Affymetrix®

User Manual

CytoScan™ Assay

P/N 703038 Rev. 4

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Cartridge Array Software: **Products may be protected by one or more of the following patents:** U.S. Patent Nos. 5,733,729; 5,795,716; 5,974,164; 6,066,454; 6,090,555; 6,185,561; 6,188,783; 6,223,127; 6,228,593; 6,229,911; 6,242,180; 6,308,170; 6,361,937; 6,420,108; 6,484,183; 6,505,125; 6510,391; 6,532,462; 6,546,340; 6,687,692; 6,607,887; 7,062,092; 7,451,047; 7,634,363; 7,674,587 and other U.S. or foreign patents

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Contents

Introduction

Topics in this chapter include:

About the Affymetrix® CytoScan™ Solution

! IMPORTANT: The CytoScan™ Assay protocol is optimized for processing from 8 to 24 samples at a time to obtain whole genome copy number and SNP information from Affymetrix® CytoScan™ Arrays. This protocol is not intended for Genome Wide Association studies.

Cytogenetics studies are performed to identify structural changes in DNA, such as copy number changes. Individuals typically have two copies of the genome in each of their cells: one inherited from the mother, and one inherited from the father. Chromosomal abnormalities are common in several disease states such as:

- *Deletions*–When one or both copies of a particular chromosome region are lost.
- *Gains*–When a chromosome or chromosomal region is duplicated or multiplied.
- *Uniparental Disomies (UPDs)*–When two copies of a chromosome or chromosomal region are present, but both have been inherited from a single parent.

Traditional cytogenetics techniques, such as karyotyping and fluorescent *in situ* hybridization (FISH) have been used to study chromosomal abnormalities for decades. However, karyotyping only detects abnormalities at low resolutions (larger than ~5 Mb), and FISH is a more focused and targeted approach without the benefit of genome-wide analysis. Further, these techniques are limited to only providing copy number information so that UPDs cannot be identified.

Together, Affymetrix® CytoScan™ Arrays and the CytoScan™ Assay, along with the Command Console and Chromosome Analysis Suite software, enable you to perform high-resolution genome-wide DNA copy number analysis. The Affymetrix solution for cytogenetics also provides genotyping information, enabling detection of loss of heterozygosity (LOH), which can be used to detect UPDs. The combined high resolution DNA copy number data and the ability to detect gains, losses, and UPDs on a single array makes the Affymetrix® CytoScan™ Solution a great tool for next generation cytogenetics studies.

About This Manual

This manual is a guide for technical personnel conducting the Affymetrix® CytoScan™ Assay experiments in the laboratory. It contains:

- Best practices that Affymetrix recommends
- Laboratory setup
- Sample preparation
- Equipment and consumables required for each step
- Step-by-step protocols for the assay
- Protocols for washing, staining, and scanning arrays
- Troubleshooting information
- Fluidics Station care and maintenance
- Guidelines for processing 16 and 24 sample formats
- Protocol for fragmentation QC using the Agilent 2100 Bioanalyzer
- Protocols for E-Gels

Best Practices

This chapter provides tips for ensuring successful performance of the protocol. Topics in this chapter include:

Controls

Using positive and negative controls is recommended to assess the performance of each run. We recommend using the Genomic DNA Control supplied in the CytoScan™ Reagent Kit as a positive control carried through the entire assay up to hybridization on the arrays. We recommend using Low EDTA TE Buffer as a negative control through the PCR gel QC stage only.

Equipment and Calibration

Keep dedicated equipment in each of the areas used for this protocol, including pipettors, ice buckets, coolers, etc. It is critical to use equipment that conforms to the guidelines and specifications detailed in this manual. To avoid contamination, do not move equipment back and forth from the Post-PCR Room to Pre-PCR Clean Room.

Lab instrumentation plays an important role in the successful execution of this assay. To help maintain consistency across samples and operators, all equipment must be well maintained and routinely calibrated per manufacturer recommendations, including:

- All thermal cyclers
- GeneChip® Hybridization Oven 645

- GeneChip® Fluidics Station
- GeneChip® Scanner 3000 7G
- Plate spectrophotometer or NanoDrop
- All single and multi-channel pipettes

Pipetting

Since the CytoScan™ Assay involves a series of ordered stages, the output of one stage directly impacts the performance of the subsequent stage.

To efficiently process samples:

- Always use pipettes that have been calibrated as per the manufacturer's specifications.
- It is essential that operators be proficient with the use of single and multi-channel pipettes.
- Always use filter tips for pipetting. This is essential to reduce sample contamination.

To familiarize yourself with the use of multi-channel pipettes, we strongly recommend practicing several times before processing actual samples. You can use water to get a feel for aspirating and dispensing solutions to multiple wells simultaneously. Take special care to observe complete evacuation of liquid from all pipette tips when using a multi-channel pipette.

Reagent Handling and Storage

IMPORTANT: Always use the 24 reaction CytoScan™ Assay Kit (P/N 901808) for this protocol. You can freeze/thaw the reagents in the 24 reaction kit ≤ 5 times.

Proper storage and handling of reagents is essential for robust performance. Follow these guidelines to ensure best results:

- Use reagents from the recommended vendors only.
- Store all reagents at the recommended temperatures and conditions. Do not use reagents that have been improperly stored. Storage methods can profoundly impact activity.
- Upon receipt of the reagent kit, store the Affymetrix® Nuclease Free water at 4 °C and the Low EDTA TE Buffer at room temperature for your convenience.
- Do not use expired reagents or reagents that have undergone more than the recommended number of freeze-thaw cycles.
- Seal all vials and bottle caps well after use to prevent evaporation.
- Do not store enzymes in a frost-free freezer.

- Store the reagents used for digestion, ligation, and PCR only in the Pre-PCR Clean Area.

When Using Reagents at the Lab Bench

- Properly chill essential equipment such as cooling blocks and reagent coolers before use.
- Unless otherwise indicated, keep all reagents (except enzymes) on ice, or in a cooling block that has been chilled to 4 °C and placed on ice during use.
- Ensure that enzymes are kept at –20 °C until needed. When removed from the freezer, immediately place in a bench top reagent cooler that has been chilled to –20 °C.
- Keep all tubes, master mixes and working solutions in chilled cooling blocks on ice.
- Since enzyme activity is a function of temperature, ensure that all temperature transitions to incubation temperatures are rapid and/or well-controlled to help maintain consistency across samples.

Master Mix Preparation

Carefully follow each master mix recipe. Use pipettes that have been calibrated as per the manufacturer's specifications. Use only the Affymetrix® Nuclease-Free water that is supplied with the kit. Do not use any other water. The enzymatic reaction in *Stage 6: Fragmentation* is particularly sensitive to pH and metal ion contamination.

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

Laboratory Workflow

- Maintain a single direction workflow. Do not re-enter the Pre-PCR Clean Area after entering the Post-PCR Area until you have showered and changed into freshly laundered clothing.
- Never bring amplified products into the Pre-PCR Clean Area.
- Keep dedicated equipment in each room or area used for this protocol. To avoid contamination, do not move equipment between the Pre-PCR Clean Area and the Post-PCR Area.

Seal, Vortex, and Spin

Unless otherwise noted, follow the instructions below when the protocol instructs you to seal, vortex and spin.

Handling the Plate Seal

NOTE: We recommend using MicroAmp® Clear Adhesive Films to seal your plates.

- To minimize sample cross contamination and to ensure tight seals, use each seal only once. NEVER REUSE A SEAL. Discard used seals immediately to avoid contaminating equipment or working surfaces with DNA.
- The seal may become loose due to high temperature in the thermal cycler. Always ensure tight sealing before vortexing a plate.
- Whenever a plate is taken out of the thermal cycler, before continuing on to the next step, ensure that the seal is tight, spin the plate in the centrifuge, then remove the seal and discard.
- Whenever a plate is taken out of the freezer, first thaw the plate, ensure that the seal is tight, centrifuge, and only then remove the plate seal.
- When reaction setup is completed, always use a new seal to seal the plate.
- When applying the seal to a plate, press the seal tightly onto the plate using an adhesive film applicator. Using a plastic lid or a plastic tube rack is a potential source of contamination. Make sure that the seal is tight around all plate/well edges.

IMPORTANT: Always ensure that your plates are tightly sealed. A tight seal will prevent sample loss and cross-well contamination, particularly when plates are being vortexed. NEVER REUSE A SEAL. ALWAYS USE A NEW SEAL.

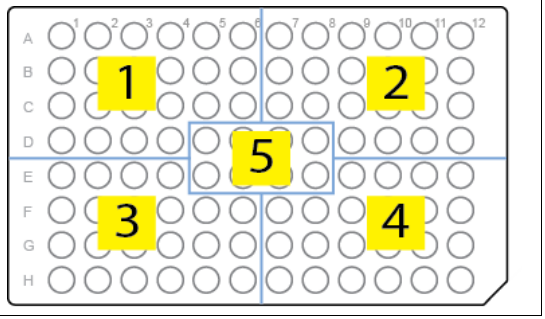
Sealing Strip Tubes

Cut adhesive seal into strips wide enough to seal 8 or 12 strip tubes. Alternatively, strip caps can also be used for sealing. Seal the strip tubes containing master mix with the adhesive strips or strip caps before spinning in the bench top quick spin microfuge.

Vortex

- **Master Mix tubes:** Vortex the master mix at high speed 3 times, 1 second each time.
- **Vortex reagents:** 3 times, 1 second each time.
- **Vortex enzyme:** Quick vortex, 1 second.
- **Vortex plates:** High speed for 1 second. in all corners and in the center (Figure 2.1).

Figure 2.1 Vortex Plates at the Corners and Center



Spin

When instructed to spin down plates or reagent vials, follow these guidelines unless otherwise instructed.

- **Plates:**
  - Spin at room temperature, except for the fragmentation step. During the fragmentation step, spin the plates at 4 °C in a refrigerated centrifuge.
  - Start the centrifuge, allow it to reach 2000 rpm and spin at that speed for 1 minute.
- **Reagent Vials:** 3 seconds using bench top mini-centrifuge
- **Enzyme Vials:** 3 seconds using bench top mini-centrifuge

Fragmentation Step

- Cool the plate centrifuge to 4 °C at least 15 to 20 minutes prior to proceeding with the fragmentation step.
- Pre-chill the reagents, empty tube for master mix and empty strip tube before starting the fragmentation step.
- Leave the Fragmentation Reagent at –20 °C until ready to use.
- All reagent additions in this step must be performed on ice.
- Always carry the sample plate to the centrifuge or the thermal cycler on the cooling block on ice.

Running Gels

- Run gels at 5 V/cm for 45 minutes or until the dye front reaches at least 75% of distance down the gel.
- Be sure to add ethidium bromide to the gel running buffer in the gel box. Add two drops of ethidium bromide per 1L of 1X TBE.

Hybridization

- Load only 6 to 8 arrays at a time. Remove the seal from the hybridization plate for only 6-8 samples at a time.
- Preheat the hybridization oven to 50 °C at least one hour prior to use.

Washing Arrays

It is important to work quickly when processing arrays for washing. Delays during this step will impact data quality. To optimize this step, we suggest the following:

- 30 minutes before hybridization is complete, prime the fluidics stations with the correct wash buffers. Start the Fluidics Protocol and follow the directions on the LCD panel of the fluidics station.
- Load Stain 1, Stain 2, and the Array Holding buffer in their respective positions on the fluidics station. Eject the wash block to avoid sensor time out.
- Process only 6-8 arrays at a time.
- Minimize delays when performing all steps after the arrays are removed from the oven, up to the time when washing begins.

Preparing the Work Area for Each Stage

Many of the stages in the CytoScan™ 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.

Thermal Cyclers, 96-Well Plate, and Adhesive Seals

The CytoScan™ Assay has been optimized using the following thermal cyclers, 96-well plate, and adhesive films.

! **IMPORTANT: Use only the 96-well plate and adhesive seals listed in Table 2.1, and only the thermal cyclers listed in Table 2.2. Using other plates and seals that are incompatible with these thermal cyclers can result in loss of sample or poor results.**

Table 2.1 96-Well Plate and Adhesive Seals Optimized For Use With the CytoScan™ Assay Protocol

Item	Vendor	Part Number
Multiplate 96-well unskirted PCR plate	Bio-Rad	MLP-9601
MicroAmp® Clear Adhesive Film	Applied Biosystems	4306311

Table 2.2 Thermal Cyclers Optimized For Use With the CytoScan™ Assay Protocol

Laboratory	Thermal Cyclers Validated for Use
Pre-PCR Clean Area Use one of these units.	Applied Biosystems Units: ▪ 2720 Thermal Cycler ▪ GeneAmp® PCR System 9700
Post-PCR Area	Applied Biosystems GeneAmp® PCR System 9700 (silver block or gold-plated silver block)

Program Your Thermal Cyclers

Use only calibrated thermal cyclers. We recommend that thermal cyclers be serviced at least once per year to ensure that they are operating within the manufacturer's specifications. The thermal cycler programs listed in Table 2.3 and Table 2.4 are used in this protocol. Enter and store these programs on the appropriate thermal cycler in the Pre-PCR Clean Area and the Post-PCR Area.

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

Table 2.3 Pre-PCR Clean Area

# of Thermal Cyclers Required	Program Name
1	CytoScan Digest
	CytoScan Ligate

Table 2.4 Post-PCR Area

# of Thermal Cyclers Required	Program Name
1	CytoScan PCR
	CytoScan Fragment
	CytoScan Label
	CytoScan Hyb

Hybridization Oven

Confirm that the GeneChip® Hybridization Oven 645 is calibrated before starting the hybridization step. Accurate hybridization temperature is critical for this assay. We recommend servicing hybridization ovens at least once per year to ensure that they are operating within the manufacturer's specifications.

Laboratory Setup and Recommendations

This chapter provides an overview of two laboratory setups that can be used when performing the Affymetrix® CytoScan™ Assay.

IMPORTANT: **If possible, we strongly recommend using two separate rooms when performing this protocol.**

Configuration 1 — Two Separate Rooms

The use of two separate rooms greatly reduces the risk of sample contamination due to previously-amplified PCR products. These rooms are referred to as the:

- Pre-PCR Clean Room
- Post-PCR Room

The high-level steps performed in each room are presented in Table 3.1.

Table 3.1 Assay Workflow When Two Separate Rooms are Used

Room	Template (Genomic DNA)	PCR Product
Pre-PCR Clean Room Assay steps: ▫ Genomic DNA preparation ▫ Digestion ▫ Ligation ▫ PCR setup only		
Post-PCR Room Assay steps: ▫ PCR thermal cycling ▫ Fragmentation ▫ Labeling ▫ Hybridization ▫ Washing and staining ▫ Scanning		

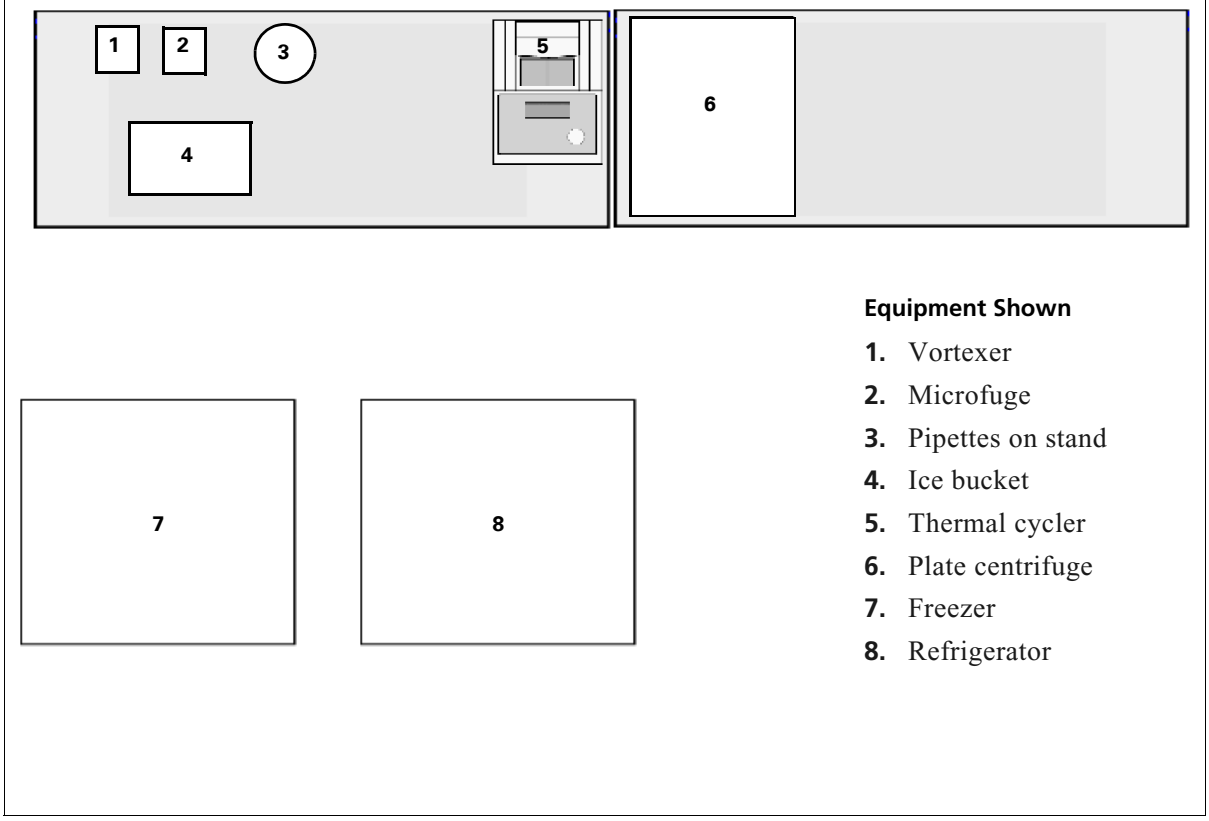
Pre-PCR Clean Room

The Pre-PCR Clean Room should be a low copy DNA template lab, and should be free of PCR product (amplicons). The major pieces of equipment required for this room are shown in Figure 3.1. The Post-PCR Room has airborne contamination with PCR product and template. After entering the Post-PCR Room, do not re-enter the Pre-PCR Clean Room without first showering and changing into freshly laundered clothes.

Activities that take place in this room include:

- Preparation of non-amplified genomic DNA.
- Digestion and ligation reactions.
- Preparation of PCR reactions.

Figure 3.1 Pre-PCR Clean Room



To help prevent sample contamination:

- All of the reagents and master stocks required for the steps performed in the Pre-PCR Clean Room should be stored in this room under the appropriate conditions.

- All of the equipment required for the steps performed in this room should be dedicated. Do not move any equipment including ice buckets and pipettes between the Pre- and the Post-PCR Rooms.
- Always wear a fresh gown, booties, and gloves to prevent PCR carryover, and to minimize the risk of trace levels of contaminants being brought into the room.

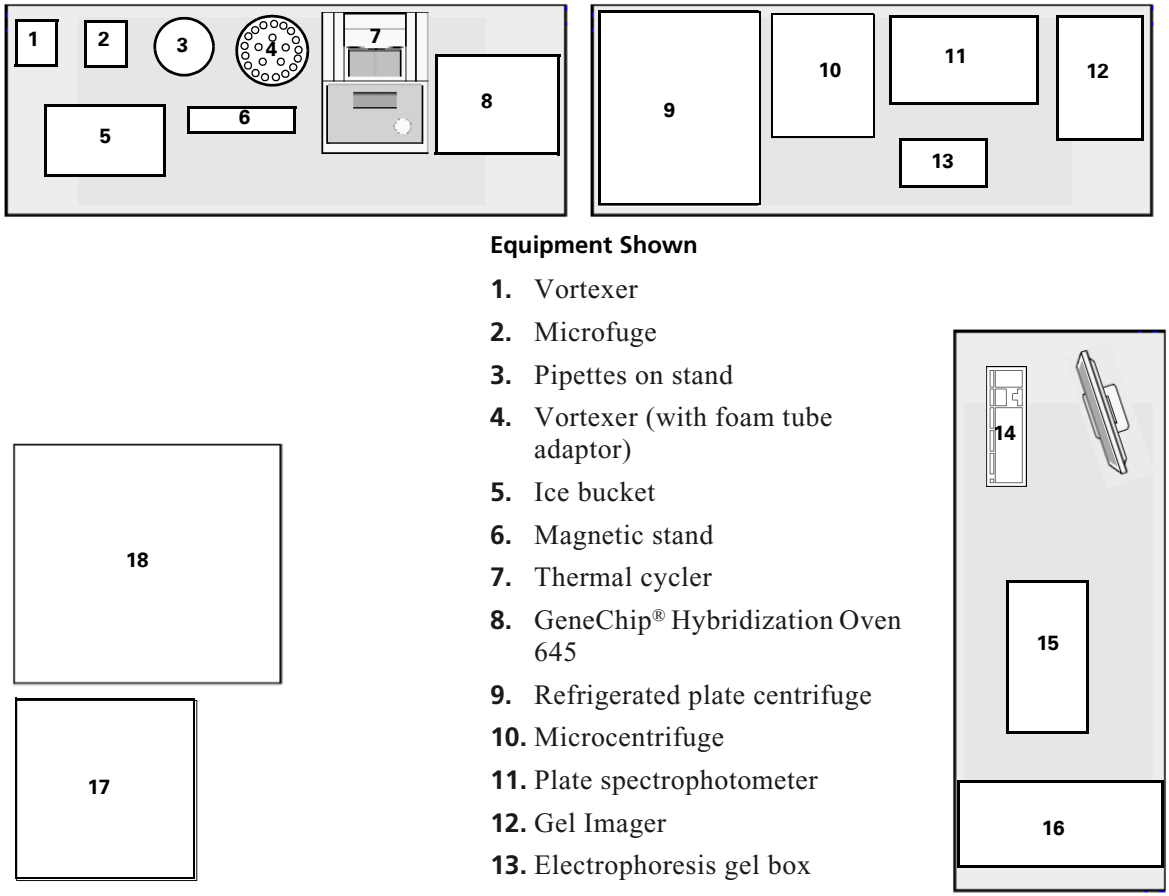
Post-PCR Room

Activities that take place in this room include:

- PCR amplification.
- PCR product purification and quantitation.
- PCR product fragmentation and labeling.
- Sample hybridization onto arrays.
- Washing and staining of arrays.
- Scanning of arrays.

The major pieces of equipment required for this room are shown in Figure 3.2.

Figure 3.2 Post-PCR Room



Equipment Shown

1. Vortexer
2. Microfuge
3. Pipettes on stand
4. Vortexer (with foam tube adaptor)
5. Ice bucket
6. Magnetic stand
7. Thermal cycler
8. GeneChip® Hybridization Oven 645
9. Refrigerated plate centrifuge
10. Microcentrifuge
11. Plate spectrophotometer
12. Gel Imager
13. Electrophoresis gel box
14. Computer, monitor, keyboard
15. Fluidics Station
16. Scanner
17. Refrigerator
18. Freezer

To help prevent sample contamination:

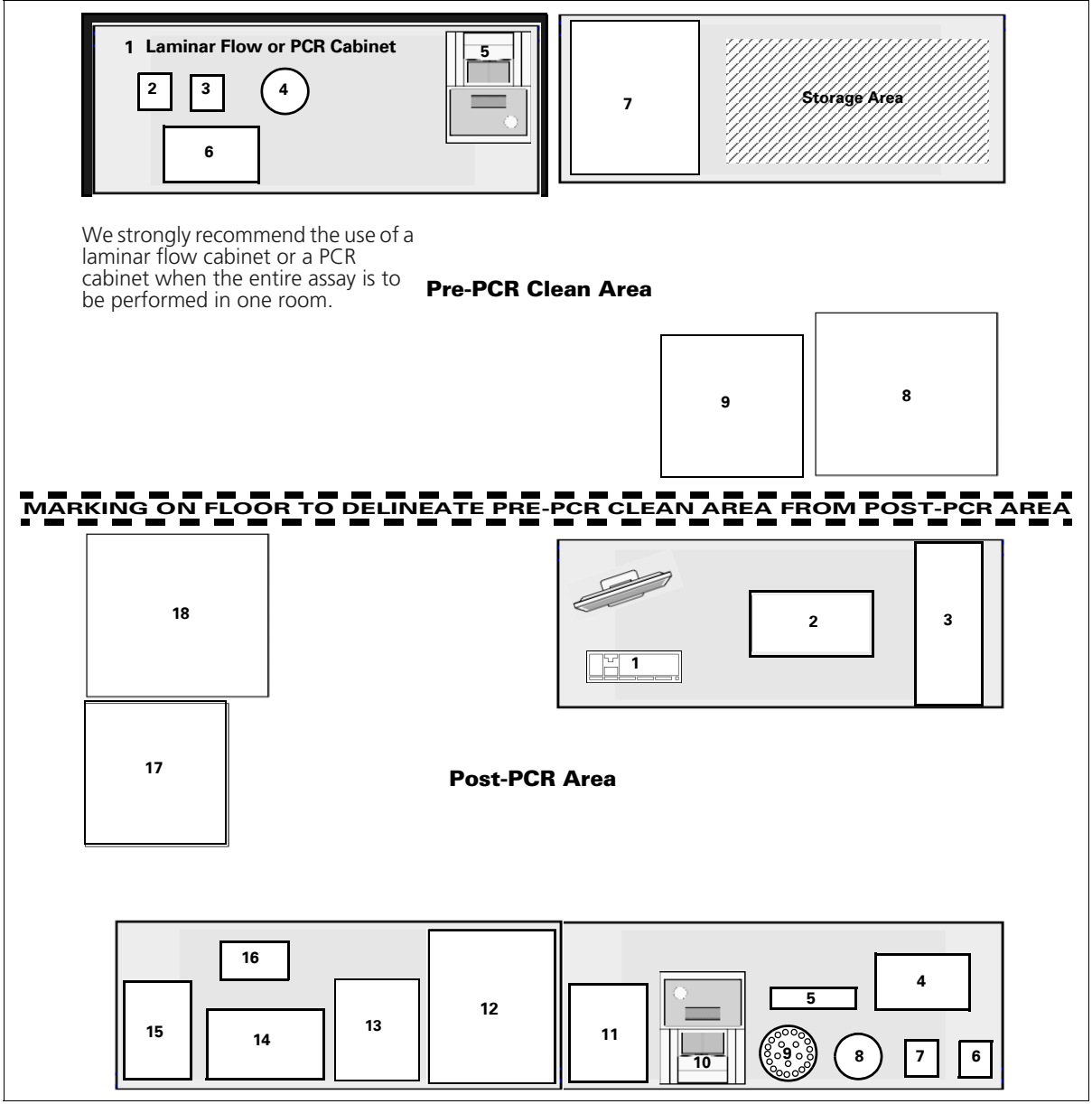
- All of the reagents and master stocks required for the steps performed in the Post-PCR Room should be stored in this room under the appropriate conditions.
- All of the equipment required for the steps performed in this area should be dedicated. Do not move any equipment including ice buckets and pipettes between the Pre- and Post-PCR Rooms.

