (Figure 7.4 on page 267) has a number of metrics that should be evaluated for each array. These metrics are defined below in Table 7.1.

It is important to check these metrics, and to create a running log for each project. The Reference Genomic DNA 103, included in the Affymetrix® Genome-Wide Human SNP Nsp/Sty Assay Kit 5.0/6.0 can serve as a positive control to ensure that all of the steps of the assay are being performed correctly. Evaluation of a particular sample should be based on QC report performance metrics.

Table 7.1 Metrics displayed in the Intensity QC Table

Column	Description
File	CEL file name
Bounds	In/Out of bounds indicates whether the CEL file met the specified Contrast QC threshold
Contrast QC	Computed Contrast QC
Computed Gender	Computed gender based on the Chr Y / Chr X ratio of the mean intensity of the X and Y copy number probes.
# CHP/CEL	Number of CHP files present in this workspace for the specified CEL file
Contrast QC (NSP)	Contrast QC for SNPs on NSP fragments
Contrast QC (NSP/STY OVERLAP)	Contrast QC for SNPs on both NSP and STY fragments
Contrast QC (STY)	Contrast QC for SNPs on STY fragments

Contrast QC

Contract QC is a metric that captures the ability of an experiment to resolve SNP signals into three genotype clusters. It uses 10,000 random SNP 6.0 SNPs. Contrast QC values are well correlated with the higher Call Rates and concordance achieved when calls are subsequently made with Birdseed or Birdseed v2. The correlation between Birdseed accuracy and Birdseed Call Rate is also very high.

After adding CEL files to a Data Set, open the Intensity QC table (Figure 7.4) by double-clicking the Intensity QC All icon in the tree. If some or all of the samples do not have QC results, right-click the All icon in the tree and select Perform QC. The information in this table indicates the overall performance of the assay for the Genome-Wide Human SNP Array 6.0.

When all steps of the assay are working as expected, the Contrast QC is typically greater than 0.4. In steady-state process, the proportion of samples that fall below the 0.4 threshold should be less than 10%. In addition, the average Contrast QC of the samples that pass the QC test should be greater than or equal to 1.7. If the proportion falling below 0.4 is greater than 10%, or if the average Contrast QC of passing samples is less than 1.7, then sample quality and process should be closely examined for possible issues.

A reduced Contrast QC may result if an error in any of the assay steps occurs, or if lower quality DNA samples are processed. Lower Contrast QC values may also be observed in situations where a new operator is learning the assay, or the number of samples processed at one time increases. In these later examples, additional practice for the operator is recommended to increase proficiency with the assay and achieve higher performance. Other factors that can lead to a reduced Contrast QC include:

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

For a sample with a lower Contrast QC, it is important to take into consideration the reasons for the lower Contrast QC 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 Contrast QC and decrease in accuracy affects the overall experimental goals. Refer to Chapter 8, *Troubleshooting* for troubleshooting tips.

	File	Bounds	Contrast QC	Contrast QC (Random)	Contrast QC (Nsp)	Contrast QC (Nsp/Sty Overlap)	Contrast QC (Sty)	Computed Gender	# CHP/CEL	File Date
1	NA06985_GW6_C.CEL	In	2.86	2.86	3.63	3.16	3.21	female	1	5/16/2007 11:30 AM
2	NA06991_GW6_C.CEL	In	2.78	2.78	3.21	2.80	2.69	female	1	5/16/2007 11:30 AM
3	NA06993_GW6_C.CEL	In	1.95	1.95	2.63	3.21	2.73	male	1	5/16/2007 11:31 AM
4	NA06994_GW6_C.CEL	In	3.01	3.01	3.75	3.34	3.56	male	1	5/16/2007 11:32 AM
5	NA07000_GW6_C.CEL	In	2.52	2.52	2.94	2.78	2.59	female	1	5/16/2007 11:32 AM
6	NA07019_GW6_C.CEL	In	2.72	2.72	3.21	3.01	3.06	female	1	5/16/2007 11:33 AM
7	NA07022_GW6_C.CEL	In	1.58	1.58	1.83	1.87	1.60	male	1	5/16/2007 11:34 AM
8	NA07029_GW6_C.CEL	In	2.60	2.60	3.29	3.18	2.87	male	1	5/16/2007 11:34 AM
9	NA07034_GW6_C.CEL	In	2.17	2.17	2.76	2.45	2.54	male	1	5/16/2007 11:35 AM
10	NA07048_GW6_C.CEL	In	2.41	2.41	2.91	2.88	2.78	male	1	5/16/2007 11:36 AM

Figure 7.4 Example of an Intensity QC Table

Genomic DNA Quality

Genomic DNA should be prepared following the guidelines in Chapter 3 of this manual. DNA prepared outside of 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 is one way to accomplish this. If an alternate genomic DNA preparation method is used, we 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.

Oligonucleotide Controls

The oligonucleotide control reagent includes oligonucleotide B2.

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 (not present on all array designs)

- The checkerboard pattern at each corner (Figure 7.5 on page 268) and throughout the array
- The array name, located in the lower left corner of the array (Figure 7.6 on page 268)

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.

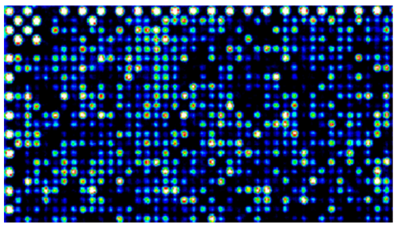


Figure 7.5 Example of Checker Board Pattern

Figure 7.6 Array name (image has been rotated for display)

Figure 7.7 on page 269 is the scanned image of the Genome-Wide Human SNP Array 6.0. Notice how the array appears to be divided into four quadrants. The genotyping probes are tiled within each quadrant. Copy number probes are tiled in the bands that form the quadrant boundaries.

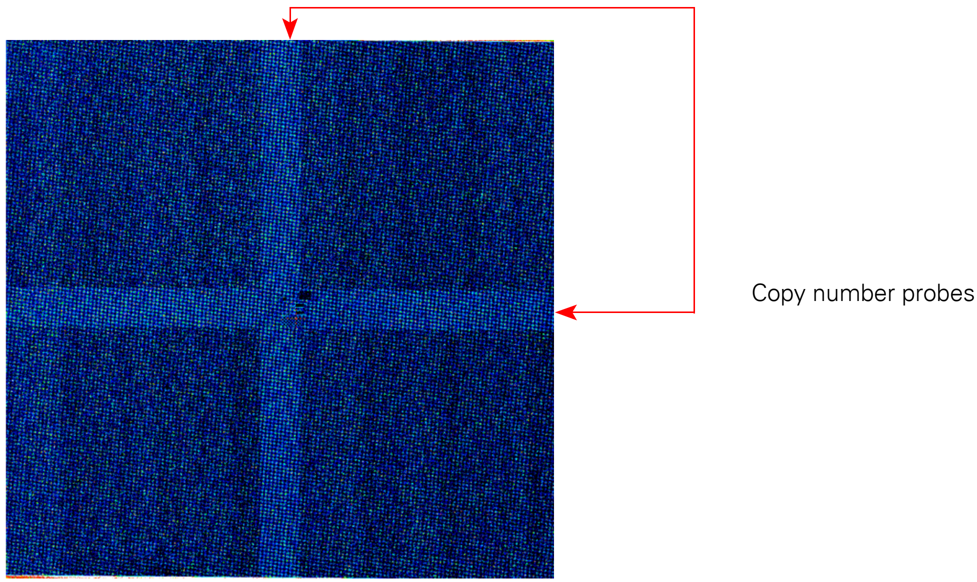


Figure 7.7 Scanned Image of the Genome-Wide Human SNP Array 6.0

Downstream Analysis Considerations

Association studies are designed to identify SNPs with subtle allele frequency differences between different populations. Genotyping errors, differences in sample collection and processing, and population differences are among the many things that can contribute to false positives or false negatives. Efforts should be made to minimize or account for technical or experimental differences. For example, randomization of cases and controls prior to genotyping can reduce or eliminate any possible effects from running cases and controls under different conditions.

Data Filtering

For many genotyping applications, poorly performing SNPs can lead to an increase in false positives and a decrease in power. Such under-performing SNPs 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 Contrast QC is greater than 0.4 for Genome-Wide SNP 6.0. This threshold assumes the use of high quality DNA (see Chapter 3, *Genomic DNA General Requirements*). Furthermore, the efficacy of this filter may be reduced if more than 10% of the experiments attempted fail to attain the QC threshold, or if the average of passed samples is less than or equal to 1.7.

As an extra guard against the inclusion of any outlier samples that pass through the Contrast QC filter, it is a good idea to reject samples that are notable outliers in terms of their Birdseed Call Rate. When using Birdseed, clustering larger batches of samples will improve the performance of the algorithm. The algorithm improvements in Birdseed v2 allow you to cluster by plate with the same performance as clustering larger batches of samples.

Prior to downstream analysis of the genotype calls generated, we highly recommended that SNP-level filters be applied to remove SNPs that are not performing ideally in the data set in question. The subject of SNP filtering is a widely-adopted practice in whole-genome genotyping studies. The specific filters and thresholds can vary somewhat from one study to another, and will depend upon the specific study context and goals. Some common filters will remove SNPs:

- With a significantly low per SNP Call Rate
- Out of HW equilibrium in controls
- With significantly different Call Rates in cases and controls
- With Mendelian errors

Studies on multiple 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. Removing these SNPs will boost overall performance, and takes out of consideration the SNPs most likely to show up as false positive associations.