- Always wear a fresh gown and gloves to minimize sample contamination.

Configuration 2 — One Room

One room with two distinctly separated areas: *Pre-PCR Clean Area* and *Post-PCR Area.*

Figure 3.3 One Room Configuration



Pre-PCR Clean Area

For the best results, adhere to the following guidelines.

- Keep the Pre-PCR Clean Area free of PCR amplicons.
- If both pre- and post-PCR operations are performed in the same room and a laminar flow cabinet is used, keep it turned on at all times.
- Keep the UV light in the laminar flow or PCR cabinet turned on when not in use.
- Always wear a gown, booties, and gloves to prevent PCR carryover, and to minimize the risk of trace levels of contaminants being brought into this area.

Equipment in Pre-PCR Clean Area

The equipment shown for the Pre-PCR Clean Area in Figure 3.3 on page 15 is listed below.

1. Laminar flow cabinet or PCR cabinet
2. Vortexer
3. Microfuge
4. Pipettes on stand
5. Ice bucket with ice
6. Thermal cycler
7. Plate centrifuge
8. Freezer
9. Refrigerator

About Laminar Flow Cabinets

The air curtain from the laminar flow cabinet prevents the introduction of contaminants from the surrounding air into work area, particularly PCR products from the Post-PCR Area. Open master stocks of PCR primer and adaptor only in the laminar flow cabinet.

! **IMPORTANT: We strongly recommend that each pre-PCR step be performed in a laminar flow or PCR cabinet, including reagent and master mix preparation. The use of this cabinet is essential for preventing sample contamination due to the introduction of PCR products from the Post-PCR Area and DNA template.**

All of the equipment required for the pre-PCR steps should be dedicated for pre-PCR and kept in the laminar flow or PCR cabinet. This equipment includes pipettes and tips, the thermal cycler, and vortexer.

Post-PCR Area

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

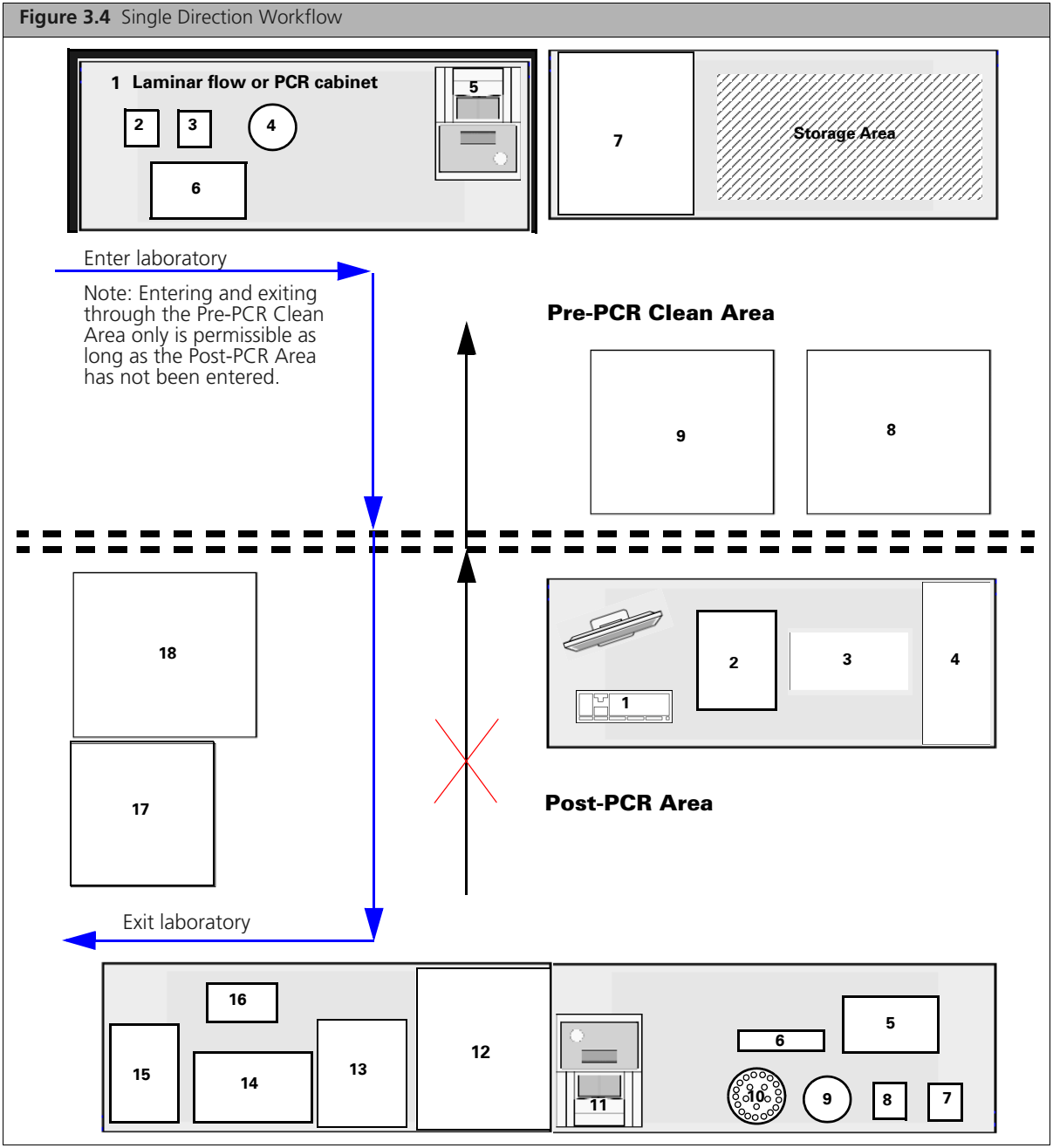
The equipment shown for the Post-PCR Area in Figure 3.3 on page 15 consists of:

1. Computer, monitor and keyboard
2. Fluidics station
3. Scanner
4. Ice bucket
5. Magnetic stand
6. Vortexer
7. Microfuge
8. Pipettes on stand
9. Vortexer with foam tube adaptor
10. Thermal cycler (one to three)
11. GeneChip® Hybridization Oven 645
12. Refrigerated plate centrifuge
13. Microcentrifuge
14. Plate spectrophotometer
15. Gel imager
16. Electrophoresis gel box
17. Electrophoresis power supply
18. Refrigerator
19. Freezer

Single Direction Workflow

To keep the Pre-PCR Clean Area as free from PCR amplicons and other contaminants as possible, always maintain a single direction workflow.

Figure 3.4 Single Direction Workflow



Contamination Prevention

Care should be taken to minimize possible sources of contamination that could interfere with copy number and genotyping analysis. To reduce the possibility of cross-contamination, Affymetrix strongly recommends that you maintain a single direction workflow: from the Pre-PCR Clean Area to the Post-PCR Area. *Do not re-enter the Pre-PCR Clean Area from the Post-PCR Area*.

The most likely potential source of contamination for the CytoScan™ Assay is previously amplified PCR product. Precautions that you can take to minimize contaminating pre-PCR steps with amplified PCR product include the following:

- Each area should contain dedicated equipment such as thermal cyclers, microfuges, pipettes and tips, ice buckets, etc.
- Once you enter the Post-PCR Area, do not return to the Pre-PCR Clean Area until you have showered and changed into freshly laundered clothing.
- Maintain an ambient laboratory environment throughout the procedure.
- Store reagents under appropriate conditions according to the box label and reagent kit insert.
- Use proper gowning procedures.
- Print separate copies of the protocol for each room.
- Use filter tips for all pipetting steps.

Safety Precautions

The Affymetrix® CytoScan™ Assay Kit as well as the Affymetrix® CytoScan™ Arrays 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.

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.

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 150 to 2000 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.

For this protocol, you will use the Affymetrix® CytoScan™ Assay Kit (24 sample, P/N 901808). This kit contains the control Genomic DNA. This control meets the requirements outlined below. The size of the starting genomic DNA can be compared with the control Genomic DNA to assess the quality. The control Genomic DNA should also be used as a routine experimental positive control 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 and Recommendations

- DNA must be double-stranded (not single-stranded). This can be verified using PicoGreen® quantitation. 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 6, *CytoScan™ Assay 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 degraded.

  The genomic DNA fragment must have Nsp 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 0.8% or 1% agarose gel using an appropriate size standard control. Control Genomic DNA 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.

Pre-amplification methods or pre-digestion with restriction enzymes other than Nsp I have not been tested by Affymetrix. If other methods are desired, we recommend conducting experiments to evaluate their performance with this 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 and Recommendations*.

- Blood
- Cell line

Blood Collection Methods

The two blood collection methods that have been shown to be compatible with the assay are EDTA and Heparin.

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:

- QIAGEN – Gentra Puregene Kit
- 5 PRIME – PerfectPure DNA Blood Kit

IMPORTANT: **The CytoScan™ Assay requires genomic DNA concentration ≥ 50 ng/uL. Therefore, the elution volumes for each of the kits will need to be adjusted accordingly to achieve the desired concentration.**

RNase Treatment

The presence of RNA and free nucleotides can interfere with some quantitation methods using specrophotometer or a NanoDrop instrument. To eliminate RNA contamination, perform RNase treatment during extraction as follows:

- QIAGEN – Gentra Puregene Kit

  Perform RNase treatment as recommended in the extraction kit manual prior to elution of genomic DNA.
- 5 PRIME – PerfectPure DNA Blood Kit

  Use only RNase-treated purification columns for extraction of genomic DNA.

The purified genomic DNA extracted using the two methods above should meet the DNA quality specifications per the manufacturer's kit extraction manual.

Assay Overview

This chapter provides an overview of the Affymetrix® CytoScan™ Assay, including information about assay configuration and workflows. It briefly explains each step of the assay and lists the required equipment and consumables.

The CytoScan™ Assay protocol is optimized for processing 8 to 24 samples at a time to obtain whole-genome copy number results and SNP information. This protocol is not intended for genome-wide association studies.

Assay and Reagent Configuration

This protocol has been optimized for processing 8 to 24 samples. The illustrations in this chapter are based on running 8 samples: 6 genomic DNA samples, plus 1 positive and 1 negative control. Use these illustrations as guidelines when processing 8 or fewer samples.

If processing more than 8 samples, refer to Appendix A, *Guidelines for Processing 16 Samples* or Appendix B, *Guidelines for Processing 24 Samples*. Important guidelines for plate layouts are included in these appendices.

CytoScan™ Assay Kit— 24 Reactions

Always use the 24 reaction CytoScan™ Reagent Kit (P/N 901808) for this protocol. This kit has been tested for multiple freeze/thaw cycles. You can freeze/thaw the reagents in the 24 reaction kit ≤ 5 times.

Equipment, Consumables, and Other Reagents

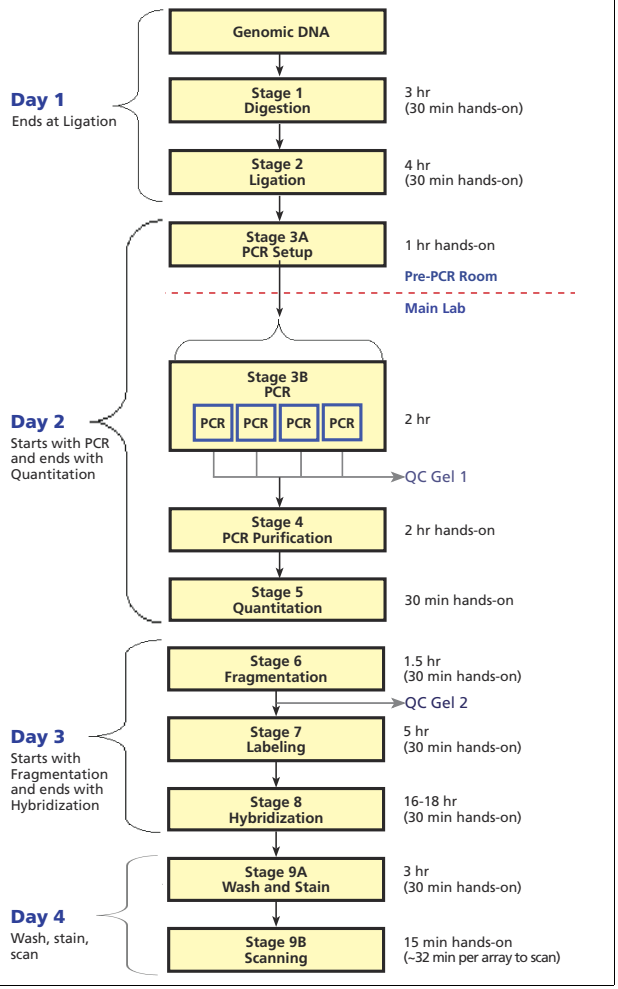
This protocol has been optimized using the equipment, consumables and reagents listed herein. For best results, we strongly recommend that you adhere to the described protocol without any deviation; do not substitute reagents.

Workflows

Recommended 4-Day Workflow

Figure 5.1 shows the recommended 4-day workflow for one operator processing 8 to 24 samples including controls.

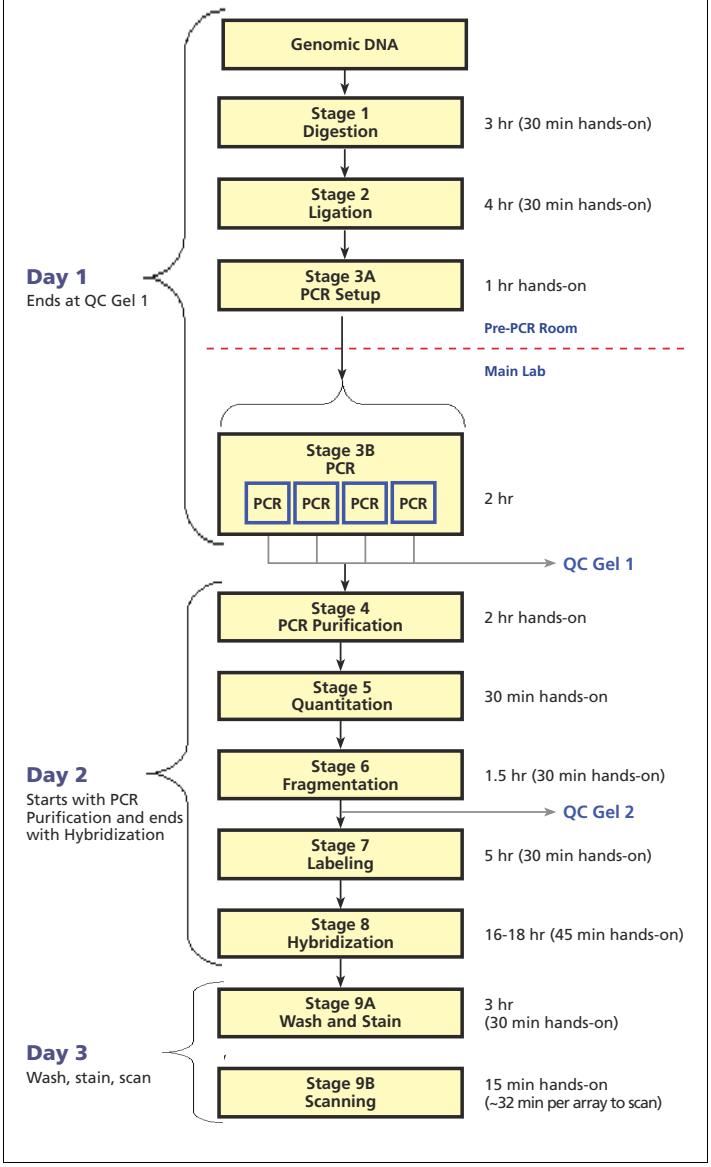
Figure 5.1 Recommended 4-Day Workflow



Optional 3-Day Workflow

Figure 5.2 illustrates the optional 3-day workflow. The difference between the 3-day workflow and the 4-day workflow is that you will hybridize your samples onto arrays at the end of day 2. This workflow may be an option if you are processing a small number of samples. If processing > 8 samples, the length of time required to complete all Day 2 activities will likely require more than an 8 hr work day.

Figure 5.2 Optional 3-Day Workflow



Overview and List of Required Reagents, Equipment and Consumables

About Genomic DNA Preparation

The human genomic DNA you will process using the CytoScan™ Assay should meet the general requirements listed in *Genomic DNA General Requirements* on page 21. During this stage, you will:

1. Determine the concentration of each genomic DNA sample (if required).
2. Dilute each genomic DNA sample to 50 ng/µL using Low EDTA TE buffer.
3. Aliquot the diluted genomic DNA samples and controls into the sample processing plate.

For the detailed protocol, see *Preparing the Genomic DNA* on page 43.

Location and Duration

- Pre-PCR Clean Area
- Hands-on time: dependent upon number of samples to be processed

Using Controls

We recommend including one positive and one negative control with every set of samples processed. For the positive control, use the Genomic DNA included in the CytoScan™ Assay Kit. For the negative control, use the Low EDTA TE Buffer included in the CytoScan™ Assay Kit.

Equipment and Consumables Required

The equipment and consumables listed in Table 5.1 are required for this stage.

Table 5.1 Equipment and Consumables Required for *Preparing the Genomic DNA*

Quantity	Item
As required	Adhesive seals for 96-well plates
1	Cooling chamber, double block, chilled to 4 °C, placed on ice (do not freeze)
1	Ice bucket, filled with ice
1	Marker, fine point, permanent
1	Mini microcentrifuge (microfuge)
1	Pipette, single channel P20
1	Pipette, single channel P100 or P200
1	Pipette, 12-channel, 2-20 µL

Table 5.1 Equipment and Consumables Required for *Preparing the Genomic DNA* (Continued)

Quantity	Item
1	Pipette, 12-channel, 20-200 µL
As needed	Pipette tips for pipettes listed above
2	Plate, Bio-Rad 96-well unskirted
As needed	Tubes, Eppendorf (Safe-Lock Tubes 1.5 mL, Natural)
1	Plate centrifuge
1	Plate spectrophotometer or NanoDrop (required only if no OD measurements available for samples)
1	Vortexer
2	GeneMate 96-Well PCR Tube Storage Rack
** **IMPORTANT** Use only the thermal cyclers, 96-well plate, and adhesive films and listed under *Thermal Cyclers, 96-Well Plate, and Adhesive Seals* on page 8.	

Reagents Required

The following reagents are required for this stage.

Table 5.2 Reagents Required for *Preparing the Genomic DNA*

Reagent
Low EDTA TE Buffer
Genomic DNA (positive control)

About Stage 1: Restriction Enzyme Digestion

During this stage, sample is digested by the Nsp I restriction enzyme. You will:

1. Prepare a Digestion Master Mix and add it to the samples.
2. Place the samples onto a thermal cycler and run the CytoScan Digest program.

For the detailed protocol, see *Stage 1: Restriction Enzyme Digestion* on page 48.

Location and Duration

- Pre-PCR Clean Area
- Hands-on time: 30 minutes
- CytoScan Digest thermal cycler program time: 2.5 hr

Input Required From Previous Stage

This stage requires a plate containing aliquots of each genomic DNA and each control prepared as instructed under *Preparing the Genomic DNA* on page 43 (5 µL at 50 ng/µL in each well).

Equipment and Consumables Required

The following equipment and consumables are required for this stage.

Table 5.3 Equipment and Consumables Required for *Stage 1: Restriction Enzyme Digestion*

Quantity	Item
As required	Adhesive seals for 96-well plates
1	Plate centrifuge
1	Cooler, chilled to –20 °C
1	Cooling chamber, double block, chilled to 4 °C placed on ice (do not freeze)
1	Ice bucket, filled with ice
1	Marker, fine point, permanent
1	Mini centrifuge (microfuge)
1	Pipette, single channel P10
1	Pipette, single channel P100 or P200
1	Pipette, 12-channel, 2-20 µL
1	Pipette, 12-channel, 20-200 µL
As required	Pipette tips for pipettes listed above
1	Thermal cycler
1	8-12 well strip tubes, 0.2 mL
As required	8-12 tube strip caps
2	Tubes, Eppendorf (Safe-Lock Tubes 1.5 mL, Natural)
1	Vortexer
2	GeneMate 96-Well PCR Tube Storage Rack
** **IMPORTANT** Use only the thermal cyclers, 96-well plate, and adhesive films and listed under *Thermal Cyclers, 96-Well Plate, and Adhesive Seals* on page 8.	

Reagents Required

The following reagents are required for this stage.

Table 5.4 Reagents Required for *Stage 1: Restriction Enzyme Digestion*

Reagent
100X BSA
10X Nsp I Buffer
Nsp I
Chilled Affymetrix® Nuclease-Free Water

About Stage 2: Ligation

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

1. Prepare a Ligation Master Mix and add it to the Nsp I digested samples.
2. Place samples onto a thermal cycler and run the CytoScan Ligate program.

For the detailed protocol, see *Stage 2: Ligation* on page 53.

Location and Duration

- Pre-PCR Clean Area
- Hands-on time: 30 minutes
- CytoScan Ligate thermal cycler program time: 3.3 hr

Equipment and Consumables Required

The following equipment and consumables are required for this stage.

Table 5.5 Equipment and Consumables Required for *Stage 2: Ligation*

Quantity	Item
1	Adhesive seals for 96-well plates
1	Plate centrifuge
1	Cooler, chilled to –20 °C
1	Cooling chamber, double block, chilled to 4 °C placed on ice (do not freeze)
1	Ice bucket, filled with ice
1	Marker, fine point, permanent
1	Mini centrifuge (microfuge)
1	Pipette, single channel P10
1	Pipette, single channel P20
1	Pipette single channel P100 or P200
1	Pipette, 12-channel, 2-20 µL
1	Pipette, 12-channel, 20-200 µL
As needed	Pipette tips for pipettes listed above
1	Thermal cycler
1	8-12 well strip tubes, 0.2 mL
As required	8-12 tube strip caps
1	Tubes, Eppendorf (Safe-Lock Tubes 1.5 mL, Natural)
1	Vortexer
2	GeneMate 96-Well PCR Tube Storage Rack
** **IMPORTANT** Use only the thermal cyclers, 96-well plate, and adhesive films and listed under *Thermal Cyclers, 96-Well Plate, and Adhesive Seals* on page 8.	

Reagents Required

The following reagents are required for this stage.

Table 5.6 Reagents Required for *Stage 2: Ligation*

Reagent
T4 DNA Ligase
10X T4 DNA Ligase Buffer
50 µM Adaptor, Nsp I

About Stage 3: PCR

During this stage, you will:

1. Dilute the ligated DNA by adding 75µL of chilled nuclease-free water.
2. Transfer 10 µl of each diluted ligated sample into *four* wells of a 96-well plate.
3. Prepare a PCR Master Mix and add it to each ligated sample.
4. Place the samples onto a thermal cycler and run the CytoScan PCR program.
5. Confirm each PCR reaction by running 3 µL of each PCR product on a gel.

For the detailed protocol, see *Stage 3: PCR* on page 58.

Location and Duration

- Pre-PCR Clean Area
  - PCR Master Mix preparation
  - PCR set up
- Post-PCR Area: samples placed on thermal cycler
- Hands-on time: 1 hr
- CytoScan PCR thermal cycler program time: 1.5 hr

  Samples can be held overnight at 4 °C in the thermal cycler.

Equipment and Materials Required

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

Table 5.7 Equipment and Consumables Required for *Stage 3: PCR*

Quantity	Item
As required	Adhesive seals for 96-well plates
1	Plate centrifuge
1	Cooler, chilled to –20 °C
1	Cooling chamber, double block, chilled to 4 °C placed on ice (do not freeze)
1	Ice bucket, filled with ice
1	Marker, fine point, permanent
1	Mini centrifuge (microfuge)
1	Pipette, single channel P20
1	Pipette, single channel P100
1	Pipette, single channel P200
1	Pipette, single channel P1000
1	Pipette, 12-channel, 2-20 µL
1	Pipette, 12-channel, 20-200 µL
As required	Pipette tips for pipettes listed above
1	Plate, Bio-Rad 96-well PCR
2	GeneMate 96-Well PCR Rube Storage Rack
1	Reagent reservoir, 25 mL
1	Thermal cycler
1	Tube, centrifuge 15 or 50 mL
1	Vortexer
1	Electrophoresis gel box
1	Electrophoresis power supply
****IMPORTANT** Use only the thermal cyclers, 96-well plate, and adhesive films listed under *Thermal Cyclers, 96-Well Plate, and Adhesive Seals* on page 8.	

Reagents Required

The following reagents are required for this stage.

Table 5.8 Reagents Required for *Stage 3: PCR*

Reagent
Chilled Affymetrix® Nuclease-Free Water
PCR Primer, 002
From the Clontech TITANIUM™ DNA Amplification Kit (300 or 400 rxn):
▪ dNTP Mixture (2.5 mM each)
▪ GC-Melt Reagent
▪ 50X TITANIUM™ *Taq* DNA Polymerase
▪ 10X TITANIUM™ *Taq* PCR Buffer

Gels and Related Materials Required

Verifying the PCR reaction is required for this stage.

Table 5.9 Gels and Related Materials Required for *Stage 3: PCR*

Reagent
DNA Marker (USB PCR Markers 50-2000bp)
Gels, 2% TBE (precast or house-made)
1X TBE Buffer
Ethidium Bromide Solution
5X RapidRun™ Loading Dye
Plates, 96-well reaction

About Stage 4: PCR Product Purification

During this stage, you will purify the PCR products as follows:

1. Pool the PCR reactions.
2. Add Purification Beads to each pooled reaction and incubate the mix.
3. Wash DNA-bound beads with Purification Wash Buffer.
4. Add Elution Buffer to elute the DNA.

For the detailed protocol, see *Stage 4: PCR Product Purification* on page 68.

Location and Duration

- Post-PCR Area
- Hands-on time: 2 hr
- DNA binding to magnetic bead: 15 to 20 minutes
- EtOH wash: approximately 10 to 20 minutes
- Elution: 15 to 30 minutes
- Total time for this stage: approximately 2.0 to 3.0 hr

Equipment and Consumables Required

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

Table 5.10 Equipment and Consumables Required for *Stage 4: PCR Product Purification*

Quantity	Item
1	Adhesive seals for 96-well plates
1	Microcentrifuge, Eppendorf 5424 with rotor for 24 – tubes, 2.0 mL
1	Magnetic stand
1	Marker, fine point, permanent
1	Microtube Foam Insert (for vortexing 2.0 mL tubes)
1	Pipette, single channel P20
1	Pipette, single channel P200
1	Pipette, single channel P1000
1	Pipette, 12-channel, 2-20 µL
1	Pipette, 12-channel, 20-200 µL
1	Pipette, 12-channel, 100-1000 µL
As needed	Pipette tips for pipettes listed above

Table 5.10 Equipment and Consumables Required for *Stage 4: PCR Product Purification*

Quantity	Item
1	Plate, Bio-Rad 96-well
One per 96-well plate	Plate holder
1	Optional: Tube, 50 mL conical
One per sample (minus neg control)	Tube, Eppendorf (Safe-Lock Tube 1.5 mL, Natural)
1	Tube holder
1	Vortexer (with foam tube adaptor attached)
1	Reagent reservoir, 25 mL
****IMPORTANT** Use only the magnetic racks listed in Table F.10 *Post-PCR Area Equipment Required* on page 150.	

Reagents Required

The following reagents are required for this stage.

Table 5.11 Reagents Required for *Stage 4: PCR Product Purification*

Reagent
Purification Wash Buffer
Elution Buffer
Purification Beads
Absolute Ethanol

About Stage 5: Quantitation

During this stage, you will quantitate each sample. For the detailed protocol, see *Stage 5: Quantitation* on page 75.

Location and Duration

- Post-PCR Room
- Hands-on time: 30 minutes

Equipment and Consumables Required

The following equipment and consumables are required for this stage.

Table 5.12 Equipment and Consumables Required for *Stage 5: Quantitation*

Quantity	Item
As required	Adhesive seals for 96-well plates
1	Marker, fine point, permanent
1	Mini centrifuge (microfuge)
1	Pipette, single channel P20
1	Pipette, single channel P200
1	Pipette, 12-channel, 2-20 µL
1	Pipette, 12-channel, 20-200 µL
As needed	Pipette tips for pipettes listed above
1	Plate, 96-well (if using NanoDrop)
1	UV Plate, 96-well, 370ul, UV-Star® (if using microplate spectrophotometer)
1	Spectrophotometer, microplate or NanoDrop
1	Reagent reservoir, 25 mL

Reagents Required

The following reagents are required for this stage.

Table 5.13 Reagents Required for *Stage 5: Quantitation*

Reagent
Affymetrix® Nuclease-Free Water

About Stage 6: Fragmentation

During this stage, the purified samples are fragmented using the Fragmentation Reagent. You will:

1. Prepare a Fragmentation Master Mix.
2. Quickly add the mix to each sample.
3. Place the samples onto a thermal cycler and run the CytoScan Fragment program.
4. Check each reaction on a gel.

For the detailed protocol, see *Stage 6: Fragmentation* on page 79.

Location and Duration

- Post-PCR Area
- Hands-on time: 30 minutes
- CytoScan Fragment thermal cycler program time: 1 hr

Equipment and Consumables Required

The following equipment and consumables are required for this stage.

Table 5.14 Equipment and Consumables Required for *Stage 6: Fragmentation*

Quantity	Item
As required	Adhesive seals for 96-well plates
1	Refrigerated plate centrifuge
1	Cooler, chilled to –20 °C
1	Cooling chamber, double block, chilled to 4 °C placed on ice (do not freeze)
1	Ice bucket, filled with ice
1	Marker, fine point, permanent
1	Mini centrifuge (microfuge)
1	Pipette, single channel P20
1	Pipette, single channel P100
1	Pipette, single channel P200
1	Pipette, 12-channel P20 (accurate to within ± 5%)
As needed	Pipette tips for pipettes listed above
1	Plate, Bio-Rad 96-well
1	Thermal cycler
1	Tube, Eppendorf (Safe-Lock Tubes 1.5 mL, Natural)
1	8-12 well strip tubes, 0.2 mL
As required	8-12 tube strip caps

Table 5.14 Equipment and Consumables Required for *Stage 6: Fragmentation* (Continued)

Quantity	Item
1	Vortexer
1	Electrophoresis gel box
1	Electrophoresis power supply
4	GeneMate 96-Well PCR Tube Storage Rack
** **IMPORTANT** Use only the thermal cyclers, 96-well plate, and adhesive films and listed under *Thermal Cyclers, 96-Well Plate, and Adhesive Seals* on page 8.	

Reagents Required

The following reagents are required for this stage.

Table 5.15 Reagents Required for *Stage 6: Fragmentation*

Reagent
10X Fragmentation Buffer
Fragmentation Reagent
Chilled Affymetrix® Nuclease-Free Water

Gels and Related Materials Required

Verifying the fragmentation reaction is required for this stage. You can use the following gels and related materials.

Table 5.16 Gels and Related Materials Required

Item/Reagent
4% TBE Gel (precast or house-made)
1X TBE Buffer
Ethidium Bromide Solution
5X RapidRun™ Loading Dye
TrackIt™ 25 bp DNA Ladder

About Stage 7: Labeling

During this stage, you will label the fragmented samples using the DNA Labeling Reagent as follows:

1. Prepare a Labeling Master Mix.
2. Add the mix to each sample.
3. Place the samples onto a thermal cycler and run the CytoScan Label program.

For the detailed protocol, see *Stage 7: Labeling* on page 87.

Location and Duration

- Post-PCR Area
- Hands-on time: 30 minutes
- CytoScan Label thermal cycler program time: 4.25 hr

Equipment and Consumables Required

The following equipment and consumables are required for this stage.

Table 5.17 Equipment and Consumables Required for *Stage 7: Labeling*

Quantity	Item
As required	Adhesive seals for 96-well plates
1	Plate centrifuge
1	Cooler, chilled to –20 °C
1	Cooling chamber, double block, chilled to 4 °C placed on ice (do not freeze)
1	Ice bucket, filled with ice
1	Marker, fine point, permanent
1	Mini centrifuge (microfuge)
1	Pipette, single channel P200
1	Pipette, single channel P1000
1	Pipette, 12-channel P20 (accurate to within ± 5%)
As needed	Pipette tips for pipettes listed above
1	Thermal cycler
1	Tube, Eppendorf (Safe-Lock Tubes 1.5 mL, Natural)

Table 5.17 Equipment and Consumables Required for *Stage 7: Labeling*

Quantity	Item
1	8-12 well strip tubes, 0.2 mL
As required	8-12 tube strip caps
1	Vortexer
4	GeneMate 96-Well PCR Tube Storage Rack
** **IMPORTANT** Use only the thermal cyclers, tubes, 96-well plates, and adhesive film and listed under *Thermal Cyclers, 96-Well Plate, and Adhesive Seals* on page 8.	

Reagents Required

The following reagents are required for this stage.

Table 5.18 Reagents Required for *Stage 7: Labeling*

Reagent
30 mM DNA Labeling Reagent
TdT
5X TdT Buffer

About Stage 8: Target Hybridization

During this stage, each sample is hybridized onto a CytoScan™ Array. You will:

1. Prepare a Hybridization Master Mix and add it to each sample.
2. Denature the samples on a thermal cycler.
3. Load each sample onto a CytoScan™ Array.
4. Place the arrays into a hybridization oven at 50 °C for 16 to 18 hr.

For the detailed protocol, see *Stage 8: Target Hybridization* on page 91.

Location and Duration

- Post-PCR Area
- Hands-on time: 45 minutes
- Hybridization time: 16 to 18 hr

Equipment and Consumables Required

The following equipment and consumables are required for this stage.

! IMPORTANT: Hybridization only in the GeneChip® Hybridization Oven 645 is recommended for this assay.

While preparing the hybridization setup, leave the samples on the cooling block on ice. The following table lists the equipment and consumables required.

Table 5.19 Equipment and Consumables Required for *Stage 8: Target Hybridization*

Quantity	Item
1	Adhesive seals for 96-well plates
1	Cooling chamber, double block, chilled to 4 °C placed on ice (do not freeze)
One array per sample	CytoScan™ Array
1	GeneChip® Hybridization Oven 645
1	Ice bucket, filled with ice
1	Pipette, single channel P200
1	Pipette, single channel P1000
1	Pipette, 12-channel 20-200 µL
As needed	Pipette tips for pipettes listed above
1	Reagent reservoir, 55 mL
1	Thermal cycler
2 per array	Tough-Spots® (1/2" diameter, PN Spot 2200, Diversified Biotech)
1	Tube, centrifuge 15 mL
1	Vortexer
4	GeneMate 96-Well PCR Tube Storage Rack
** **IMPORTANT** Use only the thermal cyclers, tubes, 96-well plate, and adhesive film and listed under *Thermal Cyclers, 96-Well Plate, and Adhesive Seals* on page 8.	

Reagents Required

The following reagents are required for this stage.

Table 5.20 Reagents Required for *Stage 8: Target Hybridization*

Reagent
Hyb Buffer Part 1
Hyb Buffer Part 2
Hyb Buffer Part 3
Hyb Buffer Part 4
Oligo Control Reagent 0100

CytoScan™ Assay Protocol

The Affymetrix® CytoScan™ Assay is designed for processing 8-24 samples (including controls). Instructions are provided for processing 8, 16, or 24 samples in parallel. The protocol is presented in the following stages:

Preparing the Genomic DNA

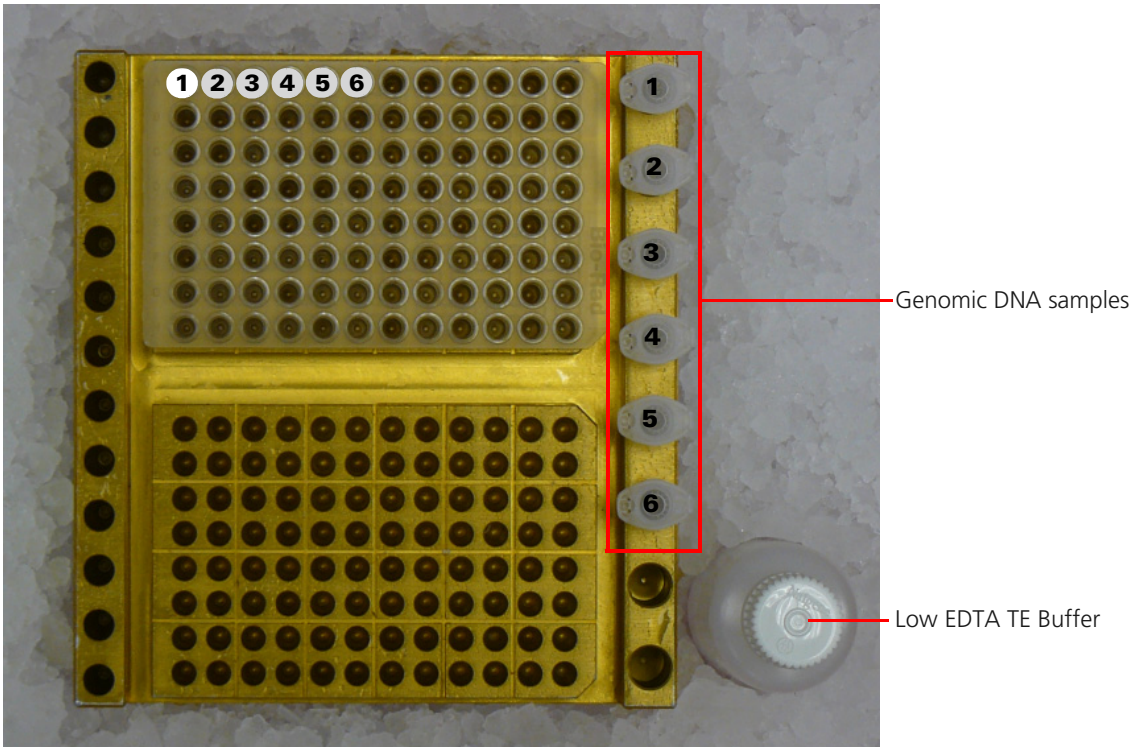
This protocol has been optimized using UV absorbance to determine genomic DNA concentrations. Other quantitation methods such as PicoGreen may give different readings.

Set Up the Work Area

To set up the work area:

1. Place a double cooling block on ice (Figure 6.1).
2. Place a 96-well plate in the upper half of the cooling block.

Figure 6.1 Diluting genomic DNA samples to 50 ng/µL



NOTE: **The illustrations in this user manual depict the setup recommended for eight samples: six genomic DNA samples plus one positive control and one negative control.**

If running less than eight samples, follow the same plate layout.

If running more than eight samples, refer to Appendix A, *Guidelines for Processing 16 Samples* or Appendix B, *Guidelines for Processing 24 Samples* for more information.

Dilute the Genomic DNA

To dilute the genomic DNA:

1. Thaw the genomic DNA (gDNA) as follows:
   - **A.** Place on the bench top at room temperature until thawed.
   - **B.** Once thawed, place in the cooling block on ice.

2. Vortex the gDNA samples at high speed for 3 seconds.
3. Spin down for 1 minute, then place back in the cooling block.
4. If sample concentration is unknown, take an OD measurement of each sample now.

   Consult your spectrophotometer handbook for more information on how to determine the sample concentration.

> **IMPORTANT:** To avoid contaminating samples with PCR product, take only an aliquot of each sample, not stock, to the plate spectrophotometer or NanoDrop.

5. Based on OD measurements, dilute each sample in a separate well of the 96-well plate to 50 ng/µL using Low EDTA TE buffer.

> **IMPORTANT:** Do NOT dilute the Genomic DNA provided in the CytoScan™ Reagent Kit; it is already at a working concentration.

6. Seal the plate, vortex at high speed for 3 seconds, then spin down for 1 minute.
7. Place back on the cooling block.

Aliquoting the Prepared Genomic DNA and Controls

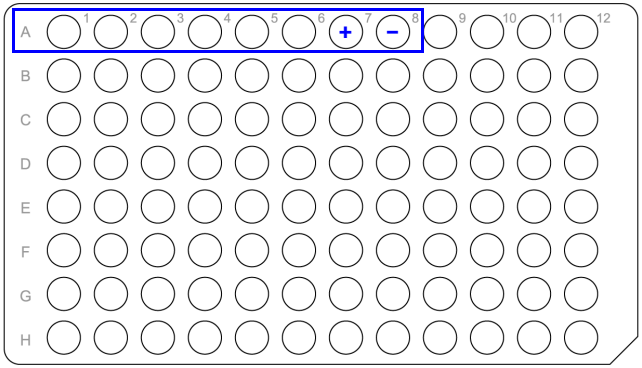
Set Up the Work Area

To set up the work area:

1. Mark a 96-well plate as shown in Figure 6.2.

   The digestion and ligation reactions will be performed in this plate.
2. Place the plate on the lower half of the cooling block (Figure 6.3 on page 47).

Figure 6.2 Marking a 96-well plate for digestion and ligation



Aliquot the gDNA and Controls

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

To aliquot the prepared genomic DNA and controls:

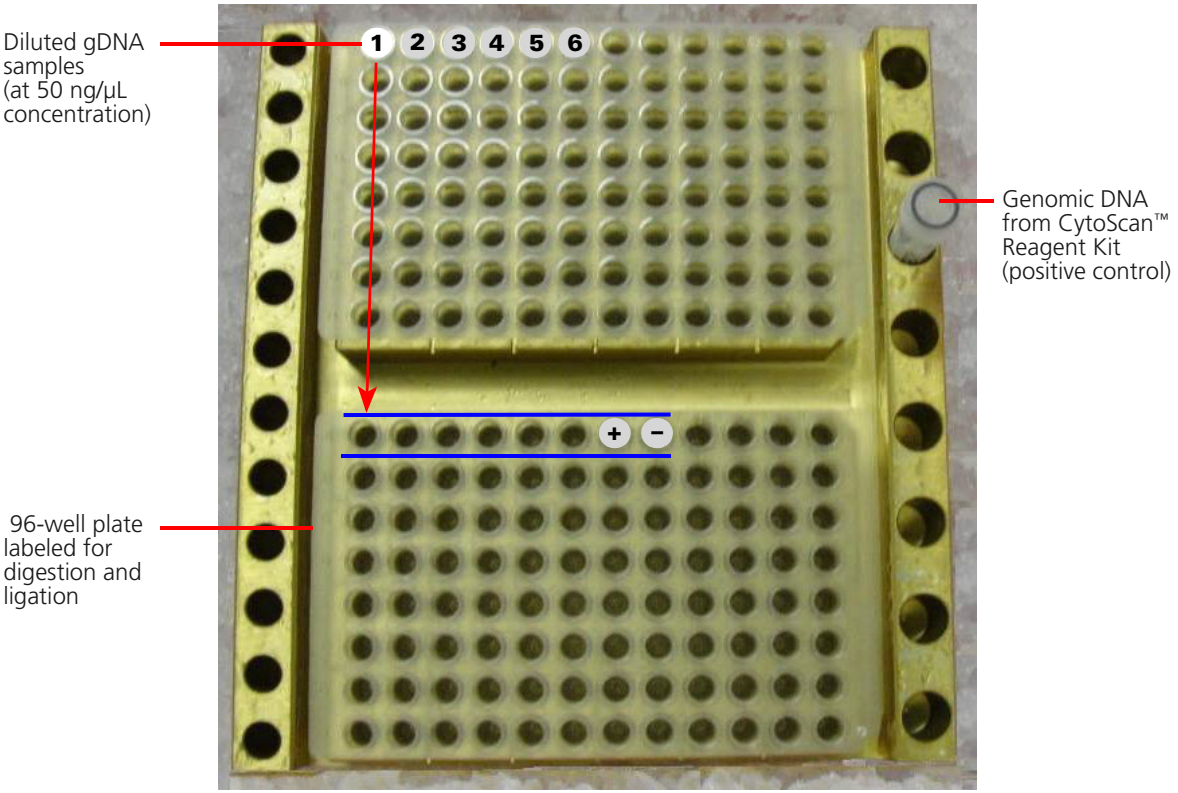
1. Thaw the Control Genomic DNA from the CytoScan™ Reagent Kit as follows:
   A. Place on the bench top at room temperature until thawed.
   B. Once thawed, place in the cooling block on ice.
2. Vortex the genomic DNA for 3 seconds, then quickly spin down for 1 minute.
3. Transfer a 5 µL aliquot of the first sample to well A1 of the digest/ligate plate (Figure 6.3).
4. Transfer 5 µL aliquots of each remaining gDNA sample in the same manner.
5. For the controls, aliquot 5 µL of:
   A. Genomic DNA from CytoScan™ Reagent Kit (+) to well A7.
   B. Low EDTA TE buffer (–) to well A8.
6. Tightly seal the digest/ligate plate with a new seal, then spin down for 1 minute at 2000 rpm.

Figure 6.3 Setup for aliquoting diluted gDNA and controls to a 96-well plate labeled for digestion/ligation

Transfer 5 µL aliquots of each diluted gDNA to the digest/ligate plate — for digestion reactions.

(+) = positive control (5 µL Genomic DNA from the CytoScan™ Reagent Kit)

(–) = negative control (5 µL Low EDTA TE Buffer)



What To Do Next

Do one of the following:

- Proceed to *Stage 1: Restriction Enzyme Digestion* *on page 48*.
- Store the prepared sample plate at –20 °C.

Stage 1: Restriction Enzyme Digestion

Prepare the Reagents, Equipment and Consumables

Turn On the Thermal Cycler

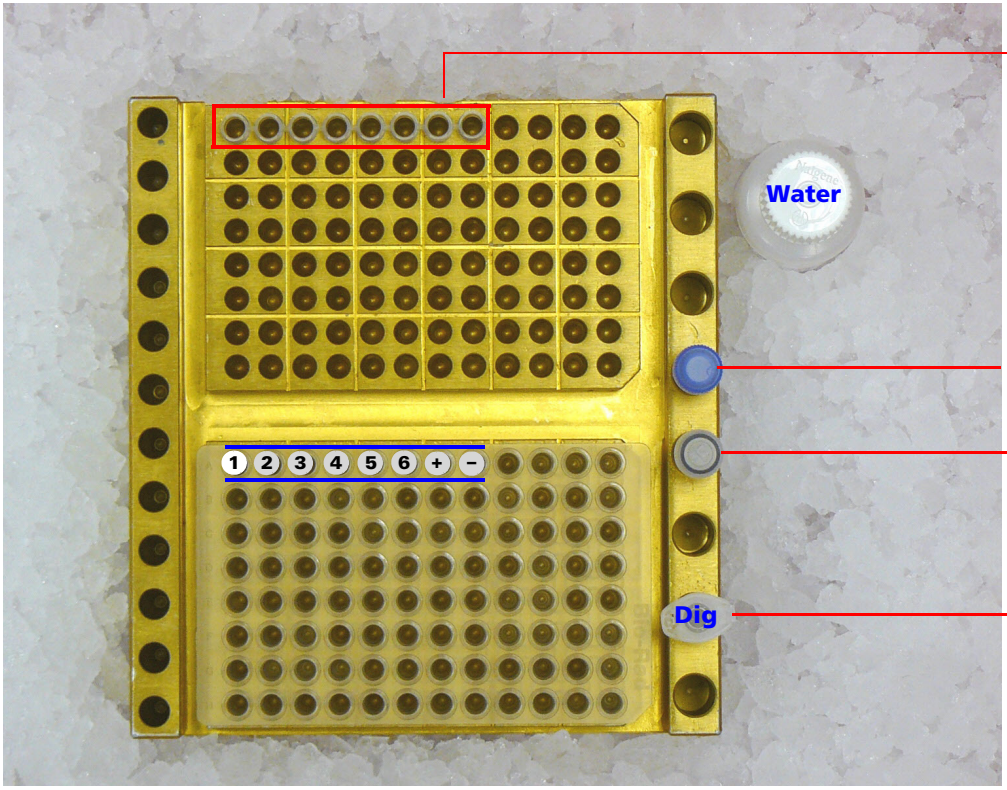
Power on the thermal cycler to preheat the lid. **Leave the block at room temperature.**

Set Up the Work Area

To set up the work area (Figure 6.4):

1. Place a double cooling block and the water on ice.
2. Place an 8-tube strip as shown in Figure 6.4 on the upper half of the cooling block
3. Label a 1.5 mL Eppendorf tube as *Dig* and place in the cooling block.
4. Cut adhesive seal into strips wide enough to seal 8 or 12 strip tubes.

Figure 6.4 Setup for Digestion (Nsp I enzyme not pictured; still at –20 °C)



Thaw the Reagents and Genomic DNA

1. Prepare the genomic DNA and controls as follows:
   A. Vortex at high speed 3 times, 1 second each time.
   B. Spin down at 2000 rpm for 1 minute.
2. If the plate of genomic DNA and controls is frozen, allow it to thaw at room temperature. Immediately spin down the plate in the centrifuge at 2000 rpm for 1 minute and place on the cooling block on ice.
3. Allow the following reagents to thaw at room temperature. Immediately place on the cooling block on ice when reagents are thawed.
   - 10× Nsp I Buffer
   - 100× BSA

   IMPORTANT: **Leave the Nsp I enzyme at –20 °C until ready to use.**
4. Prepare the 10X Nsp I Buffer and 100X BSA as follows:
   A. Vortex 3 times, 1 second each time.
   B. Pulse spin for 3 seconds.
   C. Place in the cooling block on ice.
5. Place the Affymetrix® Nuclease-Free water on ice.

Prepare the Digestion Master Mix

Keeping all reagents, tubes, and the cooling block on ice, prepare the Nsp I Digest Master Mix as follows:

1. To the 1.5 mL Eppendorf tube labeled *Dig*, add the appropriate volumes of the following reagents (see Table 6.1):
   - Chilled Affymetrix® Nuclease-Free water
   - 10X Nsp I Buffer
   - 100X BSA
2. Place the master mix in the cooling block.
3. Remove the Nsp I enzyme from the freezer and immediately place in a cooler chilled to –20 °C.
4. Vortex at high speed for 1 second.
5. Pulse spin the enzyme for 3 seconds. Keep it in the –20 °C cooler.
6. Immediately add the enzyme to the master mix.
7. Return the enzyme to the –20 °C cooler.

Table 6.1 Digestion Master Mix

Reagent	1 Sample	8 Samples (20% overage)	16 Samples (20% overage)	24 Samples (20% overage)
Chilled Affymetrix® Nuclease-Free Water	11.55 µL	110.9 µL	221.8 µL	332.6 µL
10X Nsp I Buffer	2.00 µL	19.2 µL	38.4 µL	57.6 µL
100X BSA	0.20 µL	1.9 µL	3.8 µL	5.8 µL
Nsp I	1.00 µL	9.6 µL	19.2 µL	28.8 µL
Total	**14.75 µL**	**141.6 µL**	**283.2 µL**	**424.8 µL**

8. Vortex the master mix at high speed 3 times, 1 second each time.
9. Pulse spin for 3 seconds.
10. Place in the cooling block.

Add Digestion Master Mix to Samples

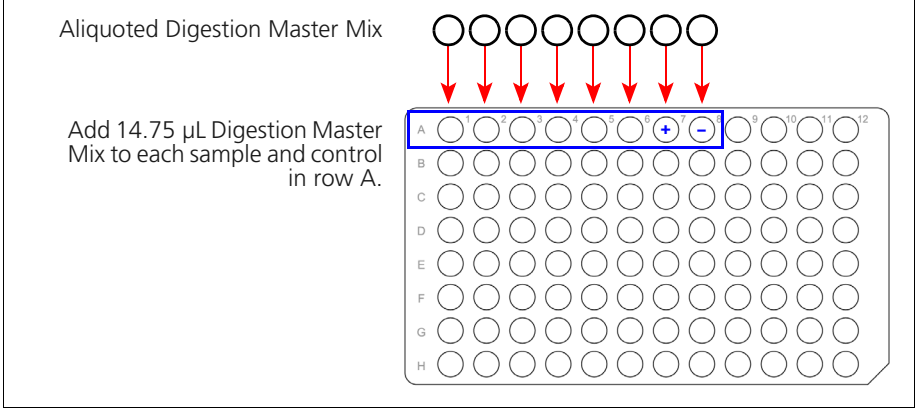
To add Digestion Master Mix to samples:

NOTE: When working with more than 8 samples, we strongly recommend dividing the master mix into strip tubes and dispensing the master mix from the strip tubes into the samples using a multi-channel pipette.