SNP Cluster Visualization

The application of per-SNP filters helps remove the majority of problematic SNPs. However, no filtering scheme is perfect. Even with a prudent level of SNP filtering, a small proportion of poorly performing SNPs will remain.

Moreover, poorly performing SNPs will often be the ones most likely to perform differently between cases and controls. The list of most significantly associated SNPs is often enriched for such problematic SNPs.

The SNP filtering process greatly reduces the occurrence of these false positives. But given their tendency to end up on the list of associated SNPs, it is likely that some will remain. Before carrying forth SNPs to subsequent phases of analysis, visual inspection of the SNPs in the clustering space is strongly recommended. Visual inspection typically helps to identify problematic cases. SNP clusters can be displayed in Genotyping Console. Refer to the *Affymetrix Genotyping Console™ Manual* for more information.

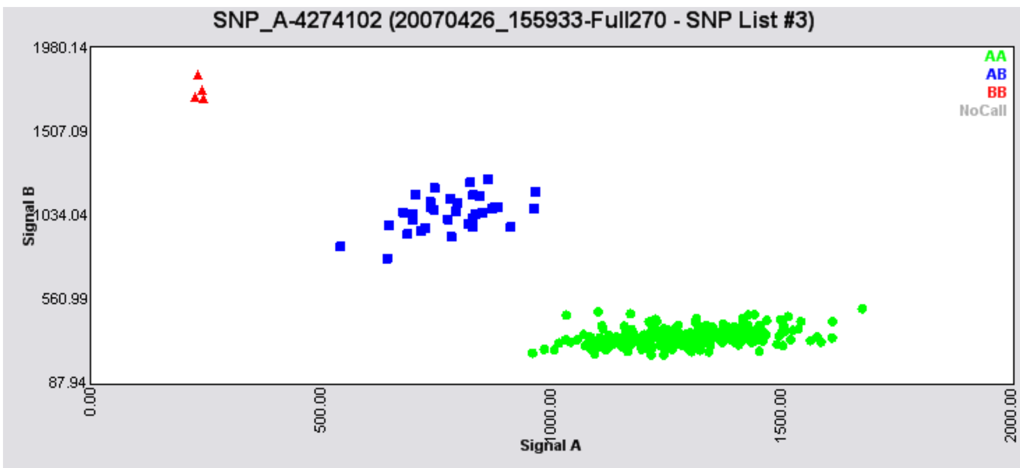


Figure 7.8 Example of a SNP cluster as displayed in Genotyping Console.

Increasing or Decreasing Accuracy and Call Rate

To increase or decrease accuracy and Call Rate:

- Adjust the default Contrast QC
- Adjust the thresholds for Birdseed

Affymetrix genotyping software provides flexible options to enable a trade off between Call Rate and genotyping accuracy.

The default Contrast QC threshold for Genome-Wide SNP 6.0 is 0.4. As long as not more than 10% of samples falls below the 0.4 threshold, and if the average Contrast QC of passing samples is greater than or equal to 1.7, its use is expected to result in high Call Rates and accuracy. Samples right at the Contrast QC threshold are expected to have a Birdseed v2 Call Rate of around 96%, with an average accuracy of ~99%. A strong correlation exists between the Contrast QC and Birdseed performance. The more a sample exceeds the threshold, the better the performance.

The default confidence score threshold for Birdseed analysis is 0.1. This default provides a good compromise between accuracy and Call Rate. Adjusting the confidence score value in Genotyping Console will result in one of the following:

- Increased Call Rates with lower genotyping accuracy
- Decreased Call Rates with greater genotyping accuracy

Refer to the *Affymetrix Genotyping Console™ Manual* for more information.

Summary of Best Practices for Data Analysis Using Birdseed v2

The following is a summary of the steps that we recommend for data analysis using Birdseed v2.

1. **Study design**

   Randomly distribute cases and controls across plates.

2. **Pre-cluster sample quality check**

   Reprocess samples with Contract QC < 0.4.

3. **Pre-cluster plate or dataset check**

   Flag datasets as potentially problematic if < 90% pass the QC test (above) or if the average Contrast QC over the dataset after filtering is < 1.7.

4. **Genotyping: Cluster samples with Birdseed v2**

   - Cluster by plate or cluster all together according to which process is most convenient for the lab workflow.
   - Each cluster should contain a minimum of 44 samples with at least 15 female samples.

5. **Genotyping: Post-cluster sample quality check**
   - Reject samples with outlier low Birdseed Call Rates.
   - Reject samples with excess predicted heterozygosity.
6. **Genotyping: Post-genotyping SNP filtration**
   - Filter SNPs with SNP Call Rates over all samples in the study in the range of 90-95%.

     The exception is Y chr SNPs which are always No Calls for female samples.
   - Optional: reject based on deviation from HW equilibrium, reproducibility, expected heterozygosity, and MAF where possible and appropriate.
7. **Genotyping: Post-association study analysis**

   Visually analyze all candidate SNPs.
8. **Copy Number: Reference Model File creation**

   The set of samples used to create the Reference Model File should contain a minimum of 44 samples with at least 15 female samples.
9. **Copy Number: CNCHP file quality check**
   - Track CNCHP quality using MAPDs. Reprocess samples with MAPDs > 0.3 when using an intralab reference (Reference Model File made from lab's own samples), or > 0.4 when using an external reference (reference generated elsewhere, such as the 270HapMap Reference).
   - If MAPDs are consistently high when using an external reference, recalculate MAPDs with an intralab reference. If the MAPDs all drop significantly, then the high MAPD is an artifact introduced by a systematic difference between current samples and the samples that made up the reference rather than a quality issue.

Chapter 8

Troubleshooting

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 Genome-Wide Human SNP Nsp/Sty 5.0/6.0 Assay has an inherent risk of contamination with PCR product from previous reactions.

In Chapter 2 *Laboratory Setup and Recommendations*, 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. 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. The assay in this manual 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 Lonza, and ligase and restriction enzymes from New England Biolabs.

Additional recommendations are as follows:

- Think ahead to ensure that the reagents and equipment you require, including pipets, are in the correct work area. Ensuring the proper equipment is available in the proper laboratory areas will make the workflow easier and will help reduce the risk of sample contamination.
- Pay particular attention to the storage and handling of reagents. Proper storage and handling is particularly important for enzymes such as DNA Ligase and the Fragmentation Reagent (DNase I). Both of these enzymes are sensitive to temperatures exceeding –20°C.

  To prevent loss of enzyme activity:

  - Immediately place the enzyme in a cooler chilled to –20 °C when removed from the freezer. Immediately return the enzyme to –20 °C after use.

- Take care when pipetting enzymes stored in glycerol, which is viscous. Do not store at –80 °C.
- Because Fragmentation Reagent (DNAse I) activity can decline over time after dilution on ice, add it to the samples as quickly as possible.

- Preparing master mixes with a 15% excess ensures consistency in reagent preparation by minimizing pipetting errors and reducing handling time of temperature sensitive reagents. The success of this assay depends on the accurate pipetting and subsequent thorough mixing of small volumes of reagents.
- 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.
- 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 gel. Product (bands) should be visible in the 200 to 1100 bp size range. See Chapter 4 *48 Sample Protocol* and Appendix D, *E-gels*. for more information and instructions.

  Following fragmentation, run samples on a gel. Successful fragmentation is confirmed by the presence of a smear of less than 200 bp in size. See Chapter 4 *48 Sample Protocol* and Appendix D, *E-gels*. for more information and instructions.
- 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. Use Reference Genomic DNA 103 as a positive control (included in the reagent kit). These controls are effective troubleshooting tools that will help you confirm the successful completion of each stage of the assay.
- Oligonucleotide controls are included in the reagent kit. These controls 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.
- For greater efficiency, we recommend using a team approach to sample processing. This approach is described *About Using Controls* on page 37.
- Regularly calibrate all multichannel pipets.
- Check that your spectrophotometer is accurately calibrated, and be sure the OD measurement is within the quantitative linear range of the instrument (0.2 to 2.0 OD).
- Hybridization ovens should be serviced at least once per year to ensure that they are operating within the manufacturer’s specifications.

Important Differences Between Genome-Wide Human SNP Arrays 6.0 and GeneChip® Expression Arrays

- For laboratories that also run GeneChip Expression arrays, always check the temperature setting on the Hybridization Oven 640.
  - **For the Genome-Wide Human SNP Array 6.0, ovens should be set to 50°C.**
  - The temperature for hybridization on expression arrays is 45°C.
- 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 Genome-Wide Human SNP Array 6.0. Also, care should be taken to ensure the fluidics station is properly maintained and primed with the correct buffers prior to use.
- Both the Genome-Wide Human SNP Nsp/Sty 5.0/6.0 Assay and Expression protocols use the same stain reagents for each staining step. However, after the last wash the Genome-Wide Human SNP Array 6.0 is filled with Array Holding Buffer.
- Genome-Wide Human SNP Arrays 6.0 are scanned once at 570 nm on the GeneChip® Scanner 3000 7G.

Troubleshooting the Genome-Wide SNP 5.0/6.0 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. Use the correct concentration of BSA.
	Failed adaptor ligation reaction.	Confirm enzyme activity.
		Ligase buffer contains ATP and should be defrosted/ held at 4°C. Vortex 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		
DNA lost during purification. Gel images show PCR product, but low OD.	Vacuum elution is not complete.	Ensure that filtering is complete for all wells (matte/dull look) before stopping vacuum elution.
Insufficient purified PCR product for quantitation		
Volume in a particular well(s) on the elution catch plate is < 2 µL after transferring 45 µL to the fragmentation plate		Do the following in this order: • Add 2 µL Buffer EB to the corresponding wells on the fragmentation plate. • Mix by pipetting up and down. • Transfer 2 µL to the corresponding well(s) on the OD plate. • Proceed to fragmentation with 45 µL in each well.
Insufficient purified PCR product for fragmentation		
Volume in a particular well(s) on the elution catch plate is < 45 µL		Do the following in this order: • Measure the actual volume using a pipettor. • Add Buffer EB to a final volume of 47 µL. • Mix by pipetting up and down. • Transfer 2 µL to the corresponding well(s) in the OD plate. • Proceed to fragmentation with 45 µL in each well.

Problem	Likely Cause	Solution
Fragmented PCR product is not the correct size		
PCR product is still visible in 200-1,100 bp size region	Failed or incomplete fragmentation due to reduced DNase activity.	Check that you have selected the correct activity of DNase from Table 4.47 on page 112 to add to fragmentation reaction. (See *Dilute the Fragmentation Reagent* on page 112)
		Ensure fragmentation reagent (DNase I) is kept at –20°C. Do not reuse diluted working stock.
.CEL file can not be generated		
GCOS or AGCC 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.	Check that you have selected the correct activity of DNase from Table 4.47 on page 112 to add to fragmentation reaction. (See *Dilute the Fragmentation Reagent* on page 112. 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.
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.
Very low call rates	Mixed up Nsp and Sty enzymes during the digestion or ligation stages.	Repeat the experiment, making sure the correct reagents are used for each digestion and ligation stage.

OD Troubleshooting Guidelines

Refer to the tables below when troubleshooting OD readings.

Table 8.1 PROBLEM: Sample OD is greater than 1.2 (6 µg/µL)

If the sample OD is greater than 1.2 (calculated concentration greater than 6 µg/µL), a problem exists with either the elution of PCR products or the OD reading. The limit on PCR yield is approximately 6 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 55 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. • Pipets 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.

Table 8.2 PROBLEM: Sample OD is Less Than 0.9 (4.5 µg/µL)

If the sample OD is less than 0.9 (calculated concentration less than 4.5 µg/µL), a problem may exist 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. 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.

Table 8.2 (Continued) PROBLEM: Sample OD is Less Than 0.9 (4.5 µg/µL)

- Pipet 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 or Tetrad 2).
- 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 Genome-Wide SNP 5.0/6.0 Assay reaction amplifies a size range of fragments that represents ~30% of the genome. The Genome-Wide Human SNP Array 6.0 is designed to detect 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, subsequently leading to lower call rates.

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

- The purified PCR product was eluted in a volume greater than 55 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.
- Pipets 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.

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. Ensure the vacuum manifold is working properly. • An error may have been made while taking the OD readings. • The PCR product may not have been adequately washed. Check the 75% EtOH wash solution.

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

Possible causes include: • Magnetic beads may have been carried over into purified sample. • Precipitate may be present in the eluted samples. • There may be defects in the OD plate. • Air bubbles in the OD plate or in solutions.

When to Contact Technical Support

Affymetrix Instruments

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 specifications

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

NOTE: **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 United Kingdom	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, Minato-ku, Tokyo 108-0014 Japan	Tel: (03) 5730-8200 Fax: (03) 5730-8201

Chapter 9

VACUUM MANIFOLD AND FLUIDICS STATION CARE AND MAINTENANCE

This chapter includes guidelines and instructions on:

- Cleaning the vacuum manifold
- General care of the fluidics station
- A cleaning (bleach) protocol that should be run once per week

Cleaning the Vacuum Manifold

Salt buildup occurs with repeated use of the vacuum manifold. The vacuum can be compromised and sample contamination may occur when too much salt is present.

Regular cleaning of the vacuum manifold is recommended.

To clean the vacuum manifold:

1. Disassemble the vacuum manifold (base, cover, cover gasket).
2. Soak the parts in warm water for 5 min.
3. Thoroughly rinse and dry each part.
4. Reassemble the vacuum manifold.

General Fluidics Station 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.
- To ensure proper functioning of the instrument, perform periodic maintenance.
- 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.

WARNING: Before performing any maintenance, turn off power to the fluidics station to avoid injury in case of a pump or electrical malfunction.

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 min 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 9.1 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 9.1 on page 288.
2. Prepare 500 mL of 0.525% sodium hypochlorite solution using deionized water.

   You can 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 fresh solution.**
- **Each fluidics station with 4 modules requires 500 mL of 0.525% sodium hypochlorite solution.**

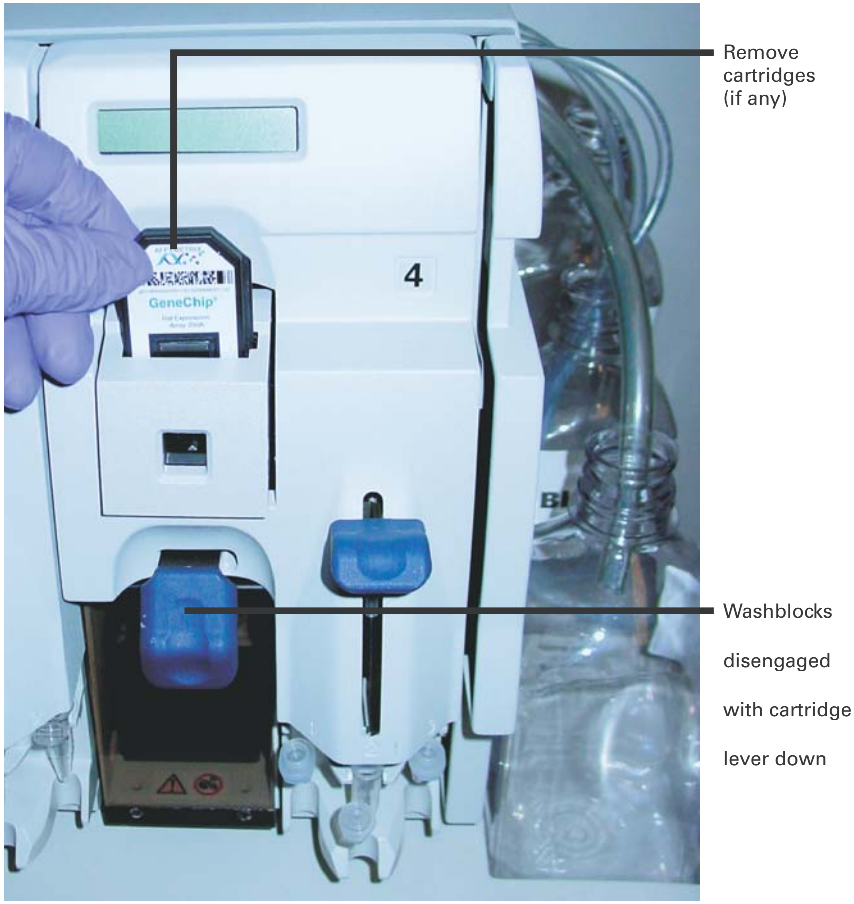


Figure 9.1 Disengaged washblocks showing cartridge levers in the down position. Remove any cartridges

3. As shown in Figure 9.2 on page 289:

A. 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.

The Bleach protocol requires approximately one liter of DI water.

B. Insert the waste line into the waste bottle.

C. Immerse all three wash and water lines into the bleach solution.

> **!** **IMPORTANT: Do NOT immerse the waste line into the bleach.**

Figure 9.2 The bleach cycle. Immerse the tubes into the 0.525% sodium hypochlorite solution. The waste line remains in the waste bottle.

4. Open the instrument control software (GCOS or AGCC).

5. Choose the current bleach protocol (as of the writing of this manual, it is BLEACHv2_450) for each module.

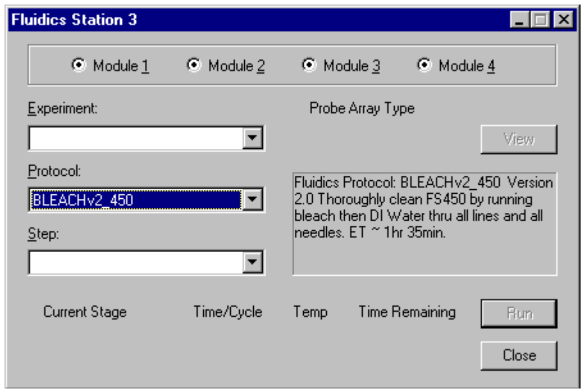


Figure 9.3 The Fluidics Station protocol window: select all modules.

6. In GCOS or AGCC, run the protocol for all modules.

NOTE: The fluidics station will not start until the needle lever is pressed down (Figure 9.4 on page 291).

The 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 9.4).

Figure 9.4 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 min, 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 9.5).