1. Divide the Digestion Master Mix equally into the 8-12 strip tubes on ice. Seal the strip tube with an adhesive seal strip (or strip caps). Spin and place back on a cooling block on ice. Remove the seal and discard.
2. Unseal the plate and discard the seal.
3. Using a multi-channel P20 pipette, aliquot 14.75 µL of Digestion Master Mix to each sample and controls in row A.
4. Seal the plate tightly with a new seal.

Genomic DNA (50 ng/µL)	5.00 µL
Digestion Master Mix	14.75 µL
Total Volume	**19.75 µL**

Figure 6.5 Adding Digestion Master Mix to Samples and Controls



Load Samples onto the Thermal Cycler

1. Vortex the plate at high speed for 1 second in all corners and in the center according to the guidelines in *Seal, Vortex, and Spin* on page 5, then spin down at 2000 rpm for 1 minute.
2. Ensure that the lid of thermal cycler is preheated.
3. Load the plate onto the thermal cycler and run the CytoScan Digest program (Table 6.2).

Table 6.2 CytoScan Digest Program

CytoScan Digest Program	
Temperature	**Time**
37 °C	2 hours
65 °C	20 minutes
4 °C	Hold

4. Return any remaining reagents to the freezer.
5. When the program is finished, remove the plate. Make sure the plate is sealed tightly and spin down at 2000 rpm for 1 minute.

What To Do Next

Do one of the following:

- Place the plate in a cooling block on ice and proceed immediately to *Stage 2: Ligation on page 53*.
- If not proceeding directly to Ligation, make sure the plate is sealed tightly and store the plate at –20 °C.

Stage 2: Ligation

Prepare the Reagents, Consumables and Other Components

Turn On the Thermal Cycler

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

Set Up the Work Area

1. Place a double cooling block on ice (Figure 6.6).
2. Label a 1.5 mL Eppendorf tube as *Lig* and place in the cooling block.
3. Place an 8-tube strip on the upper half of the cooling block (Figure 6.6).
4. Cut an adhesive seal into strips wide enough to seal 8 or 12 strip tubes.

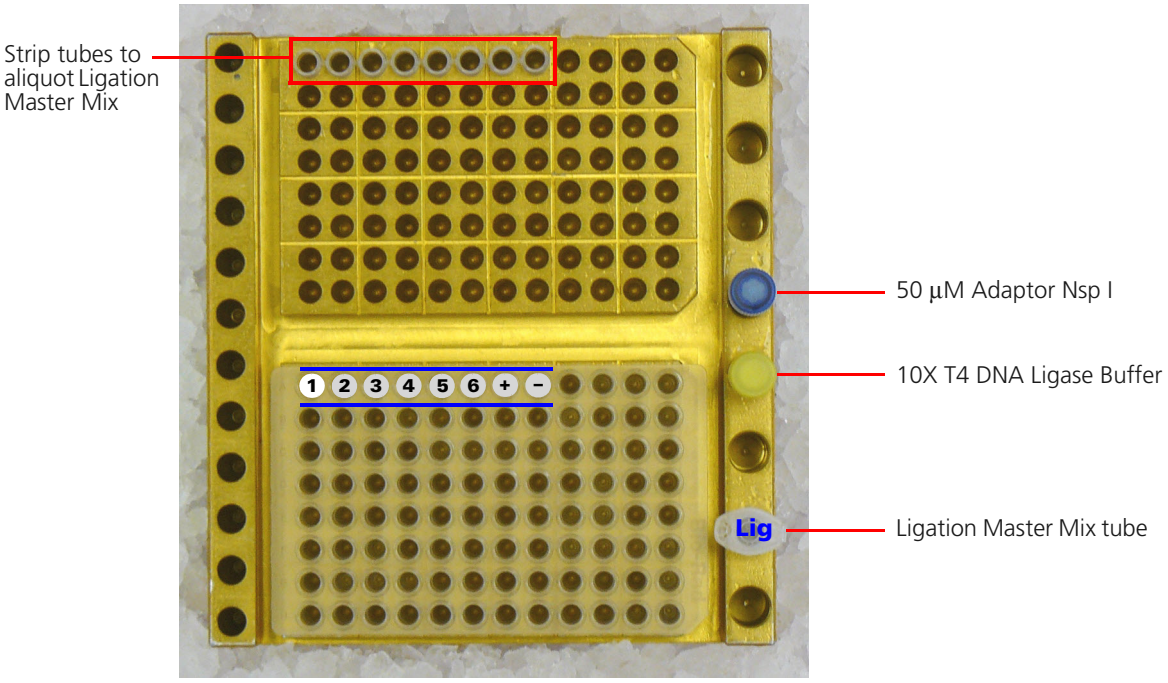
Thaw the Reagents and Digested Samples

To thaw the reagents and digested samples:

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

1. Allow the following reagents to thaw at room temperature. Immediately place on the cooling block on ice when reagents are thawed.
   - 50 µM Adaptor Nsp I
   - 10X T4 DNA Ligase Buffer (requires approximately 20 minutes to thaw)
2. If the digested samples were frozen, allow them to thaw at room temperature. Immediately spin down the plate at 2000 rpm for 1 minute and place on the cooling block on ice.

Figure 6.6 Setup for Ligation (T4 DNA Ligase enzyme not pictured; still at –20 °C)



Prepare the Digested Samples and Reagents

1. Prepare the digested samples as follows:
   - **A.** Spin down at 2000 rpm for 1 minute.
   - **B.** Place in the lower half of the cooling block on ice.
2. To prepare the reagents:

> **!** **IMPORTANT: Vortex the buffer as long as necessary before use to ensure any precipitate is re-suspended and the buffer is clear.**

   - **A.** Vortex the 10X T4 Ligase Buffer and the 50 µM Adaptor Nsp I at high speed 3 times, 1 second each time.
   - **B.** Pulse spin for 3 seconds.
   - **C.** Place in the cooling block.

Prepare the Ligation Master Mix

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

1. To the 1.5 mL Eppendorf tube labeled *Lig*, add the following reagents based on the volumes shown in Table 6.3:
   - 10X T4 DNA Ligase Buffer
   - 50 µM Adaptor Nsp I
2. Remove the T4 DNA Ligase from the freezer and immediately place in the cooler, chilled to –20 °C.
3. Vortex at high speed for 1 second.
4. Pulse spin the T4 DNA Ligase for 3 seconds. and place it in the –20 °C cooler.
5. Immediately add the T4 DNA Ligase to the master mix, then place back in the –20 °C cooler.

Table 6.3 Ligation Master Mix

Reagent	1 Sample	8 Samples (25% overage)	16 Samples (25% overage)	24 Samples (25% overage)
10X T4 DNA Ligase Buffer	2.50 µL	25.0 µL	50.0 µL	75.0 µL
50 µM Adaptor, Nsp I	0.75 µL	7.5 µL	15.0 µL	22.5 µL
T4 DNA Ligase	2.00 µL	20.0 µL	40.0 µL	60.0 µL
Total	**5.25 µL**	**52.5 µL**	**105.0 µL**	**157.5 µL**

6. Vortex the master mix at high speed 3 times, 1 second each time.
7. Pulse spin for 3 seconds.
8. Place the master mix in the cooling block on ice.
9. Proceed immediately to *Add Ligation Master Mix to Reactions*.

Add Ligation Master Mix to Reactions

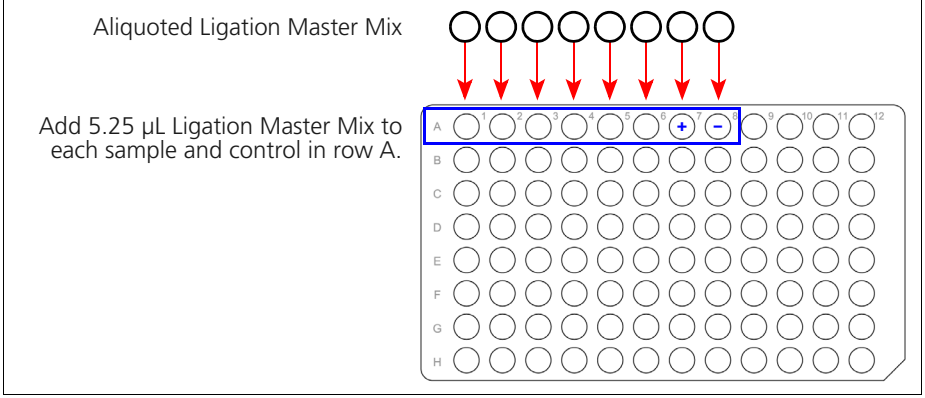
To add Ligation Master Mix to samples:

NOTE: When working with more than 8 samples, we strongly recommend dividing the master mix into strip tubes and dispensing the master mix from the strip tubes into the samples using a multi-channel pipette.

1. Divide the Ligation Master Mix equally into the 8-12 strip tubes on ice. Seal the strip tube with an adhesive seal strip (or strip caps) and pulse spin. Place back in the cooling block on ice, remove the seal and discard.
2. Unseal the digested sample plate and discard the seal.
3. Using a multi-channel P20 pipette, aliquot 5.25 µL of Ligation Master Mix to each digested sample and control (Figure 6.7).

Digested DNA	19.75 µL
Ligation Master Mix	5.25 µL
Total	**25.00 µL**

Figure 6.7 Adding Ligation Master Mix to Digested Samples and Controls



Load the Samples Onto the Thermal Cycler

1. Seal the plate tightly with a new seal.
2. Vortex at high speed for 1 second in all corners and in the center according to the guidelines in *Seal, Vortex, and Spin* on page 5, then spin down at 2000 rpm for 1 minute.
3. Ensure that the thermal cycler lid is preheated.
4. Load the plate onto the thermal cycler and run the CytoScan Ligate program.
5. Return the remaining reagents to the freezer and discard the remaining master mix.

Table 6.4 CytoScan Ligate Thermal Cycler Program

CytoScan Ligate Program	
Temperature	**Time**
16°C	3 hours
70°C	20 minutes
4°C	Hold

What To Do Next

Do one of the following:

- If following the recommended workflow (Figure 5.1 on page 24), proceed immediately to *Stage 3: PCR on page 58*.

  Samples can be stored in a cooling block on ice for up to 60 minutes.
- The sample plate can also be left in the thermal cycler at 4 °C hold over night.
- If not proceeding directly to the next step, ensure the plate is sealed tightly and then store the plate at –20 °C.

Stage 3: PCR

About Controls

To assess the presence of contamination, always include one PCR negative control with every set of samples run.

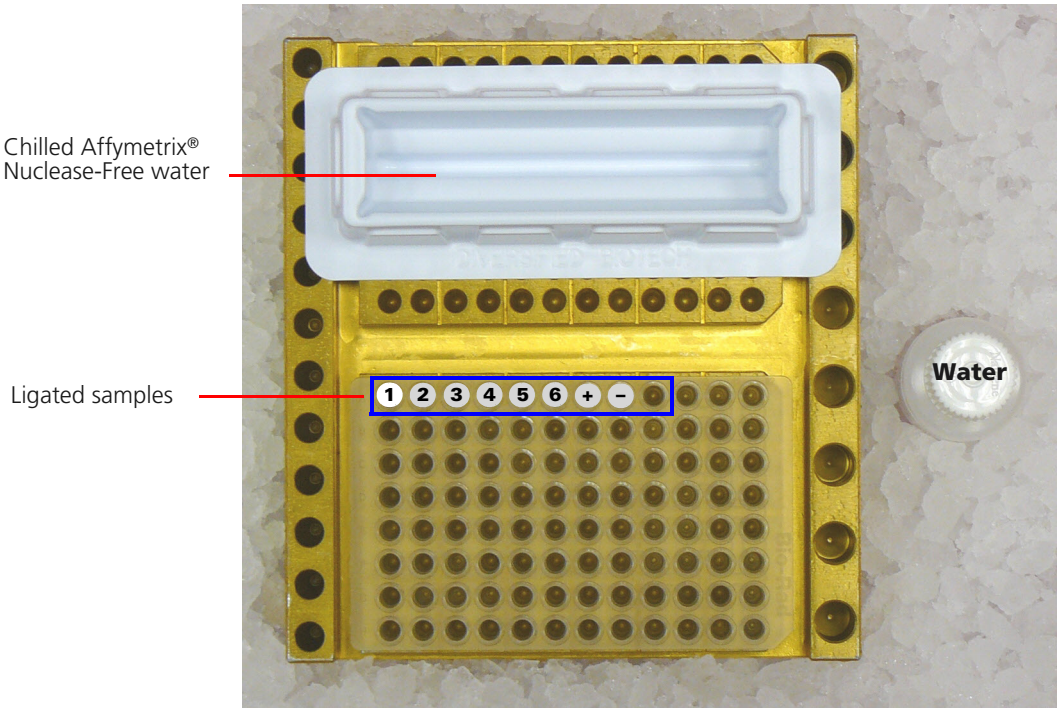
IMPORTANT: It is crucial to dilute the ligated DNA with chilled Affymetrix® Nuclease-Free water prior to PCR.

Dilute the Ligated Samples

To dilute the samples:

1. Place the Affymetrix® Nuclease-Free water on ice 20 minutes prior to use.
2. Place a double cooling block on ice (Figure 6.8).
3. Place a reagent reservoir on the upper half of the cooling block on ice.
4. Pour chilled Affymetrix® Nuclease-Free water into the reagent reservoir.
5. When the CytoScan Ligate program is finished, take the plate out. Make sure the plate is sealed tightly and spin down at 2000 rpm for 1 minute.
6. Place the plate in the lower half of the cooling block on ice.
7. Unseal the ligated sample plate and discard the seal.

Figure 6.8 Dilute the Ligated Samples



8. Using a P200 pipette, add 75 µL of water to each reaction.

Ligated DNA	25 µL
Chilled Affymetrix® Nuclease-Free water	75 µL
Total	**100 µL**

9. Tightly seal the plate with a new seal.
10. Vortex at high speed for 1 second in all corners and in the center according to the guidelines in *Seal, Vortex, and Spin* on page 5, then spin down at 2000 rpm for 1 minute.
11. If not proceeding with PCR set up, store the plate at –20 °C.

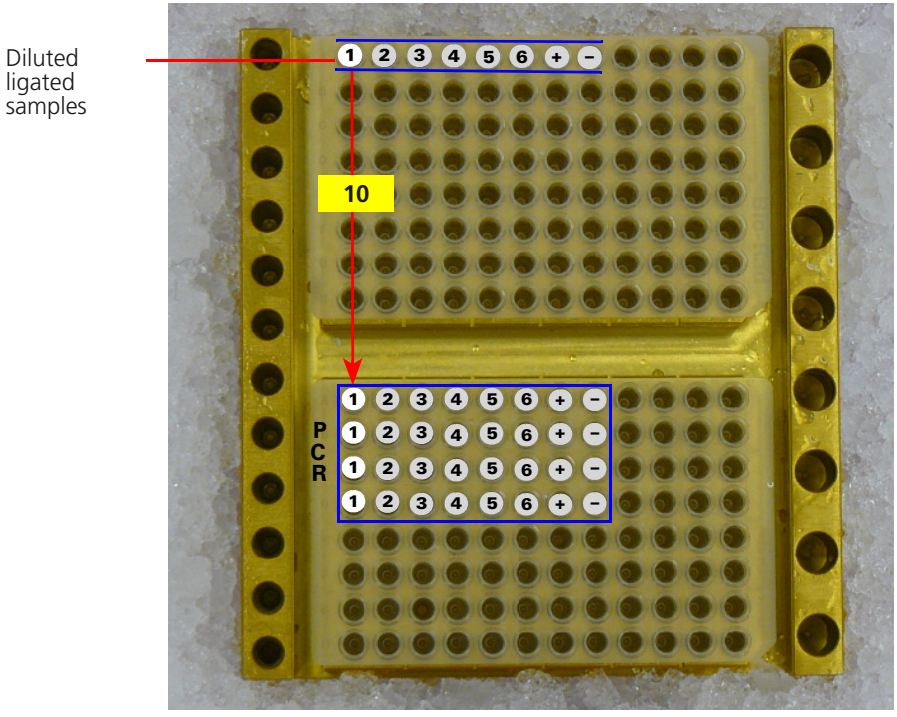
Transfer Diluted Ligated Samples to the PCR Plate

To transfer the diluted ligated samples to the PCR plate:

1. Place a double cooling block on ice.
2. Keep the diluted ligated sample plate on the upper half of the cooling block.

   If the diluted ligated samples are frozen, thaw them at room temperature. Make sure the plate is sealed tightly and vortex at high speed for 1 second in all corners and in the center according to the guidelines in *Seal, Vortex, and Spin* on page 5, then spin down at 2000 rpm for 1 minute. Immediately place the plate on the upper half of the cooling block.
3. Place a new PCR plate in the lower half as shown in Figure 6.9 and label as “PCR”.
4. Unseal the ligated and diluted sample plate and discard the seal.
5. Using a multi-channel P20 pipette, transfer 10 µL of each ligated and diluted sample to the corresponding **four** wells of the PCR plate.
6. Seal the plate tightly with a new seal and spin down at 2000 rpm for 1 minute.
7. If not proceeding immediately to PCR stage, store the plate with the remaining samples at –20 °C.

Figure 6.9 Labeling the 96-well Plate for PCR



Thaw the Reagents and Samples

IMPORTANT: Leave the 50X TITANIUM™ *Taq* DNA Polymerase at –20 °C until ready to use.

Allow the following reagents to thaw at room temperature. Immediately place on the cooling block on ice when reagents are thawed.

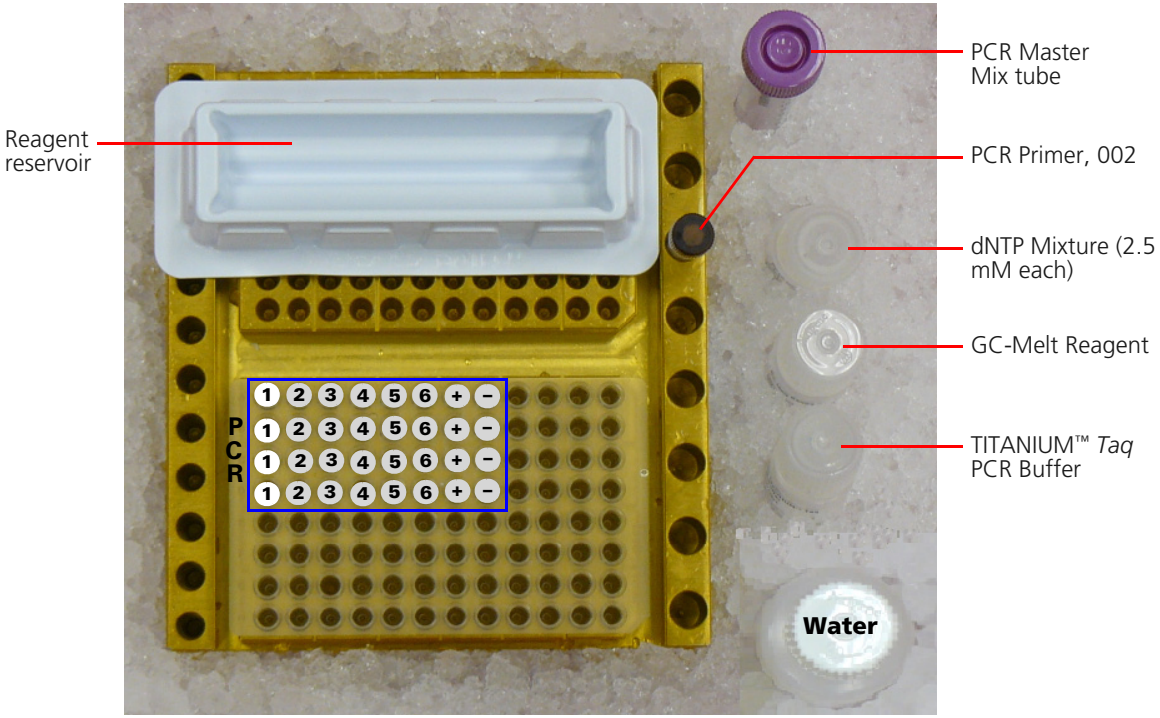
- 10X TITANIUM™ *Taq* PCR Buffer
- dNTP Mixture (2.5 mM each)
- PCR Primer, 002

Prepare the Samples and Reagents

To prepare the ligated samples and reagents:

1. Label the 15 mL centrifuge tube *PCR. (For more than 8 samples, use a 50 mL tube.)*
2. Place on ice:
   - Chilled Affymetrix® Nuclease-Free water
   - GC-Melt Reagent
   - Reagent reservoir should be placed on the upper half of the cooling block on ice.
3. If the diluted ligated samples aliquoted into the PCR plate are frozen, thaw them at room temperature. Make sure the plate is sealed tightly and vortex at high speed for 1 second in all corners and in the center according to the guidelines in *Seal, Vortex, and Spin* on page 5, then spin down at 2000 rpm for 1 minute.
4. Immediately place in the lower half of the chamber as shown in Figure 6.10.

Figure 6.10 Setup for PCR (TITANIUM Taq DNA Polymerase not shown; still at –20 °C)



5. To prepare the reagents (except enzyme):
   - **A.** Vortex the reagents at high speed 3 times, 1 second each time. Pulse spin for 3 seconds.
   - **B.** Place in the cooling block.

Turn On the Thermal Cycler (Post-PCR Area)

Have someone in the Post-PCR Area power on the thermal cycler to preheat the lid. Leave the block at room temperature.

To avoid contamination, do not go from the Pre-PCR Clean Area to the Post-PCR Area and back again.

Prepare the PCR Master Mix

IMPORTANT: Accurate pipetting of all components is critical for obtaining the correct size distribution of PCR products.

1. Keeping the 15 mL centrifuge tube on ice, add the reagents in the order shown in Table 6.5 and Figure 6.10 on page 62, **except for the 50X TITANIUM™ *Taq* DNA polymerase**.
2. Remove the 50X TITANIUM™ *Taq* DNA Polymerase from the freezer and immediately place in a cooler, chilled to –20 °C.
3. Vortex at high speed for 1 second.
4. Pulse spin the 50X TITANIUM™ *Taq* DNA polymerase for 3 seconds.
5. Immediately add the 50X TITANIUM™ *Taq* DNA polymerase to the master mix, then return the tube to the –20 °C cooler.
6. Vortex the master mix at high speed 3 times, 1 second each time.
7. Pour the master mix into the reagent reservoir, keeping the cooling block on ice.

Table 6.5 PCR Master Mix

Reagent	1 Sample	8 Samples (15% overage)	16 Samples (15% overage)	24 Samples (15% overage)
Chilled Affymetrix® Nuclease-Free Water	39.5 µL	1453.6 µL	2907.2 µL	4360.8 µL
10X TITANIUM™ *Taq* PCR Buffer	10.0 µL	368.0 µL	736.0 µL	1104.0 µL
GC-Melt Reagent	20.0 µL	736.0 µL	1472.0 µL	2208.0 µL
dNTP Mixture (2.5 mM each)	14.0 µL	515.2 µL	1030.4 µL	1545.6 µL
PCR Primer, 002	4.5 µL	165.6 µL	331.2 µL	496.8 µL
50X TITANIUM™ *Taq* DNA Polymerase (*Do not add until ready to aliquot master mix to ligated samples.*)	2.0 µL	73.6 µL	147.2 µL	220.8 µL
Total	**90.0 µL**	**3312.0 µL**	**6624.0 µL**	**9936.0 µL**

Add PCR Master Mix to Each Sample

To add the PCR Master Mix to samples:

1. Unseal the PCR sample plate and discard the seal.
2. Using a multi-channel P200 pipette, aliquot 90 µL PCR Master Mix to each sample and control on the PCR plate.

   To avoid contamination, change pipette tips after each dispensing.

   For eight samples, you may have to tilt the reagent reservoir to ensure that each pipette tip picks up 90 µL.

   After adding the master mix, the total volume in each well is 100 µL.

Ligated and diluted DNA	10 µL
PCR Master Mix	90 µL
Total	**100 µL**

3. Tightly seal the plate with a new seal according to the guidelines in *Seal, Vortex, and Spin* *on page 5*.
4. Vortex at high speed for 1 second in all corners and in the center according to the guidelines in *Seal, Vortex, and Spin* on page 5. REPEAT vortexing one more time, then spin down at 2000 rpm for 1 minute.
5. Keep the plate in the cooling block on ice until ready to load onto a thermal cycler.

Load PCR Plate onto a Thermal Cycler

! IMPORTANT: Ensure that the GeneAmp® PCR System 9700 thermal cycler you are using is equipped with silver or gold-plated silver blocks. Do NOT use thermal cyclers with aluminum blocks. It is difficult to visually distinguish silver and aluminum blocks. Instead, confirm the block type by checking the part number.

Location

Post-PCR Area

Procedure

To load the plate and run the CytoScan PCR program:

1. Transfer the plate on ice to the Post-PCR Area.
2. Ensure that the thermal cycler lid is preheated.

The block should be at room temperature.

3. Load the plate onto the thermal cycler.
4. Run the CytoScan PCR program.

Table 6.6 CytoScan PCR Thermal Cycler Program for the GeneAmp® PCR System 9700 (use only silver or gold-plated silver blocks)

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

Check the PCR Reaction by Running a Gel

To ensure consistent results, run a 3 µL aliquot from each PCR reaction on a gel.

WARNING: Use good laboratory practices and always wear the appropriate personal protective equipment when handling ethidium bromide.

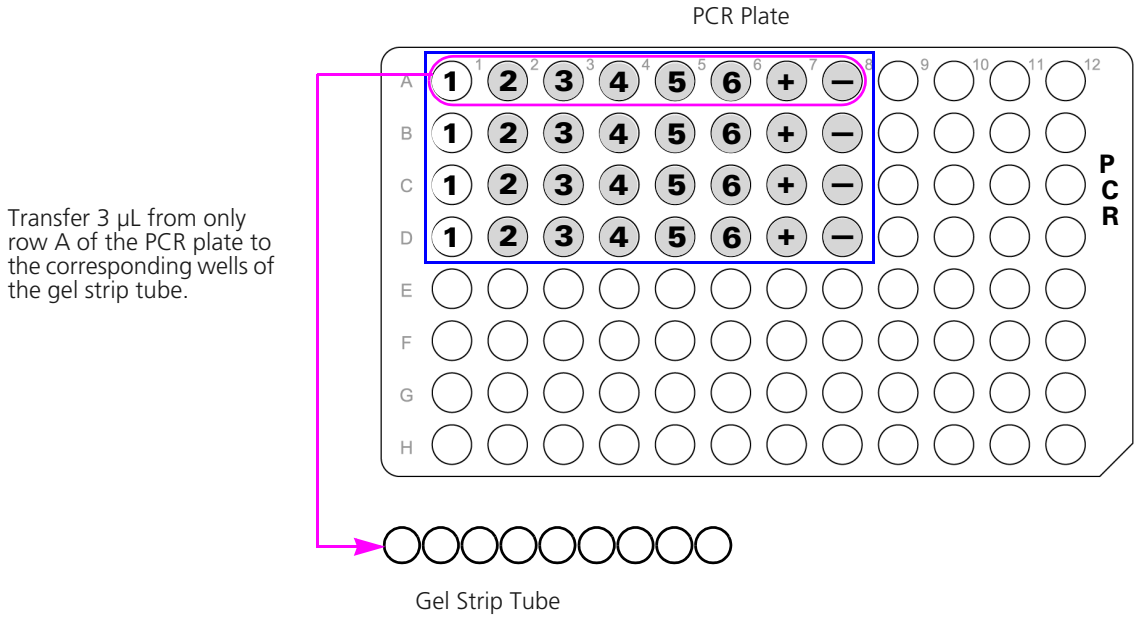
Run the Gels

When the CytoScan PCR program is finished:

1. Remove the plate from the thermal cycler.
2. Make sure the plate is sealed tightly and spin down at 2000 rpm for 1 minute.
3. Place in the cooling block on ice.
4. Label a fresh 8 to 12-well strip tube as shown in Figure 6.11.
   This is referred to as the *gel strip tube*.
5. Aliquot 5 µL of Affymetrix® Nuclease-Free water with 2 µL of 5X RapidRun™ Loading Dye to each well of the strip tube to be used.

6. Unseal the PCR plate and discard the seal.
7. Transfer 3 µL of PCR product from each well of row A only of the plate to the corresponding wells of the strip tube.

Figure 6.11 Transfer aliquots of each PCR product to the gel plate



8. Seal the PCR plate tightly with a new seal and store it at –20 °C if not proceeding to the purification step.
9. Seal the gel strip tubes tightly with an adhesive seal strip.
10. Vortex the gel strip tubes, then spin them down briefly in the strip tube microfuge.

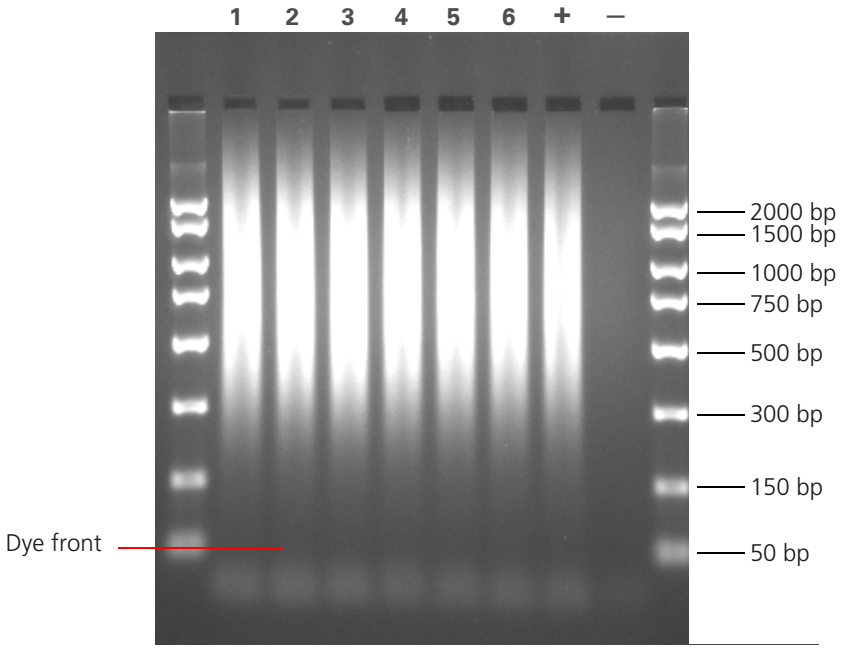
NOTE: Do not forget to add ethidium bromide to the gel running buffer. Add two drops of ethidium bromide per liter of 1X TBE.

11. Load 8 µL from each well of the gel strip tube onto a 2% TBE gel.
12. Load 5 µL USB PCR marker 50-2000bp to the first and last wells of the gel.
13. Run the gel at 5V/cm for 45 minutes or until the dye front reaches at least 75% of distance down the gel.

NOTE: Run gels at 5V/cm (5 volts × Distance in cm between electrodes). For example, run a 33 cm electrophoresis box at 165 V; run a 16 cm electrophoresis box at 80 V.

14. Verify that the PCR product distribution is between ~150 bp to 2000 bp (Figure 6.12 on page 67).

Figure 6.12 Example of PCR products run on 2% TBE agarose gel at 5 v/cm for 45 minutes. Average product distribution is between ~150 to 2000 bp.



What To Do Next

Do one of the following:

- If the PCR has been confirmed, proceed to *Stage 4: PCR Product Purification on page 68*.
- If not proceeding directly to the next stage, seal the plate with PCR product and store at –20 °C.

Stage 4: PCR Product Purification

Prepare Purification Wash Buffer

1. Add **45 mL** of **absolute ethanol** to the Purification Wash Buffer bottle.

IMPORTANT: Ensure that the correct amount of ethanol has been added to the Purification Wash Buffer bottle.

2. Cap the bottle tightly and shake.
3. Enter the preparation date on the bottle label and put a check mark in the check box.

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 frozen, thaw the PCR products in a plate holder on the bench top to room temperature.
2. Ensure the plate seal is tight. Vortex the plate at high speed for 1 second in all corners and in the center according to the guidelines in *Seal, Vortex, and Spin* on page 5, then spin down at 2000 rpm for 1 minute.
3. Mark each 1.5 mL Eppendorf tube with a sample number such as *1, 2, 3*, *4,* etc.
4. Remove and discard the plate seal.
5. Using a P200 single or multi-channel pipette, transfer all 4 aliquots of each sample to the appropriately marked 1.5 mL tube (Figure 6.13 on page 69).

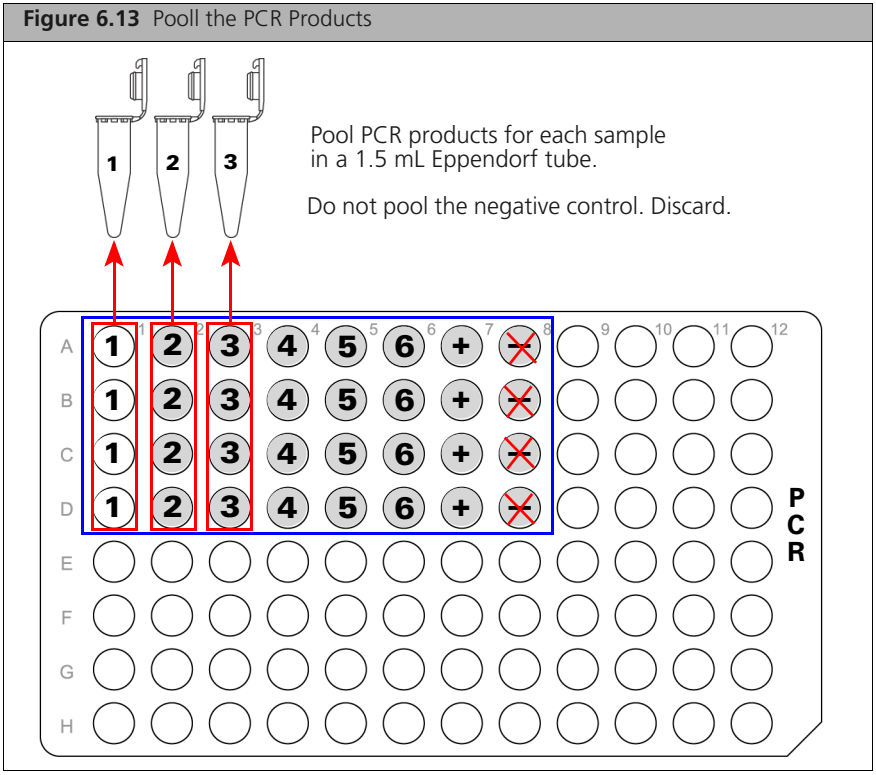
   Do not pool the negative control. Discard.

   IMPORTANT: Change pipette tips after each transfer.

PCR wells (4):	100 µL from each well	= 400 µL
Total Volume in Each 1.5 mL Eppendorf Tube		**= 400 µL/tube – 3 µl aliquoted for PCR gel**

6. When finished, examine the PCR plate and ensure that the total volume in each well has been transferred and pooled.

Figure 6.13 Pooll the PCR Products



Purify the Pooled PCR Products

Add Purification Beads and Incubate

To add the Purification Beads and incubate:

1. Thoroughly mix the Purification Beads stock by shaking and inverting the bottle. Examine the bottom of the bottle and ensure that the solution appears homogenous.
2. Open the tube caps slowly to ensure that the PCR sample does not spill out.

! IMPORTANT: The bead solution is viscous. Pipet slowly to ensure that you aspirate and dispense 720 µL.

3. Add 720 µL of Purification Beads to each pooled sample.
   - For < 8 samples: Use a single-channel P1000 pipette to add 720 µl of beads directly from the bottle to the sample. Change tips between pipetting steps.

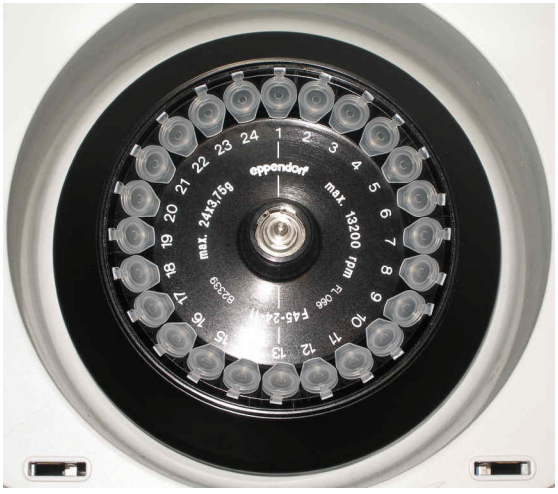
- For 16 and 24 sample workflows, 720 µl of beads may be added directly from the bottle to the sample using a single-channel P1000 pipette as described above. Alternatively, the beads can be added with a multi-channel P1000 pipette as follows:
  - 16 samples: Aliquot 15 ml of beads into a reagent reservoir.
  - 24 samples: Aliquot 21 ml of beads into a reagent reservoir.

  Using a multi-channel P1000 pipette, add 720 µL of Purification Beads to each pooled sample, three at a time. Organize your tips to enable multi-channel pipetting into the tubes to match the tube and pipette.

4. Securely cap each tube and mix well by inverting 10 times. Thorough mixing is critical to ensure that the PCR products bind to the beads.
5. Incubate at room temperature for 10 minutes.

   During incubation, the DNA binds to the Purification Beads.
6. Load the tubes – cap hinge facing out – onto the microcentrifuge and spin for 3 minutes at maximum speed (16,100 rcf; Figure 6.14).

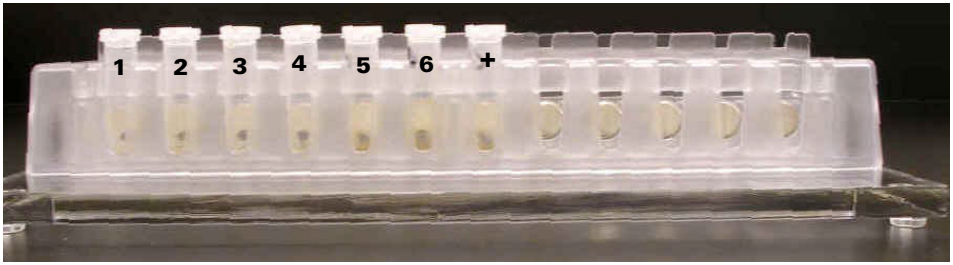
Figure 6.14 Position tubes with cap hinges out



Position tubes with the **cap hinges facing out**.
Bead pellet will be spun to the bottom and back of the tube.

7. Place the tubes on the magnetic stand until all of the pellets move to the magnet (Figure 6.15).

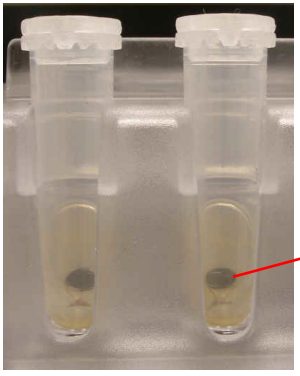
Figure 6.15 Samples with Purification Beads (MagnaRack Shown as an Example)



8. Use a P1000 pipette to pipet off the supernatant without disturbing the bead pellet. Discard the supernatant.

   For > 8 samples, use a multi-channel P1000 pipette to pipet off the supernatant without disturbing the bead pellet. Discard the supernatant. Ensure that the pipette tips are arranged to enable this. It is recommended that you remove supernatant from, at the most, 3 samples at a time.

Figure 6.16 Bead Pulled to Back and Side of Tube in Magnetic Stand



Note: The bead pellet will form and be pulled aside in all supported racks. This is an example of pellet formation in the MagnaRack.

Avoid contact with the bead pellet when pipetting off the supernatant.

Add Purification Wash Buffer

IMPORTANT: Ensure that absolute ethanol has been added to the Purification Wash Buffer bottle.

1. Using a P1000 pipette, add 1 mL of Purification Wash Buffer to each tube.

For > 8 samples, pour the wash buffer into a reservoir. Add 1 mL of Purification Wash Buffer to each tube using a multi-channel pipette, 3 samples at a time. Ensure that the pipette tips are arranged to enable this.

2. Cap the tubes and load them into the foam tube adaptor (Figure 6.17).

   Fully insert tubes into the foam to ensure they are secure. Space seven sample tubes and a balance tube adequately to balance (Figure 6.17).

3. Vortex at maximum setting for 2 minutes. The bead pellet may not be completely resuspended—that is OK.
4. Centrifuge the tubes for 3 minutes at maximum speed (position tubes with **cap hinges facing out**; 16,100 rcf).
5. Place the tubes on the magnetic stand until all of the pellets move to the magnet.

Figure 6.17 Resuspended Bead Clump and Vortexer with Foam Tube Adaptor

6. Use a P1000 pipette to pipet off the supernatant without disturbing the bead pellet and discard.

   For > 8 samples, use a multi-channel pipette to remove the supernatant from 3 samples at a time. Ensure that the pipette tips are arranged to enable this.

7. Spin the tubes for 30 seconds at maximum speed (hinges facing out; 16,100 rcf).
8. Place the tubes back on the magnetic stand.

! IMPORTANT: While pipetting out, be careful not to disturb or break off any of the bead pellet.

9. Using a single channel P20 pipette, remove the remaining drops of the Purification Wash Buffer from the bottom of each tube, one sample at a time.
10. Remove the tubes **OFF** the magnetic stand and allow the remaining Purification wash Buffer to evaporate by leaving the tubes uncapped at room temperature for 10 minutes.

Add Elution Buffer

To add Elution Buffer to each sample:

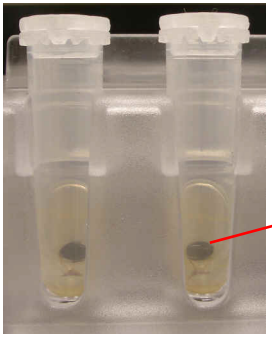
! IMPORTANT: To ensure better resuspension of the beads, add Elution Buffer directly onto the beads.

1. Using a P200 pipette, add 52 µL of Elution Buffer to each tube directly onto the beads.
2. Cap the tubes and load them into the foam tube adaptor. Make sure the tubes are balanced.
3. Vortex at maximum setting for 10 minutes.

   Vortexing will resuspend the purification beads.
4. Examine each tube to ensure that the beads are resuspended in a homogeneous slurry. If the beads are not fully resuspended, flick the tube to dislodge the pellet, and vortex an additional 2 minutes. Re-examine.
5. Centrifuge the tubes for 3 minutes at maximum speed (position tubes with **cap hinges facing out**; 16,100 rcf). For centrifuging, be sure to position all tubes with the cap hinges facing out as shown in Figure 6.14.
6. Place the tubes on the magnetic stand for 10 minutes.

   The purification beads are pulled to the side of the tube (Figure 6.18).

Figure 6.18 Bead Pulled to Back and Side of Tube in Magnetic Stand



Note: The bead pellet will form and be pulled aside in all supported racks. This is an example of pellet formation in the MagnaRack.

Avoid contact with the bead pellet when pipetting off the supernatant.

7. Check that all of the beads have been pulled to the side in each tube.

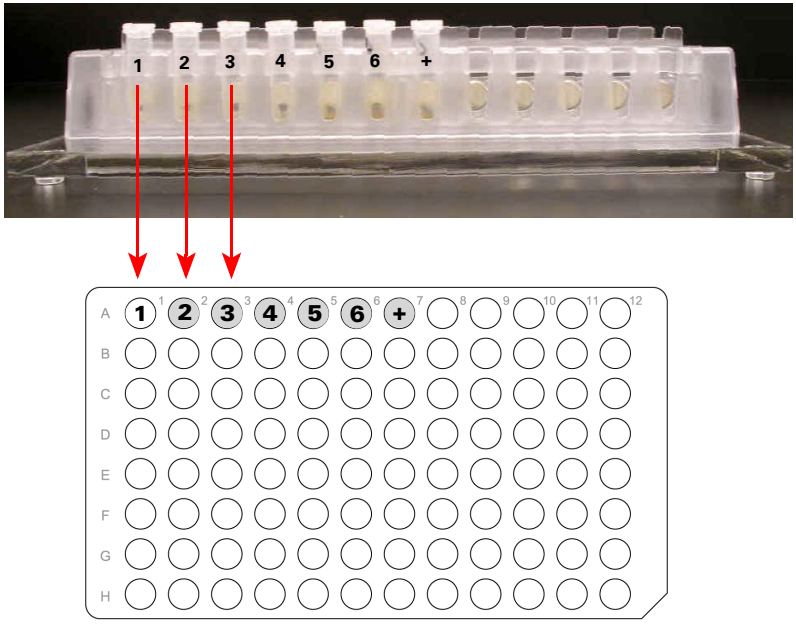
   If not, vortex the tubes to resuspend the pellet, Centrifuge the tubes for 3 minutes at maximum speed (position the tubes with the **cap hinges facing out**; 16,100 rcf). Place the tubes on the magnetic stand for 10 minutes.

NOTE: The eluate may appear yellowish.

8. Transfer 47 µL of eluted sample to the appropriate well on a fresh 96-well plate (Figure 6.19 on page 74).

   Sometimes at this step a brown residue is observed at the end of the pipette tip. It will usually remain behind on the tip when the sample is pipetted out.

Figure 6.19 Transfer Each Purified Sample to a Fresh 96-well Plate (MagnaRack Shown as an Example)



9. After transferring the eluted samples to the plate, tightly seal the plate and vortex at high speed for 1 second in all corners and in the center according to the guidelines in *Seal, Vortex, and Spin* on page 5, then spin down at 2000 rpm for 1 minute.

What To Do Next

Proceed to *Stage 5: Quantitation* on page 75.

Stage 5: Quantitation

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 linear range of the instrument.**
- **The spectrophotometer should be calibrated regularly to ensure correct readings.**
- **This protocol has been optimized using a UV spectrophotometer for quantitation.**

Prepare the Reagents, Equipment and Consumables

Turn on the Spectrophotometer

Turn the instrument ON and allow it to warm for at least 10 minutes before use.

Prepare the Work Area

To prepare the work area:

1. Place the following on the bench top:
   - Optional: conical tube or reagent reservoir
   - Affymetrix® Nuclease-Free Water
   - UV or 96-well plate
2. Ensure that the plate is sealed tightly. Vortex and spin down the purified samples at 2000 rpm for 1 minute, and put in a plate holder.

Procedure if Using a Microplate Spectrophotometer

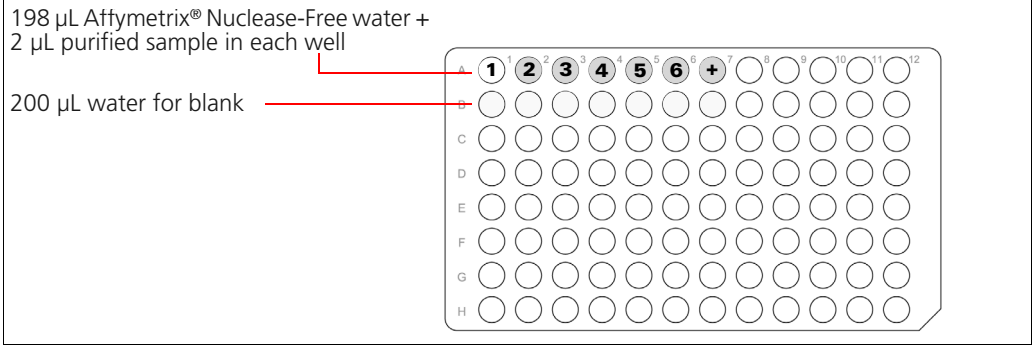
Prepare Diluted Aliquots of Purified Sample

IMPORTANT: The P20 pipette must be calibrated as per the manufacturer's specifications.

To prepare diluted aliquots of the purified samples:

1. Using a multi-channel P200 pipette, aliquot 198 µL of water to the corresponding wells of a UV plate.
2. Pipet 200 µL of water into each well of an empty row to be used as a BLANK (Figure 6.20 on page 76).
3. Using a multi-channel P20 pipette:
   A. Transfer 2 µL of each purified sample to the corresponding well of the UV plate.
   B. Pipet up and down 2 times to ensure that all of the sample is dispensed.
   The result is a 100-fold dilution.
4. Seal the plate with purified samples tightly with a new seal and store at –20 °C.
5. Seal the UV plate, and using a Kimwipe® on the adaptor surface, vortex, and spin down at 2000 rpm for 1 minute.

Figure 6.20 UV Plate Layout



Quantitate the Diluted PCR Product

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.

To quantitate the diluted purified PCR product:

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

OD280 and OD320 are used as controls.

2. Determine the OD260 measurement for the water blank and average.
3. Determine the concentration of each PCR product as follows:
   A. Calculate one OD reading for every sample:
      OD = (sample OD) – (average water blank OD)
   B. Calculate the undiluted concentration for each sample in µg/µL:
      Undiluted sample concentration = OD X 0.05 ug/uL X 100

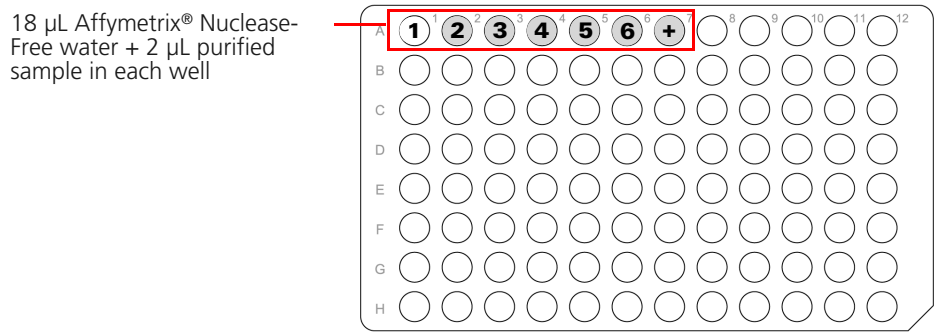
Procedure if Using a NanoDrop

! IMPORTANT: The P20 pipette must be calibrated as per the manufacturer's specifications.

To prepare diluted aliquots of the purified samples:

1. Using a P20 pipette, aliquot 18 µL of water to the corresponding wells of a 96-well plate.
2. Using a P20 pipette:
   A. Transfer 2 µL of each purified sample to the corresponding well of the 96-well plate.
   B. Pipet up and down 2 times to ensure that all of the sample is dispensed.
      The result is a 10-fold dilution.
3. Do one of the following to mix the samples:
   - Set a P20 pipette to 17 µL and pipet up and down 5 times.
   - Seal the plate tightly, vortex, and spin down at 2000 rpm for 1 minute.

Figure 6.21 96-well Plate Layout for NanoDrop



4. Blank the NanoDrop with water.
5. Take 2 µL of the diluted sample and:
   A. Measure the OD of each sample at 260, 280 and 320 nm.
      OD280 and OD320 are used as controls.
   B. Calculate the undiluted concentration for each sample as follows:
      Undiluted sample concentration in µg/µL = (Nanodrop Concentration in ng/µL × 10) ÷ (1000)

Assess the Yield

Acceptable DNA Yield

The average purification yield for 7 or more samples should be ≥ 3.0 µg/µl. If the average yield is < 3.0 µg/µL, please consult the troubleshooting section. We do not recommend further processing of samples with yields < 2.5 µg/µL.

The following OD ranges are based on the use of a conventional UV spectrophotometer plate reader and assume a path length of 1 cm.

- 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 (≤ 0.1).

If your OD readings are not within the acceptable range, refer to Chapter 8, *Troubleshooting* on page 107.

What To Do Next

Do one of the following:

- Proceed immediately to *Stage 6: Fragmentation* on page 79.
- If not proceeding immediately to the next step, seal the plate of purified samples, and store at –20 °C.

Stage 6: Fragmentation

Important Information About This Stage

The degree of fragmentation is critical. Perform this stage carefully to ensure uniform, reproducible fragmentation. To help ensure the best results, carefully read the information below before you begin this stage of the protocol.

IMPORTANT: All additions, dilutions, and mixing must be performed on ice. Ensure all reagents reach equilibrium before use.