   At this step, there is no need to be concerned about the bleach remaining in the lines.

Figure 9.5 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 used for the bleach protocol.

6. After completing the bleach protocol, follow the suggestions for storage of the Fluidics Station 450 in Table 9.2 below.

Table 9.2 Storage Suggestions for the Fluidics Station 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.

Appendix A

Alternative Purification Protocol Using a Seahorse Filter Plate

Millipore vs Seahorse Filter Plate

> **IMPORTANT: Two different filter plates can be used for the purification stage: Millipore or Seahorse. The instructions in this appendix are for using a Seahorse filter plate. To use a Millipore filter plate, follow the instructions in Chapter 4 *48 Sample Protocol* or Chapter 5 *96 Sample Protocol*.**

About this Stage

During this stage, you will:

- Pool the Sty and Nsp PCR reactions to a single deep well pooling plate
- Add beads to each pool and incubate
- Transfer each pool to a Seahorse filter plate and dry down on a vacuum manifold
- Wash the PCR products with EtOH and dry down
- Elute the PCR products using Buffer EB
- Vacuum and spin transfer the PCR products to a new 96-well plate

Location and Duration

- Main Lab
- Hands-on time: . . . . .approximately 1 hr
- Sample/bead incubation . . . . .10 min
- Initial vacuum step: . . . . .approximately 60 to 90 min
- First EtOH vacuum step:. . . . . .approximately 10 to 20 min
- Final EtOH vacuum step . . . . .10 min
- Resuspend beads in Buffer EB on Jitterbug . . . . .10 min
- Elution on vacuum manifold. . . . . .approximately 15 to 30 min

- Final elution on centrifuge . . . . .5 min
- Total time for this stage: . . . . .approximately 3.5 hr

Input Required from Previous Stage

The input required is:

Quantity	Item
3 plates	Sty PCR product
4 plates	Nsp PCR product

Equipment and Consumables Required

The following equipment and materials are required to perform this stage.

Table A.1 Equipment and Consumables Required for this Stage

Quantity	Item
1	Collar, Multiscreen, deep well
1	Jitterbug
As needed	Kimwipes
1	Pipette, 12-channel P20
1	Pipette, 12-channel P200
1	Pipette, 12-channel P1200
1	Pipette, serological
As needed	Pipette tips for pipettes listed above; full racks
1	Plate, 96-well PCR
1	Plate centrifuge with deep-well capacity (54mm H x 160g)
1	Plate, storage, 2.4 mL deep well (referred to as the *pooling plate*)
1	Plate, elution catch, 96-well V-bottom

Table A.1 Equipment and Consumables Required for this Stage

Quantity	Item
1	Plate, 2ml, 96-well format filter plate (PES 0.45 µm; Seahorse) (requires a deep well collar on the vacuum manifold; listed above)
7	Plate holders
As required	Plate seal**
1	Solution basin, 55 mL or larger
1 roll	Tape, lab
1	Vacuum Manifold, Millipore
1	Vortexer
** **IMPORTANT** Use only the PCR plate, adhesive film and thermal cyclers listed on page 29.	

Reagents Required

The following reagents are required for this stage.

Table A.2 Reagents Required for this Stage

Reagent	Volume Required for 48 Samples	Volume Required for 96 Samples
Elution Buffer (Buffer EB)	3 mL	6 mL
75% EtOH (ACS-grade ethanol diluted to 75% using AccuGENE water)	100 mL	200 mL
Magnetic Beads (AMPure or SNPClean)	50 mL	100 mL

Important Information About This Stage

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

CAUTION: Do not overdry the magnetic beads during the vacuum steps. Overdrying may inhibit elution of the purified DNA.

After adding EtOH to the wells (Step 5 on page 304), the first vacuum step should not exceed approximately 20 min.

The final EtOH vacuum step is 10 min only (Step 8 on page 304). Do not exceed 10 min.

All of the liquid in each well should be pulled through the filter. Although the beads may still be moist, there should be no standing liquid on top of the beads. The wells will appear dull (matte) – not shiny.

If any wells are clogged, do not continue filtering. Proceed with the protocol for the samples that have been successfully purified and eluted. Repeat the experiment for the samples in the clogged wells.

IMPORTANT:

- **Bring the Buffer EB and 75% EtOH to room temperature prior to use.**
- **The storage temperature for the magnetic beads is 4° C (refrigerator).**
- **To avoid cross-contamination, pipet very carefully when pooling the PCR reactions into the deep-well plate.**
- **Maintain the vacuum between 20—24 in Hg (do not exceed 24 in Hg).**
- **Inspect the vacuum manifold for salt buildup after each use, and clean regularly. Refer to Chapter 9 for cleaning instructions.**

Prepare the 75% EtOH

Dilute ACS-grade or equivalent ethanol to 75% using AccuGENE water.

Recipe for 75% EtOH

In a 1 L measuring cylinder:

1. Pour 750 mL 100% EtOH
2. Add 250 mL AccuGENE molecular biology grade water.
3. Transfer to a 1 L bottle and mix well.
4. Seal tightly and store at room temperature.

Prepare the Reagents

Allow the Buffer EB to warm to room temperature prior to use.

Prepare the Vacuum Manifold

To prepare the manifold:

1. Connect the manifold and regulator to a suitable vacuum source able to maintain 20 to 24 in Hg.

   Leave the vacuum turned off at this time.
2. Inspect the manifold for salt and other contaminants and clean if necessary.
3. Place the vacuum flask trap below the level of the manifold.

IMPORTANT: Inspect the vacuum manifold for salt buildup before each use. Clean the manifold regularly. Refer to Chapter 9 for cleaning instructions.

If the flask trap is not placed below the level of the manifold, some solution may splash back and adhere to the bottom of the filter plate.

Pool the PCR Products

CAUTION: Be very careful when pooling PCR products. Avoid cross-contaminating neighboring wells with small droplets.

To pool the PCR products:

1. If PCR products are:
   - Frozen, thaw to room temperature on the bench top in plate holders.
   - On thermal cyclers, remove them now.
2. Vortex the center of each plate at high speed for 3 sec.
3. Spin down each plate at 2000 rpm for 30 sec.
4. Place each PCR plate in a plate holder on the bench top.
5. Place a deep well pooling plate on the bench top.
6. On each PCR plate, cut the seal between each row so that it can be removed one row at a time.
7. Using a 12-channel P200 pipette set to 110 µL:

   A. Remove the seal to expose row A only on each PCR plate.

B. Transfer the reactions from row A of each PCR plate to the corresponding wells of row A on the pooling plate (Table A.3 below and Figure A.1 on page 301).

C. Change your pipette tips.

Change pipette tips after the PCR product from the same row of each PCR plate has been pooled on the pooling plate.

D. Remove the seal from each PCR plate to expose the next row.

E. Transfer each reaction from the same row of each PCR plate to the corresponding row and wells of the pooling plate.

F. Repeat steps C., D. and E. until all of the reactions from each PCR plate are pooled on the pooling plate.

8. When finished, look at the wells of each PCR plate to ensure that all of the product has been transferred and pooled.

Table A.3 Pooled Sty and Nsp PCR Products

Sty PCR plates (3):	100 µL from each well	= 300 µL/well
Nsp PCR Plate (4):	100 µL from each well	= 400 µL/well
Total Volume Each Well of Pooling Plate		**= 700 µL/well**

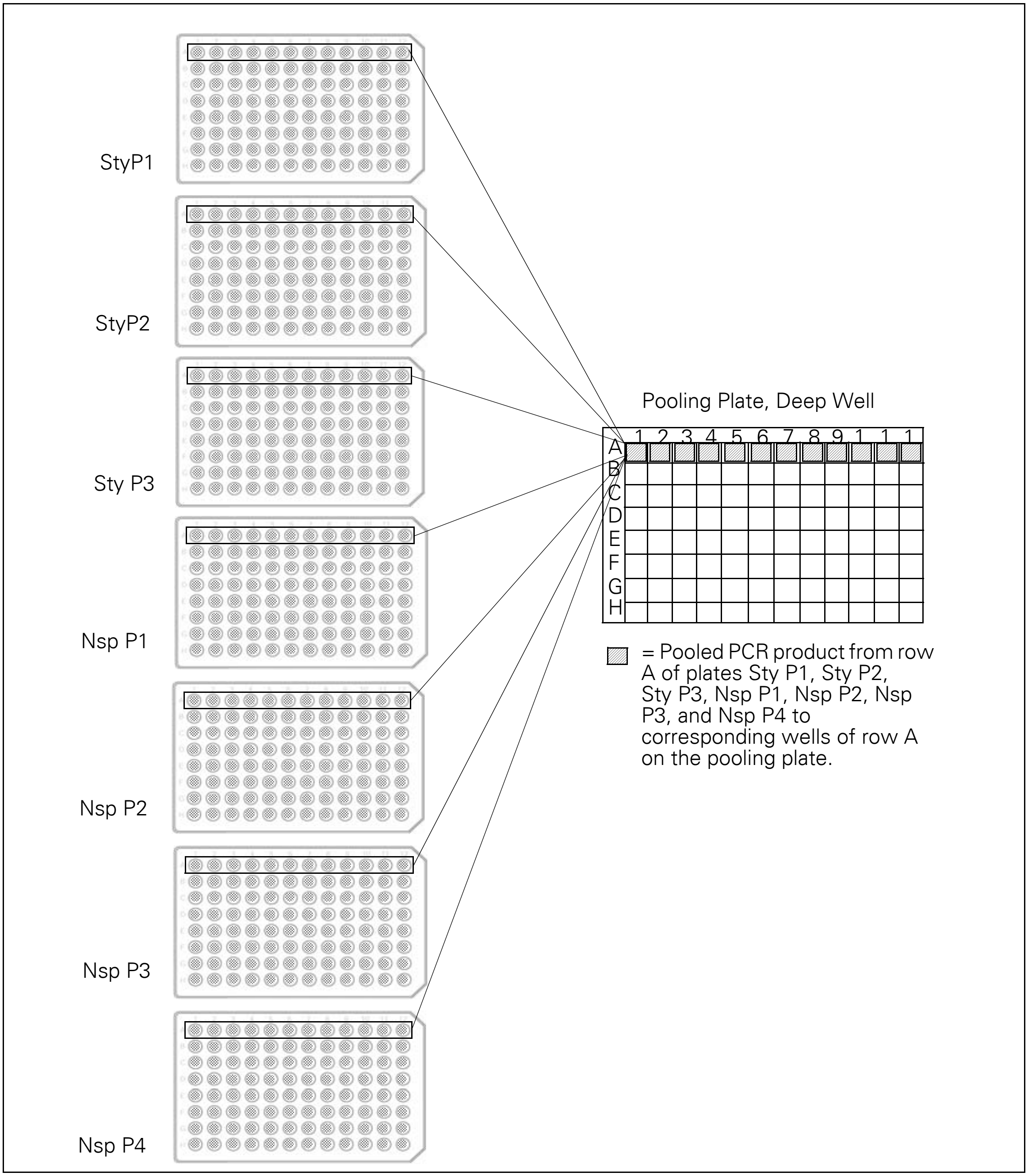


Figure A.1 Pooling Sty and Nsp PCR Products on a Deep Well Pooling Plate

Purify the Pooled PCR products

Add Magnetic Beads and Incubate

During incubation, the DNA binds to the magnetic beads.

To add magnetic beads and incubate:

1. Mix the magnetic bead stock very well by vigorously shaking the bottle.

   Beads will settle overnight. Examine the bottom of the bottle and ensure that the solution appears homogenous.

2. Pour or pipette 50 mL (48 samples) or 100 mL (96 samples) of magnetic beads to a solution basin.

   1 mL of magnetic beads is required for each reaction. You can add in multiple batches if the solution basin is not large enough.

3. Using a manual (not electronic) 12-channel P1200 pipette:

   A. Add 1.0 mL of magnetic beads to each well of pooled PCR product.

   B. Mix well by pipetting up and down 5 times using the following technique:

      Mixing Technique:

      1) Depress the plunger and place the pipette tips into the top of the solution.

      2) Move the pipette tips down – aspirating at the same time – until the tips are near the bottom of each well.

      3) Raise the tips out of the solution.

      4) Place the pipette tips against the wall of each well just above each reaction, and carefully dispense the solution.

      > **IMPORTANT: The solution is viscous and sticky. Pipette carefully to ensure that you aspirate and dispense 1 mL.**
      >
      > **Thorough mixing is critical to ensure that the PCR products bind to the beads.**

      5) Change pipette tips for each row.

4. Cover the plate to protect the samples from environmental contaminants and allow to incubate at room temperature for 10 min.

   You can use the lid from a pipette tip box to cover the wells.

Transfer Reactions to a Seahorse Filter Plate

To transfer the reactions to a filter plate:

1. Place a Seahorse filter plate on the vacuum manifold.
2. Using a 12-channel P1200 pipette, transfer each reaction from the pooling plate to the corresponding row and well of the filter plate.

> **IMPORTANT: You will need to pipette twice to transfer all of the solution from each well to the filter plate. The solution is viscous and sticky, so check to ensure that all of it has been transferred.**

3. Tightly seal any unused wells with a MicroAmp Clear Adhesive Film.

   Example for 48 samples: To ensure a tight seal, cover 1/2 to 1/3 of the wells in row D as well. Unused wells ***must be sealed*** to ensure proper vacuum pressure.

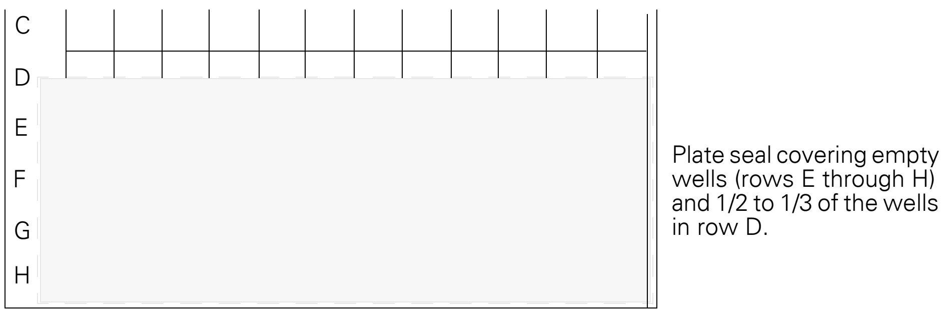


Figure A.2 Sealing empty wells on the filter plate

Purify the Reactions

To purify the reactions:

1. Turn on the vacuum to 20 to 24 in Hg and check the seals.

   Do not exceed 24 in Hg. Adjust the leak valve if necessary.
2. Ensure that any unused wells are completely sealed, and cover the plate to protect it from environmental contaminants.
3. Run the vacuum until all of the liquid has been pulled through the filter (approximately 60 to 90 min), then turn off the vacuum.
4. Examine each well.

   There should be no standing liquid in any well, and the wells should appear dull (matte). Wet wells will look shiny.

   If any of the wells are still wet, put the plate back on the vacuum and continue drying for up to 10 min (total ≤ 90 min).

5. Using a 12-channel P1200 set to 900 µL:
   - **A.** Add 900 µL of 75% EtOH to each reaction.
   - **B.** Turn the vacuum on to 20 to 24 in Hg.
   - **C.** Run the vacuum for approximately 1–2 min (or until the volume in the wells begins to decrease).
   - **D.** Add another 900 µL of 75% EtOH to each reaction (for a total of 1.8 mL EtOH).
   - **E.** Cover the plate.
   - **F.** Run the vacuum until all of the liquid has been pulled through the filter (approximately 10 to 20 min), then turn off the vacuum.
6. Examine each well.

   Again, there should be no standing liquid in any well, and the wells should appear dull (matte). Wet wells will look shiny.

   If any of the wells are still wet, put the plate back on the vacuum and continue drying for up to 5 min (total ≤ 25 min; see the Caution on on page 298).
7. Remove any excess EtOH as follows:
   - **A.** Blot the bottom of the plate on Kimwipes.
   - **B.** Wipe the bottom of the plate with a clean Kimwipe.
8. Return the filter plate to the manifold and turn on the vacuum for an additional 10 min ONLY.

   Do not exceed 10 min. Less than 10 min is OK if no excess ethanol is present in the wells or on the underside of the filter plate.

   NOTE: The purpose of this step is to remove excess EtOH so that it is not carried over into the eluate. Ten minutes is sufficient for this purpose. Leaving the vacuum on for more than 10 minutes may over-dry the beads which may inhibit elution of the purified DNA.

9. Turn off the vacuum, and blot the bottom of the plate on Kimwipes to remove any remaining EtOH.

Elute the Purified Reactions

To elute the purified reactions:

1. Attach the elution catch plate to the bottom of the filter plate using 2 strips of lab tape.

   The filter and elution plate assembly is now referred to as the *plate stack* (Figure A.3).

> **IMPORTANT: Do not completely seal with tape. Product will not elute if sealed.**

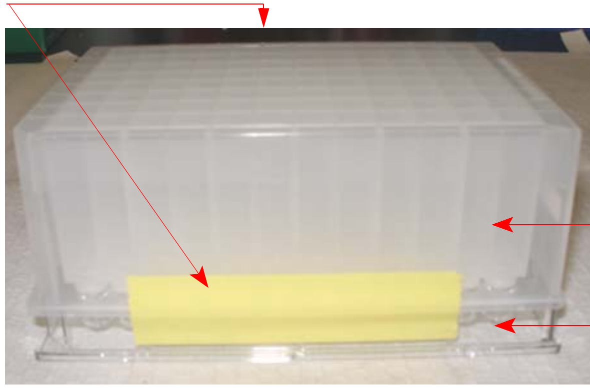


Figure A.3 Attaching the Elution Catch Plate to the Filter Plate

2. Pour 3 mL (48 samples) or 6 mL (96 samples) of Buffer EB to a solution basin.
3. Using a 12-channel P200 pipette, add 55 µL of Buffer EB to each well.

   For accurate pipetting, pre-wet pipette tips with EB before dispensing. Dispense as close to the beads as possible without touching them. Buffer EB should be applied directly on top of the beads (see Figure A.4 and Figure A.5 on page 306).

> **NOTE: If the volume of eluate in Step 13 on page 308 is < 47 µL, increase the amount of Buffer EB used in this step the next time you perform the protocol. You can increase from 55 to 60 µL (total not to exceed 60 µL).**

4. Tap the plate stack to move all Buffer EB onto the filter at the bottom of the wells.
5. Using an adhesive film, tightly seal the filter plate on the plate stack.
6. Place the plate stack on a Jitterbug for 10 min at *setting 5*.