About the Fragmentation Reagent

- This enzyme 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.
  - 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 all steps rapidly and without interruption.
- Add enzyme to the fragmentation master mix last.
- This enzyme 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 pipette tip.
  - Avoid excess solution on the outside of the pipette tip.

Prepare the Reagents, Equipment, and Consumables

Turn on the Thermal Cycler

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

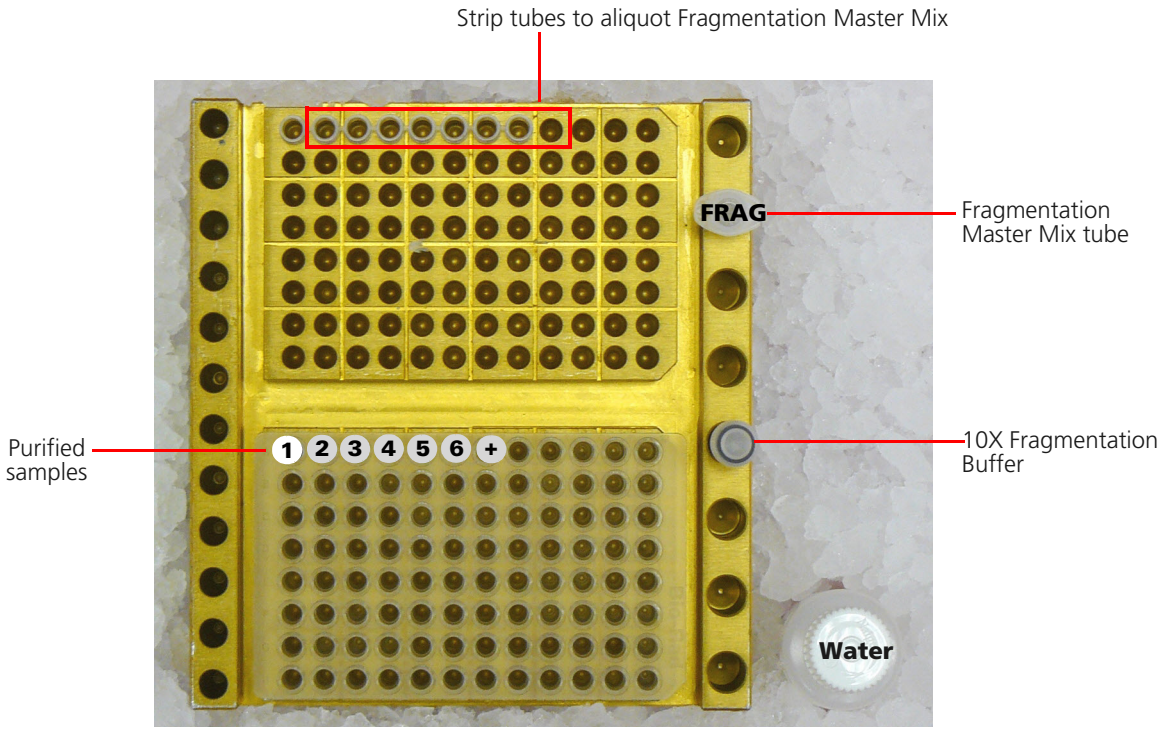
Set Up the Work Area

IMPORTANT: Always spin down the fragmentation plate in a centrifuge that has been cooled down to 4 °C.

To set up the work area (Figure 6.22):

1. Turn down the plate centrifuge to 4 °C at least 15 to 20 minutes prior to proceeding into the fragmentation step. Remember to close the centrifuge lid to facilitate effective cooling.
2. Place a double cooling block and the Affymetrix® Nuclease-Free water on ice.
3. Place an 8-tube strip in the cooling block as shown in Figure 6.22 and chill it for at least 10 minutes prior to use.
4. Label a 1.5 mL Eppendorf tube as *FRAG* for the Fragmentation Master Mix and keep it chilled in the cooling block.
5. Cut adhesive seal into strips wide enough to seal 8 or 12 strip tubes.
6. Ensure that the plate centrifuge is at 4 °C.

Figure 6.22 Setup for Fragmentation (Fragmentation Reagent not pictured, still at –20 °C)



Thaw and Prepare the Reagents

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

1. Remove the plate of purified, quantitated samples from the –20 °C freezer and thaw at room temperature. Once thawed completely, make sure the plate is sealed tightly, then vortex and spin down the plate. Place the plate on lower half of the cooling block on ice and chill for 10 minutes prior to use.
2. Thaw the Fragmentation Buffer (10X) at room temperature. Immediately place on cooling block on ice when thawed.
3. Prepare the Fragmentation Buffer as follows:
   A. Vortex 3 times, 1 second each time.
   B. Pulse spin for 3 seconds.
   C. Place in the cooling block.

Prepare the Fragmentation Master Mix

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

We strongly recommend preparing the Fragmentation Master Mix following Table 6.7 only. The Fragmentation Master Mix is sufficient for 1 to 24 samples.

IMPORTANT: Check the Fragmentation Reagent concentration before making the Master Mix. Do not make less than the recommended volume of Master Mix

1. Check the concentration of the Fragmentation Reagent stated on the tube label, then add the required volume of the water and fragmentation buffer by following Table 6.7.
2. Mix by vortexing for 1 second and pulse spin. Place back in the cooling block on ice.

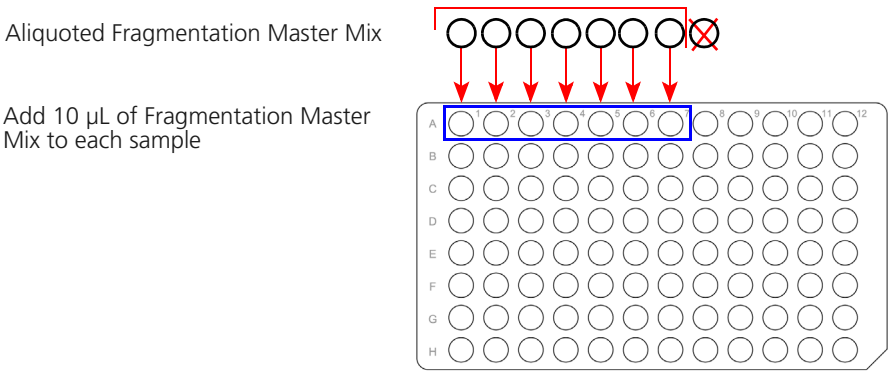
Table 6.7 Fragmentation Master Mix

Reagent	Fragmentation Reagent Concentration				
	2.0 U/µL	**2.25 U/µL**	**2.5 U/µL**	**2.75 U/µL**	**3.0 U/µL**
Chilled Affymetrix® Nuclease-Free Water	122.4 µL	123.2 µL	123.8 µL	124.4 µL	124.8 µL
10X Fragmentation Buffer	158.4 µL	158.4 µL	158.4 µL	158.4 µL	158.4 µL
Fragmentation Reagent	7.2 µL	6.4 µL	5.8 µL	5.2 µL	4.8 µL
Total	**288.0 µL**	**288.0 µL**	**288.0 µL**	**288.0 µL**	**288.0 µL**

3. Remove the Fragmentation Reagent from the freezer and immediately place it in the cooler, chilled to –20 °C.
   - **A.** Vortex the Fragmentation Reagent at high speed one time for 1 second.
   - **B.** Immediately pulse spin for 3 seconds to bring down any reagent that may be clinging to the top of the tube.
   - **C.** Immediately place in the –20 °C cooler.
4. Add the appropriate volume of Fragmentation Reagent from Table 6.7. Immediately place it back in the –20 °C cooler.
5. Vortex the master mix at high speed 3 times, 1 second each time.
6. Pulse spin for 3 seconds and immediately place in the cooling block.
7. Proceed immediately to the next set of steps, *Add Fragmentation Master Mix to the Samples*.

Add Fragmentation Master Mix to the Samples

Figure 6.23 Adding Fragmentation Master Mix to Samples



To add Fragmentation Master Mix to the samples:

1. Quickly aliquot out the Fragmentation Master Mix equally to the strip tubes placed in the cooling block on ice (Figure 6.23). Seal the strip tubes with an adhesive seal strip (or strip caps).
2. Spin down the strip tubes and place them back in the cooling block on ice. Remove the seal and discard.
3. Remove and discard the plate seal.
4. Using a multi-channel P20 pipette, transfer 10 µL of Fragmentation Master Mix to each sample — do not pipet up and down.

   Avoid introducing air bubbles at the bottom of the tubes to ensure the accurate transfer of 10 µL to each sample.

! IMPORTANT: Add the master mix to the samples as quickly as possible.

Purified PCR product	45 µL
Fragmentation Master Mix	10 µL
Total	**55 µL**

5. Seal the plate tightly with a new seal.

! IMPORTANT: Always carry the sample plate to the centrifuge or the thermal cycler on the cooling block in the ice box.

6. Vortex at high speed for 1 second in all corners and in the center according to the guidelines in *Seal, Vortex, and Spin* on page 5.
7. *Bring the sample plate to the centrifuge on the cooling block in the ice box.* Spin the plate in the pre-chilled centrifuge at 2000 rpm for 1 minute. Quickly remove the plate from the centrifuge and place in the cooling block in the ice box.
8. *Carry the sample plate on the cooling block in the ice box* and immediately load the Fragmentation plate onto the thermal cycler with preheated lid. Run the CytoScan Fragment program (Table 6.8).

Table 6.8 CytoScan Fragment Thermal Cycler Program

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

9. Remove and discard any remaining Fragmentation Master Mix. Never re-use Fragmentation Master Mix.
10. At this point the plate centrifuge may be turned back to room temperature.

What To Do Next

Check the fragmentation reaction by running gels as described under *Check the Fragmentation Reaction by Running a Gel*. If not proceeding directly to the next stage, store the samples at –20 °C.

Check the Fragmentation Reaction by Running a Gel

The instructions below are for running 4% TBE gels.

To ensure that fragmentation was successful:

1. When the CytoScan Fragment program is finished:
   A. Remove the samples from the thermal cycler.
   B. Make sure the plate is sealed tightly, then spin down at 2000 rpm for 1 minute. Place on the lower half of the cooling block on ice.
2. Label two 8-strip tubes: one as *Fragmentation QC Samples* and the other as *Gel Analysis*.
3. Remove and discard the plate seal.
4. Remove 4 µl of fragmented samples into strip tubes, labeled as *Fragmentation QC Samples*.
5. Seal the fragmented DNA plate with a new seal and keep it on the lower half of the cooling block on ice. If not proceeding immediately to Labeling step, store the plate at –20 °C.
6. Add 28 µL water to the strip tubes labeled as Fragmentation QC Samples. Seal the strip, vortex, and spin down.
7. Remove 8 µL of the diluted Fragmented QC samples and dispense into respective wells of the strip tubes labeled as *Gel Analysis*.

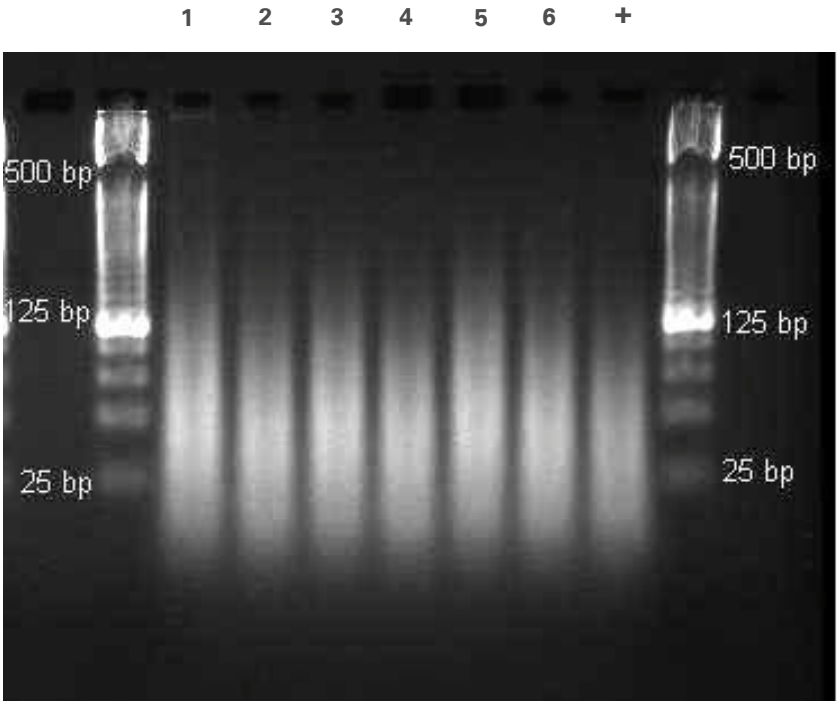
NOTE: Do not forget to add ethidium bromide to the gel running buffer in the gel box. Add 2 drops of the ethidium bromide per 1L of 1X TBE.

8. Seal and store the remaining Fragmentation QC Sample strip tubes at -20 °C for further analysis using the Agilent 2100 Bioanalyzer. For more details, see Appendix C on page 135.
9. Add 2 µL of USB 5X RapidRun™ Loading Dye to each sample in the Gel Analysis QC strip.
10. Seal the strip tubes tightly with an adhesive seal strip, vortex, and spin down.
11. Load 8 µL of the samples onto the gel.
12. Load 2 µL TrackIt™ 25 bp DNA Ladder to the first and last lanes.
13. Run the samples on a 4% TBE gel at 5 V/cm for 45 minutes or until the dye front reaches at least 75% of distance down the gel.

NOTE: Run gels at 5V/cm (5 volts × Distance in cm between electrodes). For example, run a 33 cm electrophoresis box at 165 V; run a 16 cm electrophoresis box at 80 V.

14. Inspect the gel and compare it against the example shown in Figure 6.24. The majority of fragment distribution should be between 25 to 125 bp.

Figure 6.24 Example of Fragmented PCR Products Run on 4% TBE Agarose Gel at 5 V/cm for 45 minutes. Fragmentation is confirmed by majority of distribution between 25 to 125 bp.



Stage 7: Labeling

Prepare the Reagents, Equipment, and Consumables

Turn on the Thermal Cycler

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

Set Up the Work Area

1. Place a double cooling block on ice (Figure 6.25 on page 88).
2. Place an 8-tube strip in the upper half of the cooling block on ice.
3. Label the 1.5 mL eppendorf tube as *LBL*, and place in the cooling block.
4. Cut adhesive seal into strips wide enough to seal 8 or 12 strip tubes.

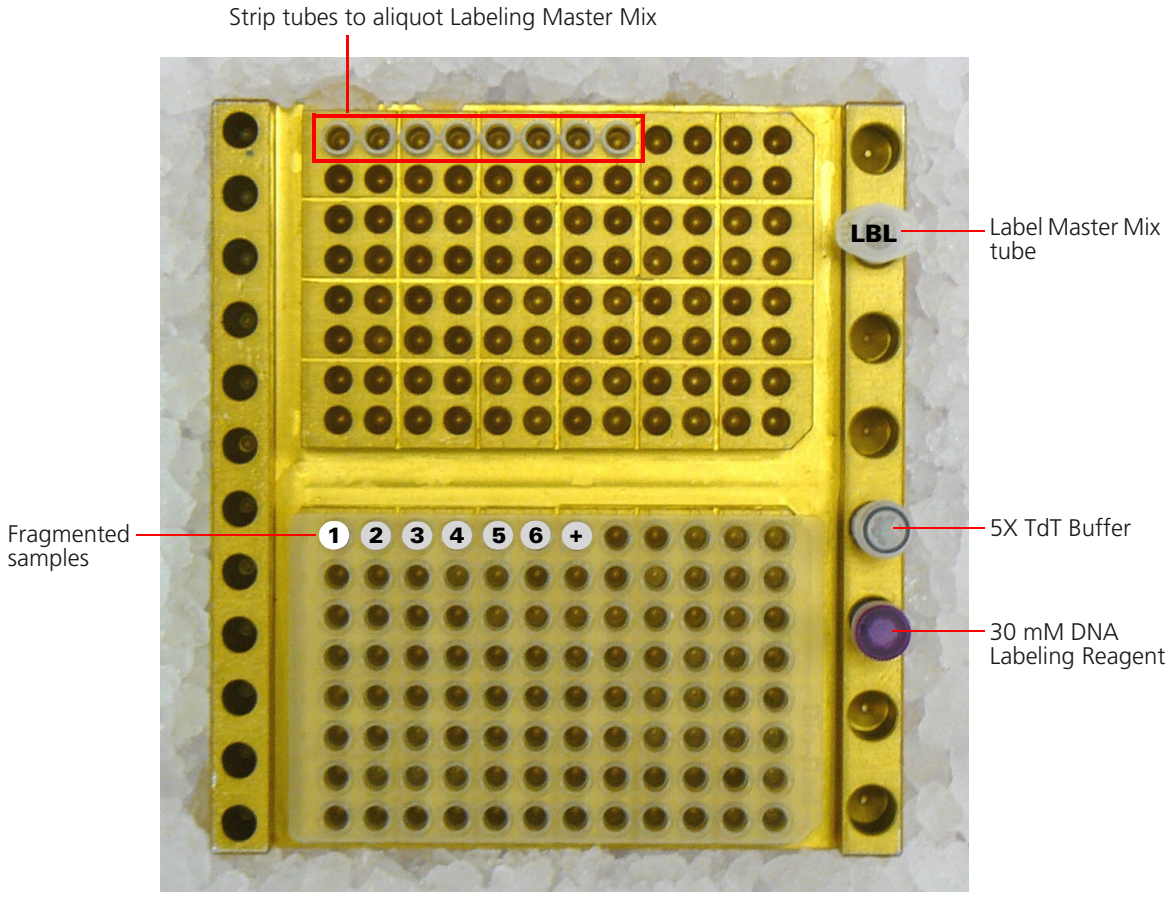
Thaw and Prepare the Reagents

1. Thaw the following reagents at room temperature. Immediately place on cooling block on ice when thawed.
   - 5X TdT Buffer
   - 30 mM DNA Labeling Reagent

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

2. Prepare the 5X TdT Buffer and 30 mM DNA Labeling Reagent as follows:
   A. Vortex each reagent at high speed 3 times, 1 second each time.
   B. Pulse spin for 3 seconds, then place in the cooling block.
3. If the fragmented samples were frozen, allow them to thaw at room temperature. Immediately spin down the plate at 2000 rpm for 1 minute and place on the lower half of the cooling block on ice.

Figure 6.25 Setup for Labeling (TdT enzyme not pictured, still at –20 °C)



Prepare the Labeling Master Mix

Preparation

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

To prepare the Labeling Master Mix:

1. Add the following to the 1.5 mL Eppendorf tube on ice using the volumes shown in Table 6.9:
   - 5X TdT Buffer
   - 30 mM DNA Labeling Reagent

Table 6.9 Labeling Master Mix

Reagent	1 Sample	8 Samples (20% overage)	16 Samples (20% overage)	24 Samples (20% overage)
5X TdT Buffer	14.0 µL	134.4 µL	268.8 µL	403.2 µL
30 mM DNA Labeling Reagent	2.0 µL	19.2 µL	38.4 µL	57.6 µL
TdT	3.5 µL	33.6 µL	67.2 µL	100.8 µL
Total	**19.5 µL**	**187.2 µL**	**374.4 µL**	561.6 **µL**

2. Remove the TdT enzyme from the freezer and immediately place in the cooler, chilled to –20 °C.
3. Vortex the enzyme at high speed one time for 1 second.
4. Pulse spin the enzyme for 3 seconds, then immediately place back in the –20 °C cooler.
5. Add the TdT enzyme to the master mix. Place the enzyme back in the –20 °C cooler.
6. Vortex the master mix at high speed 3 times, 1 second each time.
7. Pulse spin for 3 seconds.

Add the Labeling Master Mix to the Samples

To add the Labeling Master Mix to the samples:

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

1. Aliquot the Labeling Master Mix equally into strip tubes that are pre-chilled on the cooling block on ice. Seal the strip with an adhesive seal strip (or strip caps) and pulse spin. Place back in the cooling block, remove the seal and discard.

NOTE: When working with more than 8 samples, we strongly recommend dividing the master mix into strip tubes and dispensing the master mix from the strip tubes into the samples using a multi-channel pipette.

2. Remove and discard the plate seal.
3. Using a P20 multi-channel pipette, aliquot 19.5 µL of Labeling Master Mix to each sample.

Fragmented DNA (less 4.0 µL for gel analysis)	51.0 µL
Labeling Mix	19.5 µL
Total	**70.5 µL**

4. Seal the plate tightly with a new seal.
5. Vortex at high speed for 1 second each in all corners and in the center according to the guidelines in *Seal, Vortex, and Spin* on page 5, then spin down for 1 minute at 2000 rpm.
6. Place the labeling plate in the pre-heated thermal cycler block and run the CytoScan Label program.

Table 6.10 CytoScan Thermal Cycler Program

CytoScan Label Program	
Temperature	**Time**
37°C	4 hr
95°C	15 minutes
4°C	Hold (OK to hold overnight)

7. When the CytoScan Label program is finished, remove the plate from the thermal cycler and spin down at 2000 rpm for 1 minute.

What To Do Next

Do one of the following:

- Proceed to the next stage.
- If not proceeding directly to the next stage, you can:
  - Hold at 4 °C on the thermal cycler overnight.
  - Freeze the samples at –20 °C.

Stage 8: Target Hybridization

Important Information About This Stage

To help ensure the best results, carefully read the information below before you begin this stage of the protocol. Since this manual is intended as an assay protocol manual, there is no specific section on all of the various features and workflows available in the Command Console software. If you would like to learn more about Command Console, please refer to the Affymetrix® GeneChip® Command Console® 3.2 User Manual (P/N 702569).

Prepare the Equipment

Turn On the Thermal Cycler

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

Preheat the Hybridization Oven 645

NOTE: Confirm that the Hybridization Oven 645 is calibrated. The hybridization oven should be serviced at least once per year to ensure operation within specification.

To preheat the hybridization ovens:

1. Turn on the oven at least 1 hour before hybridization with the temperature set to 50 °C.
2. Set the rpm to 60.
3. Turn the rotation on and allow to preheat for 1 hr before loading arrays.

Prepare the Arrays and Create a Batch Registration File

To prepare the arrays:

1. Unwrap the arrays and place on the bench top, septa-side up.
2. Mark the front and back of each array with a designation that will identify which sample is loaded onto each array (Figure 6.27).

Allow the arrays to warm to room temperature on the bench top for 10 to 15 minutes. During this time you can scan the barcode which will be used in batch registration.

Create a Batch Registration File

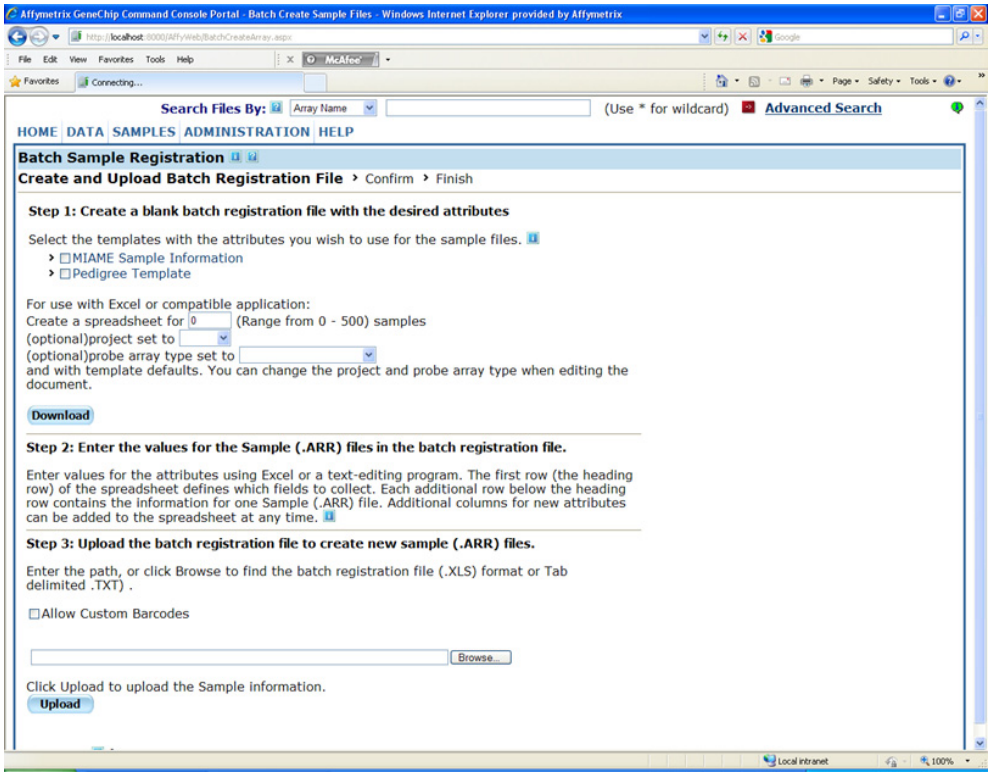
To register a new sample using AGCC:

1. From the Command Console, launch the AGCC Portal.

IMPORTANT: Confirm that you are running AGCC v.3.2.2 or higher. If not, please update your version of AGCC to v.3.2.2 or latest available.

2. Under the “Samples” tab, select “Batch Registration”.
   The Batch Registration window opens (Figure 6.26).

Figure 6.26 Barch Registration Window



3. Within Step 1:
   - **A.** Enter the number of samples for which a spreadsheet needs to be created under "Create a Spreadsheet for".
   - **B.** Select "Default" from the "Project Set to" drop-down list.

C. Select the appropriate array type from the "Probe Array type set to" drop-down list:
   - If using the CytoScan™ HD Array, select "CytoScanHD_Array".
   - If using the CytoScan™ 750K Array, select "CytoScan750K_Array".

D. Click **Download**.

An Excel spreadsheet will open.

4. Within Step 2:
   A. Name the experiment file using the following convention: "SampleName_PlateCoordinate_ExperimentDescriptionString_ArrayType_OperatorInitials_yyyymmdd".
   B. The sample file name and the 'Array name' would be identical.
   C. Scan the corresponding barcodes for each Sample name.
   D. Save the Excel file in "Excel 97-2003 workbook" format.
5. Within Step 3:
   A. Browse to the location of the Batch registration file that was saved.
   B. Upload the Batch registration file by clicking the tab to create new sample (.ARR) files.