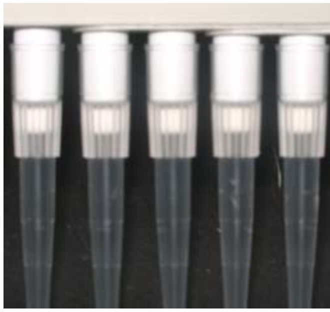


Figure A.4 Ridge on Rainin Pipette Tips

Figure A.5 Adding Buffer EB to Reactions on the Filter Plate

7. Inspect each well to verify that the beads are thoroughly resuspended.

The beads must be thoroughly resuspended in Buffer EB so that the DNA can come off the beads.

8. Remove the plate stack from the Jitterbug and remove the adhesive seal.

9. Continue elution on the vacuum manifold as follows:

A. Remove the manifold cover and insert the plate stack.

B. Seal any empty wells with adhesive film.

C. Place the ***deep well*** collar over the plate stack (Figure A.6 on page 307).

D. Turn the vacuum on to 20 to 24 in Hg and ensure that a seal has been formed between the collar and the base of the manifold.

E. Ensure that the unused wells are completely sealed.

F. Cover the plate stack to protect it from environmental contaminants.

G. Run the vacuum until all of the liquid has been pulled through the filter (approximately 15 to 30 min).

H. Turn off the vacuum.

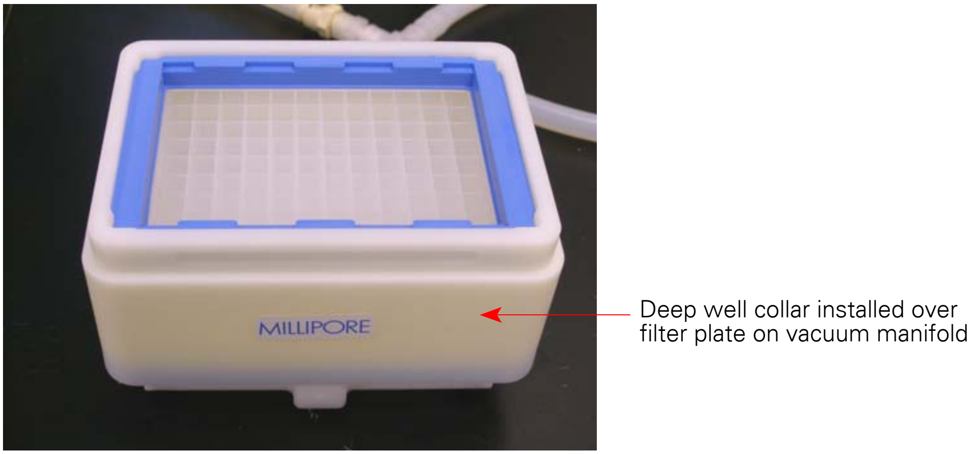


Figure A.6 Plate Stack Inside Vacuum Manifold with Deep Collar

10. Examine each well.

Again, there should be no standing liquid in any well, and the wells should appear dull (matte). Wet wells will look shiny.

If any of the wells are still wet, continue filtering for up to 15 additional min.

11. Seal the plate stack with an adhesive film, and spin it down at room temperature for 5 min at 1400 rcf.

Use the following formula to convert relative centrifugal force (rcf) to revolutions per minute (rpm):

rpm = 1000 × square root(rcf/1.12r)

The radius, *r*, is equal to the distance in millimeters between the axis of rotation of the centrifuge and the bottom of the plate bucket.

For example, on the Eppendorf 5804R, spinning at 3100 rpm gives an rcf of 1400 (assuming r = 133 mm).

12. Remove the elution catch plate from the filter plate.

13. Using a 12-channel P200 pipette, transfer 45 µL of eluate to a new PCR plate for fragmentation.

> **NOTE: If the volume of eluate is < 47 µL, increase the amount of Buffer EB used for elution the next time you perform the protocol. You can increase from 55 to 50 µL (total not to exceed 60 µL).**
>
> **See also the Caution on on page 298, and on page 279 of Chapter 8 *Troubleshooting* for more information.**

What To Do Next

Take an OD measurement using 2 µL from the remaining eluate as described below.

Do one of the following:

- If following the recommended workflow, seal the plate containing the eluate and store it overnight at –20 °C.
- Proceed directly to *Stage 9: Fragmentation* for 48 or 96 samples.

Appendix B

Reagents, Equipment, and Consumables

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 Affymetrix® Genome-Wide Human SNP Nsp/Sty 5.0/6.0 Assay.

> **IMPORTANT: Use only the 96-well plate and adhesive seals listed in Table B.6, and only the thermal cyclers listed in Table B.7. Using other plates and seals that are incompatible with these thermal cyclers can result in loss of sample or poor results.**

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

Reagents

Affymetrix Reagents Required

The Affymetrix® Genome-Wide Human SNP Nsp/Sty Assay Kit 5.0/6.0 is required to perform this protocol. The kit is available in two sizes: 100 or 50 reactions.

Table B.1 Affymetrix® Genome-Wide Human SNP Nsp/Sty Assay Kit 5.0/6.0

Kit Contents	Part Number
Reference Genomic DNA 103, 50 ng/μL (use as a positive control)	Included
Box 1:	100 reactions: 901015 50 reactions: 901152
• Adaptor Nsp I, 50 μM	
• PCR Primer 002, 100 μM	
Box 2:	
• Adaptor Sty I, 50 μM	
• PCR Primer 002, 100 μM	
Box 3:	
• Oligonucleotide Control Reagent, 0100	
• GeneChip® DNA Labeling Reagent, 30 mM	
• Terminal Deoxynucleotidyl Transferase, 30 U/μL	
• 5X Terminal Deoxynucleotidyl Transferase Buffer	
• 10X Fragmentation Buffer	
• GeneChip® Fragmentation Reagent (see label on tube for U/μL concentration)	

New England Biolabs Reagents Required

Table B.2 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 • NEBuffer 2; NEB P/N B7002S The BSA and NEBuffer 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 • NEBuffer; NEB P/N B7003S The BSA and NEBuffer 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 B.3 Other Reagents Required for the Genome-Wide Human SNP Nsp/Sty 5.0/6.0 Assay

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 rxns (enough for 96 Sty samples) 639243 – 400 rxns (enough for 96 Nsp samples)
TITANIUM™ *Taq* DNA Polymerase (50X) and TITANIUM™ *Taq* PCR Buffer	Clontech	Contains: • 50X Clontech TITANIUM™ *Taq* DNA Polymerase • 10X Clontech TITANIUM™ *Taq* PCR Buffer	P/N 639209 (also in kit P/N 639240 or 639243 above)
GC-Melt	Clontech	5 M	639238 (also in kit P/N 639240 or 639243 above)
Beads, Magnetic	Agencourt	SNPClean	A31944, 75 mL
Buffer EB (250 mL)	Qiagen	250 ml Elution Buffer	19086
dNTPs*	Included in the Clontech TITANIUM DNA Amplification Kit listed above.		
	Takara	mixture of dATP, dCTP, dGTP, dTTP at 2.5 mM each	4030
	Fisher Scientific		TAK 4030
Denhardt's Solution	Sigma-Aldrich		D2532
DMSO	Sigma-Aldrich		D5879
Ethanol	Sigma-Aldrich	ACS reagent, ≥ 99.5% (200 proof), absolute	459844
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
Tetramethyl Ammonium Chloride (TMACL; 5M)	Sigma-Aldrich	5M	T3411

Table B.3 (Continued) Other Reagents Required for the Genome-Wide Human SNP Nsp/Sty 5.0/6.0 Assay

Reagent	Vendor	Description	Part Number
Tween-20, 10%	Pierce	10%, diluted to 3% in molecular biology-grade water	28320 (Surfact-AmpsQ®)
Water, AccuGENE®	Lonza	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

This protocol has been optimized using the following equipment and software.

Affymetrix Equipment and Software Required

Table B.4 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
Instrument control software (one of the following applications): • GeneChip® Operating Software • Affymetrix GeneChip® Command Console	Latest version
Affymetrix Genotyping Console™	—

* *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 B.5 Other Equipment Required to Run the Genome-Wide Human SNP Nsp/Sty 5.0/6.0 Assay

Equipment		Quantity	Manufacturer/ Distributor	Part Number	Laboratory Location
Collar, Multiscreen	Deep well for vacuum manifold	1	Millipore	MSVMHTS0D	Main Lab
Cooler (–20 °C)	StrataCooler® Lite Benchtop	2	Stratagene	400012	Pre-PCR and Main Lab
	StrataCooler® II Benchtop			400002 (blue) 400008 (red)	
Cooling chamber/ block Either BioSmith or Diversified Biotech can be used.	BioSmith96-well aluminum block	7	BioSmith	81001	5 in PCR Staging Area; 2 in Main Lab
	single gold block	3 double 1 single	Diversified Biotech	CHAM-1000 (single)	2 double and 1 single in PCR Staging Area; 1 double in Main Lab
	double gold block			CHAM-1020 (double)	
Ice bucket (4 to 9 liters)	—	2	—	—	Pre-PCR and Main Lab
Jitterbug™ Microplate Incubator Shaker		1	In the U.S.A.: Fisher Scientific	11-701-13	Main Lab
			In the U.S.A.: VWR	35821-065	
			In the U.S.A. and all other countries: Boekel Scientific	130000 (115V) 130000-2 (230V)	
Vacuum Manifold, MultiScreenHTS		1	Millipore	MSVMHTS00	Main Lab
Microcentrifuge, PicoFuge® (maximum rotation 6000 rpm)		2	Stratagene	400550	Pre-PCR and Main Lab
Pipet-Lite™ , Magnetic-Assist single channel P20		2	Rainin	L-20	Pre-PCR and 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

Table B.5 (Continued) Other Equipment Required to Run the Genome-Wide Human SNP Nsp/Sty 5.0/6.0 Assay

Equipment	Quantity	Manufacturer/ Distributor	Part Number	Laboratory Location
Pipet, 12-channel P20 (accurate to within ± 5%)	2	Rainin	P/N L12-20	Pre-PCR and Main Lab
Pipet, 12-channel P100	2	Rainin	P/N L12-100	Pre-PCR and Main Lab
Pipet, 12-channel P200	2	Rainin	P/N L12-200	Pre-PCR and Main Lab
Pipet, 12- or 8 channel P1200	1	Rainin	P/N	Main Lab
Plate Centrifuge, multipurpose (must be deep well in Main Lab)	1	Eppendorf	5804 or 5810	Pre-PCR
Plate Centrifuge, multipurpose, deep well (must accommodate plates 54mm height; 160g weight)	1	Eppendorf	5804 or 5810	Main Lab
Plate holders	9	USA Scientific	2300-9602	7 Main Lab
Spectrophotometer, high throughput microplate spectrophotometer	1	Molecular Devices	SpectraMax Plus384	Main Lab
Thermal Cyclers – see Table B.7 on page 316				
Vortexer, for plates and tubes (must have plate pad)	2	VWR	58816-12	Pre-PCR and Main Lab

Thermal Cyclers, PCR Plates and Plate Seals

Quantity Required

Five thermal cyclers are required for this protocol:

- One in the PCR Staging Room
- Four in the Main Lab

Vendor and Part Number Information

This protocol has been optimized using the following thermal cyclers, PCR plate and adhesive films.

! IMPORTANT: Use only the 96-well plate and adhesive seals listed in Table B.6, and only the thermal cyclers listed in Table B.7. Using other plates and seals that are incompatible with these thermal cyclers can result in loss of sample or poor results.

Table B.6 96-well plate and adhesive seals optimized for use with this protocol

Item	Vendor	Part Number
Multiplate 96-well unskirted PCR plate	Bio-Rad	MLP-9601
Adhesive seals:		
• Microseal 'B' Adhesive Seal	Bio-Rad	MSB1001
• MicroAmp® Clear Adhesive Film	Applied Biosystems	4306311

Table B.7 Thermal cyclers optimized for use with this protocol

Laboratory	Thermal Cyclers Validated for Use
Pre-PCR Clean Area	Applied Biosystems units: • 2720 Thermal Cycler • GeneAmp® PCR System 9700
	Bio-Rad units: • MJ Tetrad PTC-225 • DNA Engine Tetrad 2
Post-PCR Area	Applied Biosystems: • GeneAmp® PCR System 9700 (silver block or gold-plated silver block)
	Bio-Rad units: • MJ Tetrad PTC-225 • DNA Engine Tetrad 2

Consumables Required

Arrays Required

This protocol requires the use of the Affymetrix® Genome-Wide Human SNP Array 6.0.

Table B.8 Affymetrix® Genome-Wide Human SNP Array 6.0

Arrays/Pack	Part Number
50	901153
100	901150

Gels and Gel Related Materials Required

Use either standard gels (Table B.9) or E-Gels (Table B.10 on page 318).

Table B.9 Standard Gels and Related Materials

Item	Vendor	Part Number
Gel, Reliant® Gel System, precast agarose gel (2% SeaKem Gold, TBE) or 4% NuSieve 3:1 Plus, TBE Buffer, 8 bp = 1 kb 2 x 12 wells, ethidium bromide	Lonza	54939 57226 (100 wells) 54929 (24 wells) 57225 (100 wells)
All Purpose Hi-Lo DNA Marker	Bionexus	BN2050
Gel Loading Buffer	Sigma-Aldrich	G2526

Table B.10 E-Gels and Related Materials

Item	Vendor	Part Number
Mother E-Base™	Invitrogen	EB-M03
Daughter E-Base™	Invitrogen	EB-D03
E-Gel® *48* 2% agarose gel, 8 pack	Invitrogen	G8008-02
E-Gel® *48* 4% agarose gel, 8 pack	Invitrogen	G8008-04
25 bp DNA Ladder (used with E-Gel *48* 4%)	Invitrogen	10597-011
5X SB loading medium (used with E-Gel *48* 4%)	Faster Better Media	SB5N-8
All Purpose Hi-Lo DNA Marker (used with E-Gel *48* 2%)	Bionexus	BN2050
Gel Loading Buffer (used with E-Gel *48* 2%)	Sigma-Aldrich	G2526

Other Consumables Required

Table B.11 Other Consumables Required for the Genome-Wide Human SNP Nsp/Sty 5.0/6.0 Assay

Item	Manufacturer/ Distributor	Part Number	Laboratory Location
Pipet tips As needed for pipets listed in Table B.5.	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 B.6 on page 316			Pre-PCR and Main Lab
Plates, 96-well PCR – see Table B.6 on page 316			Pre-PCR and Main Lab
Microplate, 96-well, conical bottom (Elution Catch Plate)	In the U.S.A. only: E & K Scientific	EK-21101	Main Lab
	All other countries: Greiner Bio-One	651101	

Table B.11 (Continued) Other Consumables Required for the Genome-Wide Human SNP Nsp/Sty 5.0/6.0 Assay

Item	Manufacturer/ Distributor	Part Number	Laboratory Location
Filter Plate - use one of the following:			Main Lab
• Multiscreen Deep Well Solvinert philic PTFE 0.45 µ	Millipore	MDRLN0410	
• Plate, 2ml, 48 or 96 Well Format Filterplate (PES 0.45 µm) Hydrophilic, Long Drip Director	In the U.S.A. only: E & K Scientific All other countries: Seahorse Bioscience	XP0251 (48) XP0228 (96)	
Deep Well Storage Plate, 2.4 mL (Pooling Plate)	In the U.S.A. only: E & K Scientific	EK-22280	Main Lab
	All other countries: Greiner Bio-One	780280	
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, Falcon, 50 mL	VWR	21008-940	Pre-PCR

Supplier Contact List

Table B.12 Supplier Contact List

Supplier	Web Site Address
Affymetrix	www.affymetrix.com
Agencourt Bioscience Corp.	agencourt.com
Applied Biosystems	www.appliedbiosystems.com
Bionexus Inc.	www.bionexus.net
Bio-Rad	bio-rad.com
Boekel Scientific	www.boekelsci.com
CLP Direct	clpdirect.com
Clontech	www.clontech.com
Diversified Biotech	divbio.com
E&K Scientific	eandkscientific.com
Eppendorf	eppendorf.com
Faster Better Media	fasterbettermedia.com
Fisher Scientific	www.thermofisher.com
Greiner Bio-One	www.gbo.com
ISC Bioexpress	iscbioexpress.com
Invitrogen Life Technologies	invitrogen.com
Labcor	labcorproducts.com
Lonza	www.lonza.com
Millipore	millipore.com
Molecular Devices	moleculardevices.com
New England Biolabs	www.neb.com
Pierce Biotechnology (part of Thermo Fisher Scientific)	piercenet.com
Promega	www.promega.com
Rainin	www.rainin.com
Seahorse Bioscience	www.seahorselabware.com

Table B.12 (Continued) Supplier Contact List

Supplier	Web Site Address
Sigma-Aldrich	www.sigma-aldrich.com
Stratagene	stratagene.com
Takara Bio Inc.	www.takara-bio.com
Teknova	teknova.com
USA Scientific	www.usascientific.com
VWR	vwr.com

Appendix C

THERMAL CYCLER PROGRAMS

This appendix includes the thermal cycler programs required for the Affymetrix® Genome-Wide Human SNP Nsp/Sty 5.0/6.0 Assay.

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

GW5.0/6.0 Digest

GW5.0/6.0 Digest Program	
Temperature	Time
37°C	120 min
65°C	20 min
4°C	Hold

GW5.0/6.0 Ligate

GW5.0/6.0 Ligate Program	
Temperature	Time
16°C	180 min
70°C	20 min
4°C	Hold

GW5.0/6.0 PCR

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

GW5.0/6.0 PCR Program for GeneAmp® PCR System 9700		
Temperature	Time	Cycles
94°C	3 min	1X
94°C	30 seconds	30X
60°C	45 seconds	
68°C	15 seconds	
68°C	7 min	1X
4°C	HOLD (Can be held overnight)	

For the MJ Tetrad PTC-225 and Tetrad 2

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

GW5.0/6.0 PCR Program for MJ Tetrad PTC-225 and Tetrad 2		
Temperature	Time	Cycles
94°C	3 min	1X
94°C	30 seconds	30X
60°C	30 seconds	
68°C	15 seconds	
68°C	7 min	1X
4°C	HOLD (Can be held overnight)	

GW5.0/6.0 Fragment

GW5.0/6.0 Fragment Program	
Temperature	**Time**
37°C	35 min
95°C	15 min
4°C	Hold

GW5.0/6.0 Label

GW5.0/6.0 Label Program	
Temperature	**Time**
37°C	4 hours
95°C	15 min
4°C	Hold
Samples can remain at 4 °C overnight.	