      A new window opens.
6. Click **Save** to save the new sample files.

Prepare the Arrays

1. Place the arrays on a clean bench top area designated for hybridization.
2. Insert a 200 µL pipette tip into the upper right septum of each array.
3. Paste two 1/2” Tough-Spots on the top edge of the array for later use (Figure 6.27).

IMPORTANT: To ensure that the data collected during scanning is associated with the correct sample, mark each array in a meaningful way. It is critical that you know which sample is loaded onto each array.

Figure 6.27 Arrays Prepared for Sample Loading

Prepare the Reagents and Consumables

Set Up the Work Area

To set up the work area:

1. Place a double cooling block on ice (Figure 6.28 on page 95).
2. Place a reagent reservoir on the upper half of the cooling block on ice.
3. Label the 15 mL centrifuge tube as Hyb Master Mix, and place on the ice.

Prepare the Samples

1. If the labeled samples from the previous stage were frozen. allow them to thaw on the bench top to room temperature and spin down at 2000 rpm for 1 minute.
2. Immediately place the plate in the lower half of the cooling block on ice.

Thaw and Prepare the Reagents

Thaw the following reagents at room temperature. Immediately place on cooling block on ice when

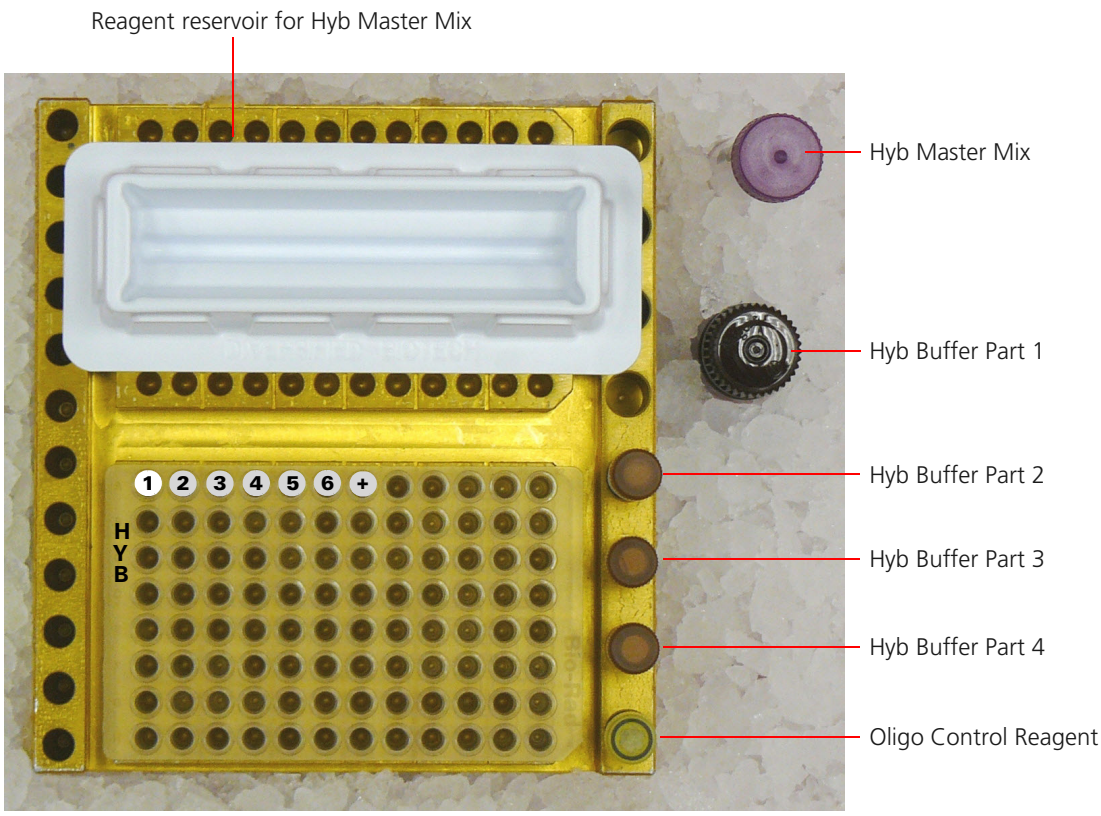
thawed.

- Hyb Buffer Part 1
- Hyb Buffer Part 2
- Hyb Buffer Part 3
- Hyb Buffer Part 4
- Oligo Control Reagent 0100

Prepare the reagents as follows:

1. Vortex each reagent at high speed 3 times, 1 second each time.
2. Pulse spin for 3 seconds, then place in the cooling block as shown in Figure 6.28.

Figure 6.28 Hybridization Setup



Prepare the Hybridization Master Mix

1. To the 15 mL Hyb Master Mix centrifuge tube on ice, add the appropriate volume of each reagent in the order shown in Table 6.11.

IMPORTANT: Some of the Hyb Buffer components are viscous; carefully pipette and dispense when preparing the master mix.

2. Mix well by vortexing the master mix at high speed 3 times, 3 seconds each time (until the mixture is homogeneous).

Table 6.11 Hybridization Master Mix*

Reagent	1 Sample (20% Overage)	8 Samples (20% Overage)	16 Samples (20% Overage)	24 Samples (20% Overage)
Hyb Buffer Part 1	165.0 µL	1584.0 µL	3168.0 µL	4752.0 µL
Hyb Buffer Part 2	15.0 µL	144.0 µL	288.0 µL	432.0 µL
Hyb Buffer Part 3	7.0 µL	67.2 µL	134.4 µL	201.6 µL
Hyb Buffer Part 4	1.0 µL	9.6 µL	19.2 µL	28.8 µL
Oligo Control Reagent 0100	2.0 µL	19.2 µL	38.4 µL	57.6 µL
Total	**190 µL**	**1824 µL**	**3648 µL**	**5472 µL**

IMPORTANT: Make sure the Hybridization Master Mix is adequately vortexed.

Add Hybridization Master Mix and Denature

NOTE: When working with more than 8 samples, we strongly recommend transferring the master mix to a reservoir and dispensing the master mix from the reservoir into the samples using a multi-channel pipette.

To add Hybridization Master Mix and denature the samples:

1. Remove and discard the plate seal.
2. Pour the Hybridization Master Mix into a reagent reservoir placed on the upper half of the cooling chamber on ice. Use a multi-channel pipette to add 190 µL of Hybridization mix to the samples.

IMPORTANT: The Hybridization Master Mix is viscous; pipette carefully when dispensing to samples.

3. Tightly seal the plate with a new seal and carefully check to confirm that the plate is well sealed.

IMPORTANT: The volume in the hybridizaton plate is full. Ensure that the plate is vortexed to mix sample and hybridization buffer well.

4. Vortex the plate at high speed for 1 second each in all corners and in the center. **REPEAT** vortexing to ensure that the plate is well mixed, then spin down for 1 minute.
5. Place the plate onto the pre-heated thermal cycler and run the CytoScan Hyb program.

Table 6.12 CytoScan Hyb Thermal Cycler Program

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

Load the Samples onto Arrays

To load the samples onto arrays:

1. When the thermal cycler reaches 49 °C, leave the samples at 49 °C for at least one minute, and then open the lid.

IMPORTANT: Load only 6 to 8 arrays at a time. Remove the seal from the hybridization plate for only 6 to 8 samples at a time.

2. If you are hybridizing more than eight samples, cut and remove the seal from 6 to 8 samples at a time only.

   Leave the remaining wells covered. Keeping these wells covered helps prevent cross-contamination and evaporation.

Figure 6.29 Loading Samples onto Arrays

IMPORTANT: **The hybridization mix is very viscous. Pipette slowly to ensure that all of the volume is loaded into the chip.**

3. Using a P200 pipette, remove 200 µL of the first sample and immediately inject it into an array.
4. Cover the septa on the array with the 1/2" Tough-Spots that were previously placed on the top edge of the array (Figure 6.29).

   Press firmly to ensure a tight seal to prevent evaporation and leakage.
5. When 6 to 8 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.
6. Repeat this process until all samples are loaded onto arrays and are placed in the hybridization oven.

   All samples should be loaded within 30 minutes.
7. Allow the arrays to rotate at 50 °C, 60 rpm for 16 to 18 hr.

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

Washing, Staining and Scanning Arrays

This chapter describes how to wash, stain and scan the Affymetrix® CytoScan™ Arrays. The instruments that you will use include the:

- Fluidics Station 450 to wash and stain the arrays
- GeneChip® Scanner 3000 7G to scan the 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 7.1 Equipment and Consumables Required for Washing, Staining and Scanning Arrays

Item	Vendor	Part Number
GeneChip® Scanner 3000 7G	Affymetrix	—
GeneChip® Fluidics Station 450	Affymetrix	—
The instrument control application: Affymetrix GeneChip® Command Console v.3.2.2 or higher	Affymetrix	—
Tube, Safe-Lock Tube 1.5 m, Amber	Eppendorf	022363221
Tube, Safe-Lock Tube 1.5 mL, Blue	Eppendorf	022363247
Tube, Safe-Lock Tube 1.5 mL, Natural	Eppendorf	022363352
Pipets, (P-2, P-20, P-200, P-1000)	Rainin Pipetman® (or equivalent)	—
Sterile-barrier pipette tips and non-barrier pipette tips	—	—
Tygon® Tubing, 0.04" inner diameter	Cole-Parmer	H-06418-04
Tough-Spots®, Label Dots (3/8")	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.

Table 7.2 Reagents Required for Washing and Staining Arrays

Reagent
Stain Buffer 1
Stain Buffer 2
Affymetrix® GeneChip® Array Holding Buffer
Affymetrix® GeneChip® Wash A
Affymetrix® GeneChip® Wash B

Fluidics Station and Scanner Control Software

You will use the Affymetrix GeneChip® Command Console (AGCC, Version 3.2.2 or higher) to operate the fluidics station and the scanner. For more information on the AGCC application, refer to the Affymetrix *GeneChip® Command Console™ User's Guide*

Prime 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

The Fluidics Station 450 is used to wash and stain the arrays; it is operated using AGCC software.

To prime the Fluidics Station:

1. Turn on the Fluidics Station.
2. Prime the Fluidics Station.
   - From Affymetrix Command Console application, start the 'Affymetrix Launcher'.
   - From the Affymetrix Launcher, open 'AGCC Fluidics Control' application.

- From the AGCC Fluidics Control panel, select 'PRIME_450' script for the specific fluidics stations and the modules.

! IMPORTANT: Use the Affymetrix® GeneChip® Wash A and Wash B buffers that are designated for the CytoScan™ Assay only. These wash and stain buffers differ from the GeneChip® expression buffers.

- Intake buffer reservoir A: use Wash A.
- Intake buffer reservoir B: use Wash B.

3. To initiate the fluidics script, click the "Run" icon for each module or click the "Run All" icon, for all the selected stations and modules.

Washing and Staining Arrays

1. Briefly vortex the stain bottles before aliquoting the reagents.
2. Aliquot the following reagents into 1.5 mL microfuge tubes for each array:
   A. Aliquot 500 µL Stain Buffer 1 into 1.5 mL microfuge tubes (use amber color tubes as Stain Buffer 1 is light sensitive).
   B. Aliquot 500 µL Stain Buffer 2 into 1.5 mL microfuge tubes (clear/natural tubes).
   C. Aliquot 800 µL Array Holding Buffer into 1.5 mL microfuge tubes (blue tubes).
3. Select a protocol from the AGCC Fluidics Control Panel:
   - If using the CytoScan™ HD Array, select "CytoScanHD_Array_450".
   - If using the CytoScan™ 750K Array, select "CytoScan750K_Array_450".
4. 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).

   Eject the wash block to avoid sensor time out.
5. Remove any previously loaded empty vials.
6. When prompted to "Load vials 1-2-3":
   A. Place one vial containing 500 µL Stain Buffer 1 in position 1.
   B. Place one vial containing 500 µL Stain Buffer 2 in position 2.
   C. Place one vial containing 800 µL Array Holding Buffer in position 3.
7. After 16 to 18 hrs of hybridization, remove no more than 8 arrays at a time from the oven. Remove the Tough-Spots from the arrays.

> **IMPORTANT:** **Once the arrays are removed from the hybridization oven, quickly load them onto the Fluidics Station. Delays during this step will impact data quality.**

8. Immediately insert the arrays into the designated modules of the fluidics station while the cartridge lever is in the Down or Eject position.
9. Press down on the needle lever to snap needles into position and to start the run.

   The fluidics protocol begins. The Fluidics Station dialog box at the workstation terminal and the LCD window display the status of the washing and staining steps.
10. When the wash and stain procedure is completed, remove the arrays from the fluidics station by first pressing down the cartridge lever to the Eject position.
11. Check the array window for bubbles or air pockets. If air bubbles are present, return the array to the fluidics station. Follow the instructions on the LCD panel of the fluidics station. Pull the lever up and load to remove bubbles.
12. If air bubbles are still present after repeating the above process a few times, use the manual process.
    A. Insert a 200 µL pipette tip into the upper right septum of the array.
    B. Using a pipette, remove half of the solution.
    C. Manually fill the array with Array Holding Buffer.
13. If the array has no bubble, it is ready for scanning. Proceed to *Scanning Arrays* *on page 102*.

    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 hr.
14. Pull up on the cartridge lever to engage wash block. Remove the microcentrifuge vials containing stain and replace with three empty vials as prompted.
15. When washing and staining are complete, shut down the fluidics station following the procedure on page 105.

Scanning Arrays

The GeneChip Scanner 3000 7G is controlled by AGCC software.

Prepare the Scanner

Turn on the scanner at least 10 minutes 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

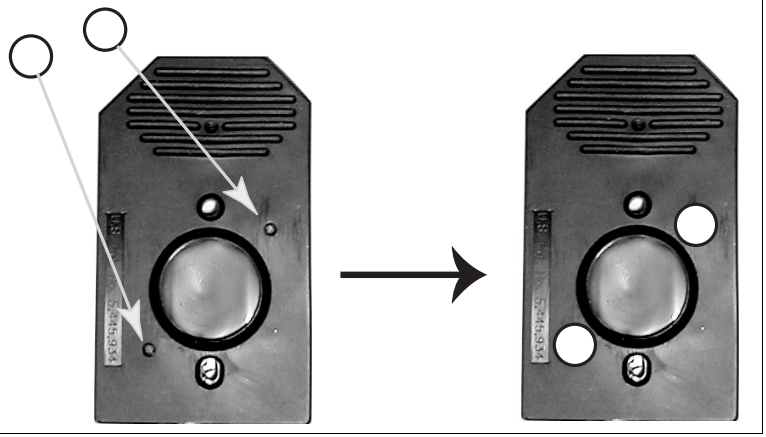
To prepare arrays for scanning:

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 surface.
3. On the back of the array cartridge, clean excess fluid from around the septa.
4. Carefully cover both septa with Tough-Spots (Figure 7.1).

   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.

Figure 7.1 Applyilng Tough-Spots® to Arrays



Scanning the Array

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

To scan arrays:

1. Open the 'AGCC Scan Control' application from the 'Affymetrix Launcher'.
2. Load the arrays onto the Autoloader of the scanner.
3. Once all the arrays are loaded, click the "Start" icon to initiate the scan.
4. Select the check box "arrays in carousel positions 1-4 at room temperature". If the arrays are not at room temperature, do not select this option. The scanner will wait 10 minutes before scanning begins to allow the arrays to reach room temperature.

   Only one scan per array is required. Pixel resolution and wavelength are preset and cannot be changed.

WARNING: The door is locked while the instrument is scanning. Do not attempt to open the door manually.

Adding Arrays During an Autoloader Run

To add arrays while an AutoLoader run is in progress:

1. Click the Add Chips icon 

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   The GeneChip Scanner message appears.

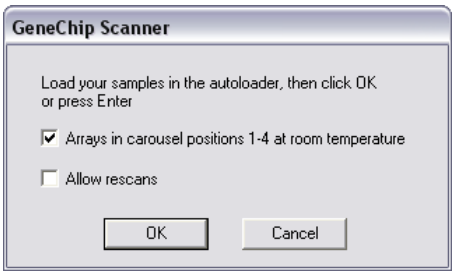
Figure 7.2 GeneChip Scanner Message

2. Click **Add after Scan**.

IMPORTANT: Do not use the Add Now feature. Use only the Add after Scan feature when working with CytoScan™ Arrays.

3. When the status on the scanner reads **Autoloader Door Unlocked**, open the scanner and add the arrays.
4. Close the scanner.
5. When the following message is displayed, click **OK**.

Figure 7.3 GeneChip Scanner Message



6. After you click OK, click the **Resume** icon.
7. If any arrays in the carousel are to be rescanned, select the check box **Allow rescans**.

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.

   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 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. Empty the waste bottle.

! **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 (see **Chapter 9 *Fluidics Station Care and Maintenance* on page 119**.

Troubleshooting

General Assay Performance Recommendations

As with any assay using PCR, the CytoScan™ Assay has an inherent risk of contamination with PCR product from previous reactions. In Chapter 3, *Laboratory Setup and Recommendations* on page 11, we strongly recommend two separate work areas be used to minimize the risk of cross contamination during the assay procedure. It is essential to adhere to workflow recommendations. PCR reactions should be set up in the Pre-PCR Area only. Personnel should not re-enter the Pre-PCR Clean Area once exposed to PCR products without first showering and changing into clean clothes.

Carefully reading and following the protocol as written is essential. The CytoScan™ Assay has been validated using the reagents and suppliers listed. Substitution of reagents and taking shortcuts are not recommended as your results could be suboptimal. For example, always use Affymetrix® Nuclease-Free water and PCR reagents from Clontech.

Additional recommendations are as follows:

- Think ahead to ensure that the reagents and equipment you require, including pipettes, 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 T4 DNA Ligase and the Fragmentation Reagent. Both of these enzymes are sensitive to temperatures exceeding –20°C.

  To prevent loss of enzyme activity:

  - Store the enzymes in a cooler placed in a –20 °C freezer to preserve activity. When taking out enzymes for reaction setup, always use a cooler chilled to –20 °C.
  - Take care when pipetting enzymes stored in glycerol, which is viscous. Do not store enzymes at
    –80 °C.
  - Because Fragmentation Reagent activity can decline over time after dilution on ice, add it to the samples as quickly as possible.
- Preparing the Ligation Master Mix with 20% to 25% overage and the PCR Master Mix with 15% overage 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 the specified thermal cyclers. We highly recommend that your PCR thermal cyclers be calibrated regularly. Take care when programming your thermal cycler and use the recommended 96-well plate.
- It is essential to run gels to monitor both the PCR and the fragmentation reactions.

  For the PCR reaction, individual PCR products are run on a gel. Product (bands) should be visible in the 150 to 2000 bp size range. See *Check the PCR Reaction by Running a Gel* on page 65 for more information. See Appendix D on page 137 for E-Gel® information.

  Following fragmentation, run your samples on a gel. Successful fragmentation is confirmed by the presence of a majority of the distribution between 25 to 125 bp. See *Check the Fragmentation Reaction by Running a Gel* on page 85 for more information. Alternatively, the fragmented samples can be analyzed using the Agilent 2100 Bioanalyzer. See Appendix D on page 137 for E-Gel® information.
- Always run positive and negative controls in parallel with each group of samples.

  The absence of bands on your PCR gel for the negative control confirms no previously amplified PCR product has contaminated your samples. Use Genomic DNA from the CytoScan™ Reagent Kit as a positive control. 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 scanning of the array.
- Regularly calibrate all single channel and multi-channel pipettes.
- Check that your spectrophotometer or Nanodrop is accurately calibrated, and be sure the OD measurement is within the linear range of the instrument as per the manufacturer's recommendations.
- Hybridization oven temperature is critical to the performance of the assay. Use the GeneChip® Hybridization Oven 645 only. Hybridization ovens should be serviced at least once a year to ensure that they are operating within specification.

Troubleshooting the CytoScan™ Assay

PCR Gel QC Step	Likely Cause	Solution
Faint or no PCR product visible on gel. Both samples and positive control affected.	Failed restriction digest or adapter ligation.	▪ Repeat the assay from the beginning with Genomic Control DNA after reviewing best practices, ensuring that all equipment is correctly calibrated, and reagents are handled and stored properly. ▪ If available, include ligated material from a previous successful experiment as a positive control for the PCR step. If it fails again, repeat with fresh reagents.
		Ensure that the ligation buffer is thoroughly resuspended before use.
		Ensure that the reaction plates are sealed tightly in all steps.
	Non-optimal PCR conditions.	Use only calibrated thermal cyclers. Double-check PCR programs to ensure that they have been entered correctly.
		Check the PCR reagents. Use only those reagents recommended by Affymetrix.
		Verify pipette calibration and function. Repeat PCR from the remaining digestion / ligation material if available, otherwise restart from the beginning.
		Take care with preparation of master mixes. Ensure accurate pipetting and thorough mixing.
		▪ Use the recommended 96-well PCR plates and plate seals. ▪ Ensure that the plates are sealed tightly in all steps.
	Ligation reaction not diluted or diluted ligation reaction not mixed properly prior to PCR.	Be sure to correctly dilute the ligation reaction with the water provided in the kit and mix properly before proceeding with PCR.

PCR Gel QC Step	Likely Cause	Solution
Faint or no PCR product visible on the gel. Samples are affected but positive control is OK.	Insufficient or degraded genomic DNA.	▪ Starting amount of 250 ng genomic DNA should be used. ▪ Confirm the concentration using a calibrated spectrophotometer. ▪ Confirm that the genomic DNA sample meets the quality and integrity guidelines. See Chapter 4 on page 21.
	Sample DNA contains enzymatic or chemical inhibitors. Nsp I can be inhibited by high concentrations of salts.	Ensure that genomic DNA is extracted using one of the recommended procedures. See Chapter 4 on page 22.
Wrong size distribution of PCR product.	Mispipetting of PCR primer volume in the master mix.	▪ Verify pipette calibration and function. ▪ Repeat PCR from the remaining digestion/ligation material if available; otherwise restart from the beginning.
	Mispipetting of Taq polymerase in the master mix.	
PCR product evident in the negative control.	Reagents or equipment contaminated with ligated product or amplified product.	▪ Always use filter tips. ▪ Clean the pre-PCR lab area and equipment thoroughly using 10% bleach. ▪ Decontaminate the pipettes following manufacturer's recommendation. ▪ Retrain personnel on pre-lab best practices. ▪ Repeat the assay using fresh reagents and sample.

Purification Yield QC Step	Likely Cause	Solution
Low eluate volume (<47µl)	Insufficient volume due to pipetting error or pipet out of calibration.	Check pipette calibration. Make sure 52 µL of elution buffer is added to the beads for elution and the tubes are centrifuged before placing on the magnet.
Low yields (the average purification yield of 7 or more samples is <3.0 µg/µl or individual yield is <2.5 µg/µl)	Loss of sample prior to purification.	If the yield is not adequate, repeat the assay.
	Possible problems with input genomic DNA.	▪ Use the recommended collection and purification procedures to avoid carryover of inhibitors such as heme, EDTA, etc. ▪ Starting amount of 250 ng genomic DNA should be used. ▪ Confirm the concentration using a calibrated spectrophotometer. ▪ Confirm that the genomic DNA sample meets the quality and integrity guidelines provided in Chapter 4.
	Purification Wash Buffer was prepared incorrectly.	Verify that the correct volume of absolute ethanol was added to the Purification Wash Buffer before use.
	Inadequate mixing of Purification Beads and PCR reactions during binding.	Take care to completely mix the PCR reactions and the Purification Beads during sample binding.
	Inadequate bead washing prior to elution.	Repeat purification with attention towards complete removal of the binding eluate before the bead wash.
	Excess Elution Buffer added to beads.	Verify pipette calibration and function.
	Incorrect buffer was used for elution.	Verify that the Elution Buffer was used during the elution step and not the Purification Wash Buffer.
	Purification Beads were over dried.	Do not dry Purification Beads longer than the recommended time.
	The eluted DNA plate was inadequately vortexed before taking an aliquot for an OD reading.	Eluted DNA can be heterogeneous. Repeat the dilution followed by an OD reading, making sure to vortex the eluted DNA and the OD plate thoroughly at each step.

Purification Yield QC Step	Likely Cause	Solution
	PCR reaction volume was inaccurate.	Repeat the assay and confirm that the PCR reaction is set up correctly.
High yields (> 4.5 µg/µl)	Too little Elution Buffer added to the Purification Beads.	Verify pipette calibration and function. Make sure 52 µL of Elution Buffer is added to the Purification Beads for elution.
	Eluted DNA plate inadequately vortexed before OD reading is taken.	Eluted DNA can be heterogeneous. Repeat the dilution followed by OD reading, being sure to vortex the eluted DNA and the OD plate thoroughly at each step.
	Instruments or pipettes may be out of calibration or incorrectly set.	Verify instrument and pipette calibration and settings during operation.
	Yield calculation formula within the software template may be incorrect.	Verify the formula used to calculate the yields from a given O.D.
OD 260/280 ratio is not between 1.8 and 2.0	PCR product may not have been adequately washed.	Ensure that proper volume of absolute ethanol is added to the Purification Wash Buffer and follow the procedure provided in Chapter 6 on page 68.
	An error may have been made while taking the O.D readings.	Retake the O.D following the instructions provided in Chapter 6 on page 75.
OD 320 measurement is > 0.1	Purification beads may have been carried over into purified samples.	Spin down the sample for 5 minutes. Place on the MagnaRack and pipette out the eluate. Retake the OD measurement.
	Scratches or dust particles on the OD plate.	Ensure that the bottom surface of the OD plate is clean and scratch-free.
	Air bubbles are present in the diluted DNA within the OD plate.	Vortex the OD plate, spin it down again following the guidelines provided in Chapter 6 on page 75, and retake the OD.