GW5.0/6.0 Hyb

GW5.0/6.0 Hyb Program	
Temperature	**Time**
95°C	10 min
49°C	Hold

Appendix D

E-GELS

This appendix describes the use of E-GelS® for confirming:

- Sty and Nsp PCR reactions
- Fragmentation reactions

Before Using E-Gels

When Using the E-Gel *48* 2%

Use the following reagents:

- Loading solution: Gel Loading Buffer from Sigma-Aldrich

  Dilute this solution to 1:20 or 1:30 using H_2O before use.
- DNA Marker: All Purpose Hi-Lo DNA Marker from Bionexus

  Dilute this marker 1:3 with H_2O before use.

For more information, refer to Appendix B, *Reagents, Equipment, and Consumables*.

When Using the E-Gel *48* 4%

Use the following reagents:

- Loading solution: 5xSB Loading Medium from Faster Better Media

  Dilute this solution to 1:20 or 1:30 with H_2O before use.
- DNA Marker: 25 bp DNA Ladder from Invitrogen

  5xSB Loading Medium contains Orange-G. Because Orange-G is known to affect DNA migration slightly, and because E-Gels are salt sensitive, dilute the ladder and samples with the same loading solution.

For more information, refer to Appendix B, *Reagents, Equipment, and Consumables*.

Modifications for Stage 3: Sty PCR

Follow the Stage 3 instructions listed in *Stage 3: Sty PCR* on page 49 with the modifications listed below.

Gels and Related Materials Required

Reference Table 4.17 on page 51**.** The amounts listed are sufficient to process 48 Sty samples.

Table D.1 E-Gels and Related Materials Required for *Stage 3: Sty PCR*

Quantity	Reagent
180 µL	All Purpose Hi-Lo DNA Marker, diluted 1:3 with H_2O (See *When Using the E-Gel 48 2%* on page 327)
As needed	Gel loading buffer, diluted 1:20 or 1:30 with H_2O (See *When Using the E-Gel 48 2%* on page 327)
3	E-Gel *48* 2% agarose gel
3	Plates, 96-well reaction

Running Gels

Before Running Gels

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

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

Run the Gels

When the GW5.0/6.0 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 12 µL of diluted gel loading buffer to each well in rows A through D of the fresh, labeled P*X*Gel plates.

6. Using a 12-channel P20 pipet, transfer 3 µL of each PCR product from the 3 Sty PCR plates to the corresponding plate, row and wells of the P*X*Gel plates.

   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 the P*X*Gel plates.
8. Vortex the center of each P*X*Gel plate, then spin them down at 2000 rpm for 30 sec.
9. Load the total volume of 15 µL from each well of each PXGel plate onto E-Gel *48* 2% agarose gels.
10. Run the gels for 22 min.
11. Verify that the PCR product distribution is between ~250 bp to 1100 bp (Figure D.1).

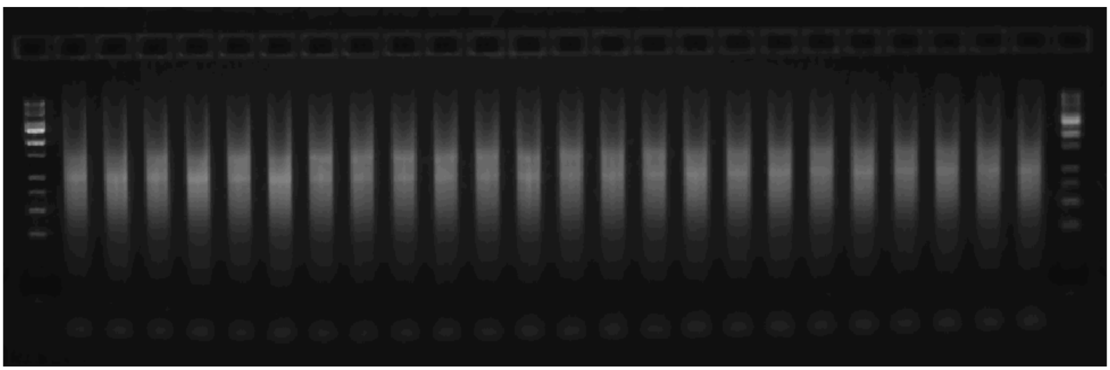


Figure D.1 Example of PCR Products Run on E-Gel *48* 2% Agarose Gel for 22 min. Average Product Distribution is Between ~250 to 1100 bp.

Modifications for Stage 6: Nsp PCR

Follow the Stage 3 instructions in *Stage 6: Nsp PCR* on page 73 with the modifications listed below.

Gels and Related Materials Required

Reference Table 4.31 on page 75. The amounts listed are sufficient to process 48 samples.

Table D.2 E-Gels and Related Materials Required for *Stage 6: Nsp PCR*

Quantity	Reagent
240 µL	All Purpose Hi-Lo DNA Marker, diluted 1:3 with H_2O (See *When Using the E-Gel 48 2%* on page 327)
As needed	Gel loading buffer, diluted 1:20 or 1:30 with H_2O (See *When Using the E-Gel 48 2%* on page 327)
4	E-Gel *48* 2% agarose gel
4	Plates, 96-well reaction

Running Gels

Reference the instructions on page 82.

Before Running Gels

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

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

Run the Gels

When the GW5.0/6.0 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 four fresh 96-well reaction plates *P1Gel*, *P2Gel*, *P3Gel,* and *P4Gel.*
5. Aliquot 12 µL of diluted gel loading buffer to each well in rows A through D of the fresh, labeled P*X*Gel plates.

6. Using a 12-channel P20 pipet, transfer 3 µL of each PCR product from the 4 Nsp PCR plates to the corresponding plate, row and wells of the P*X*Gel plates.

   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 the P*X*Gel plates.
8. Vortex the center of each P*X*Gel plate, then spin them down at 2000 rpm for 30 sec.
9. Load the total volume of 15 µL from each well of each PXGel plate onto E-Gel *48* 2% agarose gels.
10. Run the gels for 22 min.
11. Verify that the PCR product distribution is between ~250 bp to 1100 bp (see Figure D.1 on page 329).

Modifications for Stage 9: Fragmentation

Follow the Stage 9 instructions in *Stage 9: Fragmentation* on page 107 with the modifications listed below.

Gels and Related Materials Required

Reference Table 4.46 on page 109. The amounts listed are sufficient to process 48 samples.

Table D.3 E-Gels and Related Materials Required

Quantity	Reagent
60 µL	25 bp DNA Ladder, diluted 1:15 with pre-diluted 5xSB Loading Medium (See *Before Using E-Gels* on page 327)
As needed	5xSB Loading Medium, diluted (See *Before Using E-Gels* on page 327)
1	E-Gel *48* 4% agarose gel (Invitrogen; P/N G8008-04)

Check the Fragmentation Reaction

Reference the instructions on page 115.

To ensure that fragmentation was successful:

1. When the GW5.0/6.0 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 1.5 µL of each fragmented PCR product with 13.5 µL of diluted 5xSB Loading Medium.
3. Run on E-Gel *48* 4% agarose gels with the 25 bp DNA Ladder for 22 min.

   The colorless 25 bp DNA ladder is diluted 1:15 with diluted 5xSB Loading Medium. Use 15 µL diluted ladder for each marker lane.
4. Inspect the gel and compare it against the example shown in Figure D.2 on page 333.

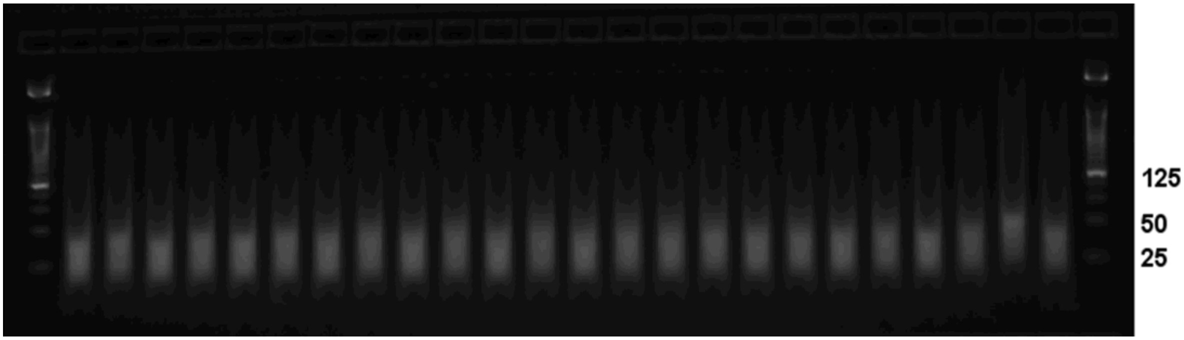


Figure D.2 Typical Example of Fragmented PCR Products Run on an E-Gel *48* 4% Agarose Gel for 22 min.