Fragmentation QC Step (Gel or Bioanalyzer)	Likely Cause	Solution
Over fragmentation: Majority of fragmented sample appears < 50 bp on a 4% agarose gel.	Excess Fragmentation Reagent was added during preparation of the Fragmentation Master Mix.	▪ Carefully observe the pipette tip and the shaft during pipetting of the fragmentation reagent. Touch the tip to the inside of the vial to help remove any droplets of enzyme clinging to the exterior of the tip. ▪ Make sure pipettes are calibrated.
	Purified samples or assembled reactions were allowed to warm to room temperature during reaction assembly or prior to incubation.	Ensure that the plate centrifuge is completely chilled to 4° C before spinning the assembled fragmentation plate.
		Keep the master mix, samples, and reaction components on ice or in a cooling block at all times during master mix assembly and dispensing of the master mix to the samples.
		Check that the fragmentation reaction temperature and incubation time are correctly programmed on the thermal cycler and that the fragmentation mix is made correctly as per the guidelines.
		Ensure that the master mix tube and strip tubes are pre-chilled before reaction setup.
	Incorrect fragmentation reagent unit was used to prepare the master mix.	Verify the unit activity on the label of the Fragmentation Reagent tube and formulate the master mix appropriately.
Under fragmentation: PCR product is still visible in 150-2000 bp size region on a 4% agarose gel.	Improper storage or handling of the Fragmentation Reagent.	▪ The Fragmentation Reagent should be stored at -20 °C at all times. ▪ Handle the Fragmentation Reagent as minimally as possible, holding the vial at the cap rather than the center. Return the Fragmentation Reagent to the cooler as soon as the reagent has been dispensed. We recommend storing the Fragmentation Reagent at -20 °C inside a cooler to preserve its activity. ▪ Do not over vortex the Fragmentation Reagent.

Fragmentation QC Step (Gel or Bioanalyzer)	Likely Cause	Solution
	▪ Insufficient Fragmentation Reagent or 10X Fragmentation Buffer was added during assembly of the Fragmentation Master Mix. ▪ Improper mixing of the Fragmentation Master Mix.	Verify pipette calibration and function. Take care when preparing the master mix to ensure accurate pipetting and thorough mixing.
	The Fragmentation Master Mix was not made fresh or was allowed to warm to room temperature before use.	▪ Keep the Fragmentation Master Mix on ice at all times to preserve activity. ▪ Work quickly during reaction assembly. ▪ Do not save or reuse a previously assembled Fragmentation Master Mix.
	Samples were frozen during fragmentation reaction assembly or centrifugation.	▪ Make sure that cold blocks are not chilled to -20 °C as sample freezing can occur. ▪ Before centrifugation, ensure that the interior of the chilled plate centrifuge is not lower than 2 °C.
	Incorrect Fragmentation Reagent units were used to prepare the master mix.	Verify the unit activity on the label of the Fragmentation Reagent tube and formulate the master mix appropriately.
	Thermal cycler was not programmed correctly or is out of calibration.	▪ Confirm that the fragmentation reaction temperature and incubation time are correctly programmed on the thermal cycler. ▪ Confirm that the fragmentation mix is made correctly as per the guidelines. ▪ Verify that the thermal cycler is within calibration.

.CEL File Generation	Likely Cause	Solution
.CEL file is not generated	Signal from the corner checkerboards is absent.	Verify that the Oligo Control Reagent was added to the Hybridization Master Mix during assembly. The Oligo Control Reagent must be present during hybridization to ensure proper grid alignment.
	Signal from corner checkerboards is dim.	▪ Verify that the correct amount of the Oligo Control Reagent was added to the Hybridization Master Mix during assembly. ▪ Ensure that GeneChip® Hybridization Oven 645 is calibrated and set to the correct temperature. ▪ Ensure that Hybridization Master Mix was correctly assembled and added at the correct volume to the fragmented samples. ▪ Confirm that Stain Buffer 1 and Stain Buffer 2 are placed in the correct order on the fluidics station. Stain Buffer 1 is light sensitive. Be sure to store Stain Buffer 1 in the dark when not in use. ▪ Use only those staining reagents provided by Affymetrix.
	Bright hybridization artifact(s) obscure gridding oligo locations on the array.	Try to manually align the grid. See the Affymetrix® GeneChip® Command Console® User Manual (P/N 702569) for instructions. If manual grid alignment fails to produce a .CEL file, repeat the experiment.

Data QC Failures	Likely Cause	Solution
Low or failing SNPQC	▪ Cross-contamination between samples within a plate ▪ Contaminated reagents, equipment, or input DNA.	▪ Repeat assay using a control sample of known integrity, such as Ref103. ▪ Review and follow best practices. ▫ Ensure a tight plate seal at every step. ▫ Use fresh filter tips at each pipetting step. ▫ Use caution when pooling PCR product. ▪ If the problem persists use fresh reagents and fresh input DNA. ▪ Decontaminate the pre-PCR room and equipment if necessary.
		Process only 6 - 8 arrays at a time. When processing arrays for washing it is important to work quickly as delays in this step will impact data quality.
		Perform all steps after removal of arrays from the oven to the time the washing begins with minimal delays.
	Over or under fragmentation of the PCR product	See above.
	Hybridization oven out of calibration or oven model is not compatible with this assay.	▪ Ensure that only the GeneChip® Hybridization Oven 645 is used for this assay. ▪ Have the oven serviced.
Elevated or failing MAPD	Assay drift due to variation in assay execution.	▪ Recalibrate pipettes to ensure accurate delivery of reagent volumes. ▪ Consider operator retraining or review by an Affymetrix® Field Applications Scientist if the problem persists. ▪ Review Chapter 2, *Best Practices on page 3*.
	Over fragmentation	See above.
	Degraded starting material.	▪ Perform a QC gel of input DNAs to assess samples for degradation. ▪ Ensure that the DNA samples are of high quality (for example, run in a 1 to 2% agarose gel and compare to the Genomic DNA Control provided in the CytoScan™ Reagent Kit).
	Reference is inappropriate for the sample.	Use only the recommended sample types (peripheral blood and cell line DNA).

Data QC Failures	Likely Cause	Solution
High waviness-SD	Degraded genomic DNA.	Confirm that the genomic DNA sample meets the quality and integrity guidelines in Chapter 4 on page 21.
	Incompatible sample type.	Use only cell line or blood derived genomic DNA.
	Incompatible genomic DNA extraction method used.	Only use the recommended extraction methods. See Chapter 4 on page 22.
	Sample-specific effect.	See the *Chromosome Analysis Suite User Manual* (P/N 702943).
High MAPD with low SNPQC	Error during washing the array.	▪ Ensure that the Wash A and B lines of the Fluidics Station are placed in the correct wash buffers during priming and array washing. ▪ Ensure that the Fluidics Stations are maintained according to the guidelines in the Fluidics Station User's Guide.

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

NOTE: Make sure you have the model and serial number available when calling Affymetrix Technical Support.

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

Fluidics Station Care and Maintenance

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 minutes to complete. Affymetrix recommends running this protocol weekly, regardless of the frequency of use. The current version of the protocol can be found at:

www.affymetrix.com/support/technical/fluidics_scripts.affx

The Bleach Cycle

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

Table 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 121.
2. Prepare 500 mL of 0.525% sodium hypochlorite solution using deionized water.

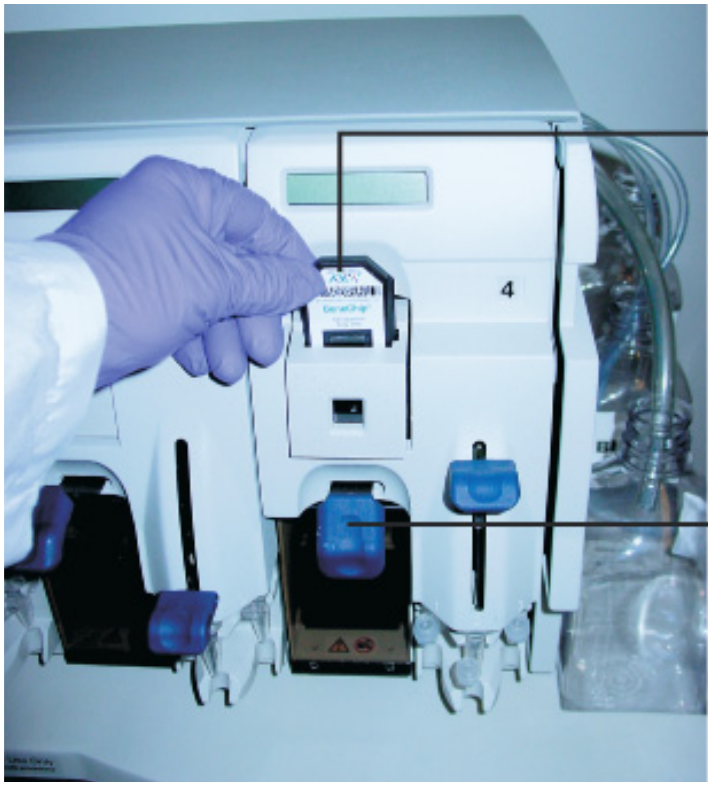
   You can follow these directions to make 500 mL of bleach:

   In a 1 liter plastic or glass graduated cylinder, combine 43.75 mL of commercial bleach (such as Clorox® bleach, which is 6.15% 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 hr. 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 122:
   - **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.

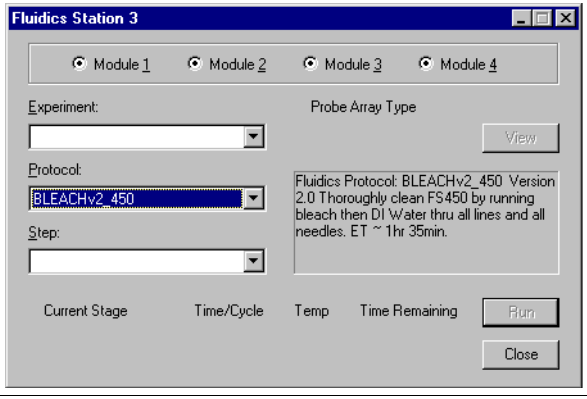
     ! IMPORTANT: Do NOT immerse the waste line into the bleach.
4. Open the instrument control software (AGCC).
5. Choose the current bleach protocol for each module.

Figure 9.2 Bleach Cycle

Immerse the tubes into the 0.52% sodium hypochlorite solution.

The waste line remains in the waste bottle.

Figure 9.3 Fluidics Station Protocol Window: Select All Modules



6. In 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 123). 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 minutes, the LCD will prompt you when the bleach cycle is over and the rinse cycle is about to begin.

The Rinse Cycle

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

1. Follow the prompts on the LCD for each module. Lift up on the needle levers and remove the bleach vials. Load clean, empty vials onto each module.
2. Remove the three wash and water lines from the bleach bottle and transfer them to the DI water bottle (Figure 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.

Table 9.2 Storage Suggestions for the Fluidics Station 450

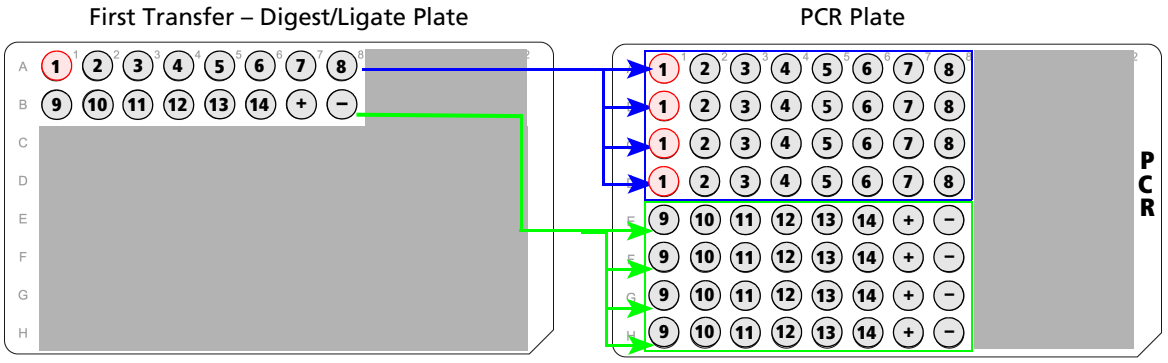
If:	Then do this:
Planning to use the system immediately	After running the bleach protocol, remove the DI water supply used in the rinse phase and install the appropriate reagents for use in the next staining and washing protocol (including fresh DI water). • Perform a prime protocol without loading 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.

Guidelines for Processing 16 Samples

This appendix illustrates the plate layouts recommended for processing 16 reactions (14 samples plus one positive and one negative control). It also provides a high level overview of the workflow.

Digestion, Ligation and PCR

Figure A.1 16 Reaction Workflow — Digest/Ligate Plate to 96-well Plates



To avoid transfer mistakes, keep all wells capped *except* for:

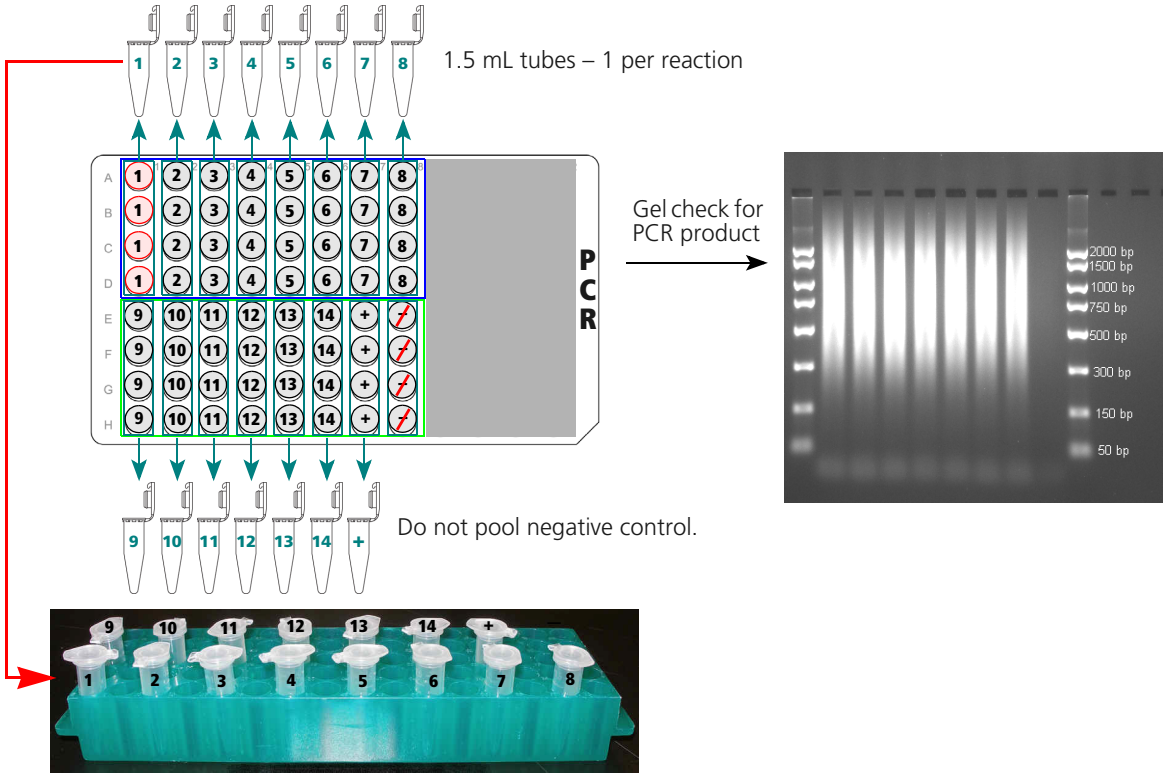
- One row on the Digest/Ligate plate.
- The rows to which you are transferring to on the PCR plate.

Example: When transferring samples from row A of the Digest/Ligate plate to PCR plate:

- Cap all wells in row B through row H on the Digest/Ligate plate.
- Cap all wells in row E through row H on PCR Plate.

PCR to Purification

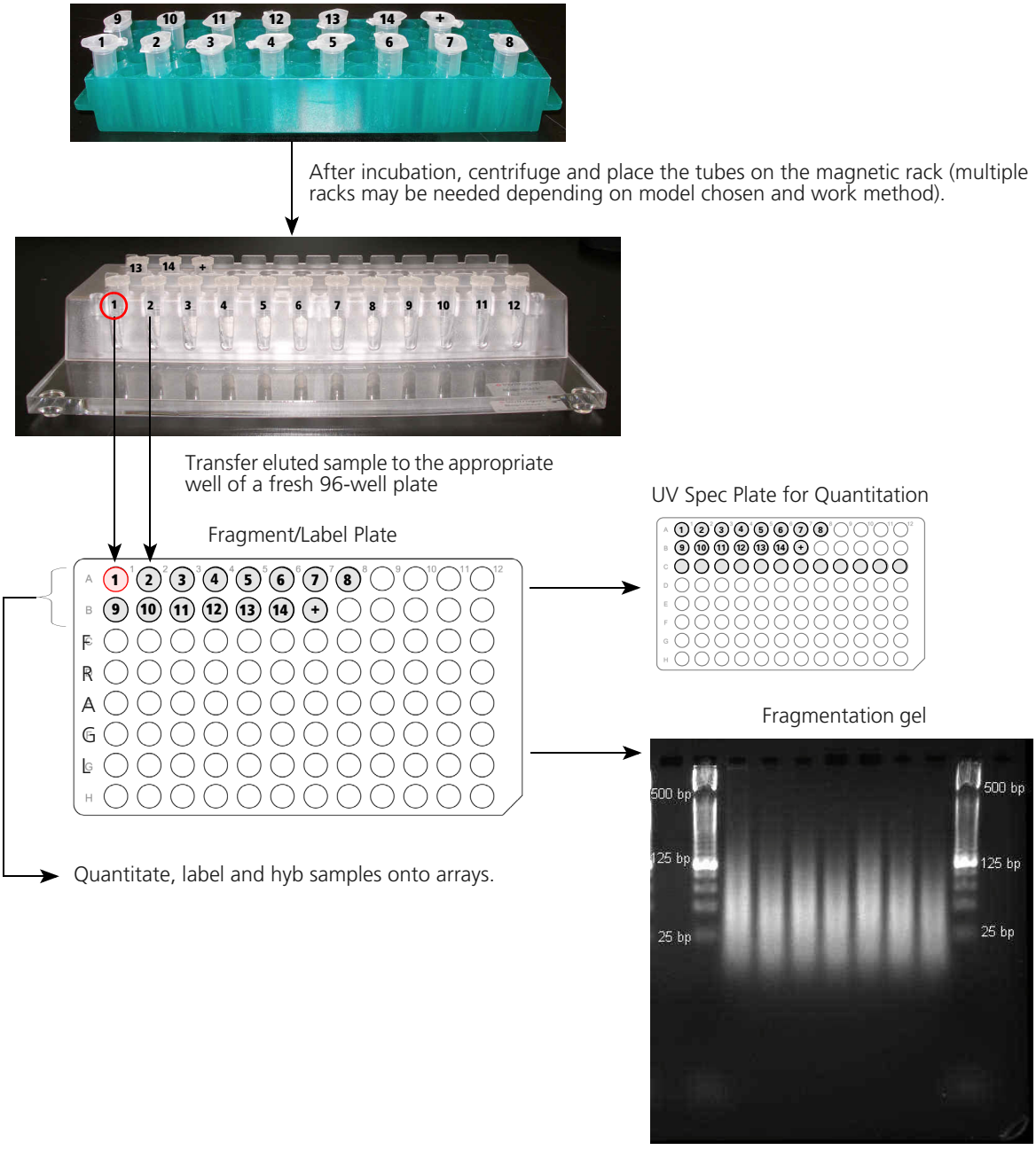
Figure A.2 16 Reaction Workflow — PCR to Purification



Add purification beads to each tube and incubate in the tube rack.

Purification Continued to Fragmentation and Labeling

Figure A.3 16 Reaction Workflow — Purification to Labeling

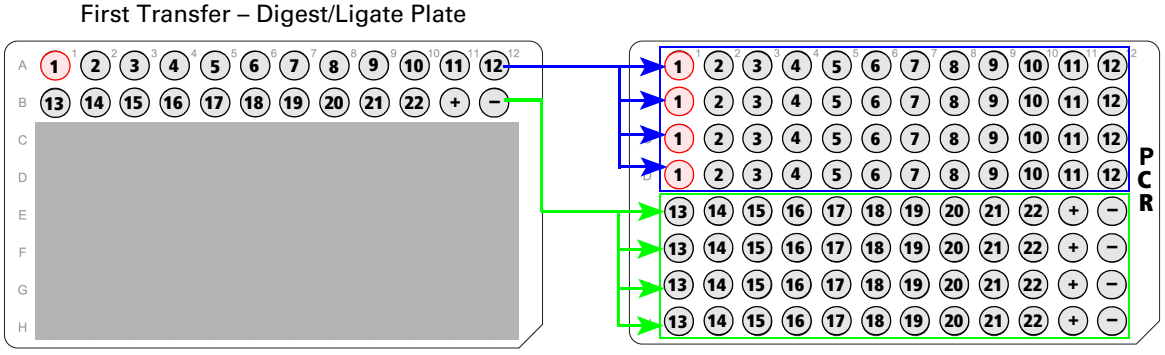


Guidelines for Processing 24 Samples

This appendix illustrates the plate layouts recommended for processing 24 reactions (22 samples plus one positive and one negative control). It also provides a high level overview of the workflow.

Digestion, Ligation, and PCR

Figure B.1 24 Reaction Workflow — Digest/Ligate Plate to PCR Plates



To avoid transfer mistakes, keep all wells capped *except* for:

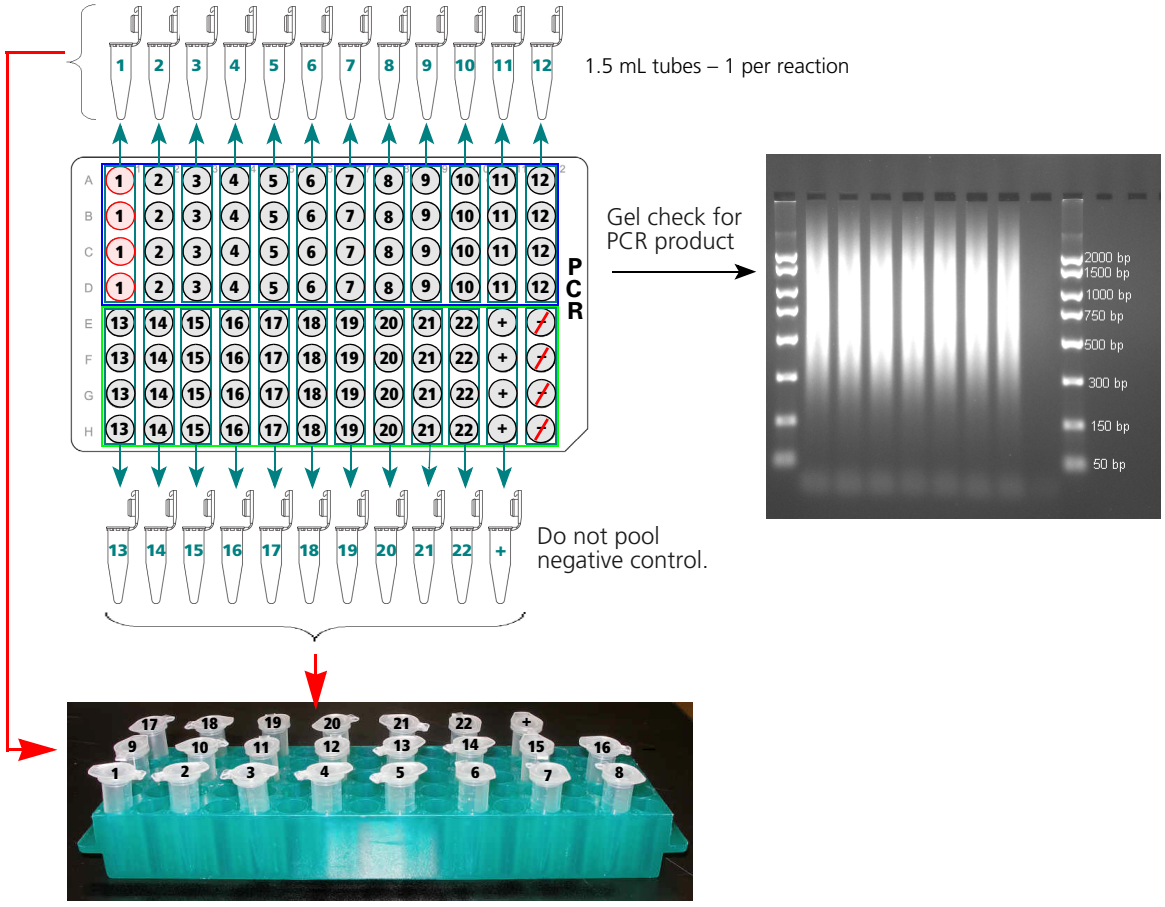
- One row on the Digest/Ligate plate.
- The rows to which you are transferring to on the PCR plate.

Example: When transferring samples from Digest/Ligate plate row A to PCR plate:

- Cap all wells in row B through row H on the Digest/Ligate plate.
- Cap all wells in row E through row H on PCR Plate.

PCR to Purification

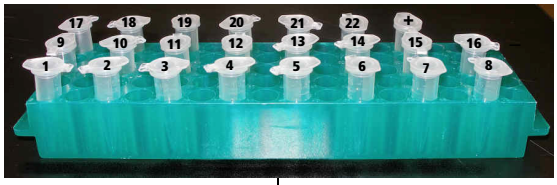
Figure B.2 24 Reaction Workflow — PCR to Purification



Add purification beads to each tube and incubate in the tube rack.

Purification Continued to Fragmentation and Labeling

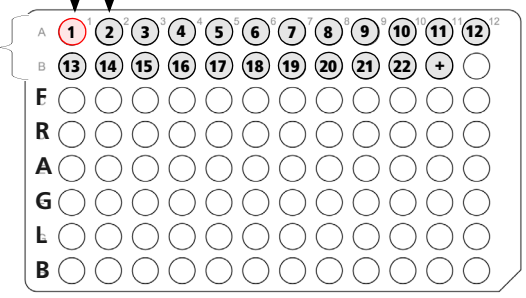
Figure B.3 24 Reaction Workflow — Purification Continued to Fragmentation and Labeling



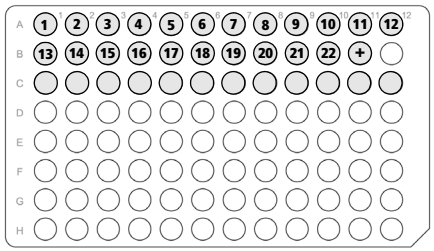
After incubation, centrifuge and place the tubes on the magnetic rack (multiple racks may be needed depending on model chosen and work method).

Transfer eluted sample to the appropriate well of a fresh 96-well plate.

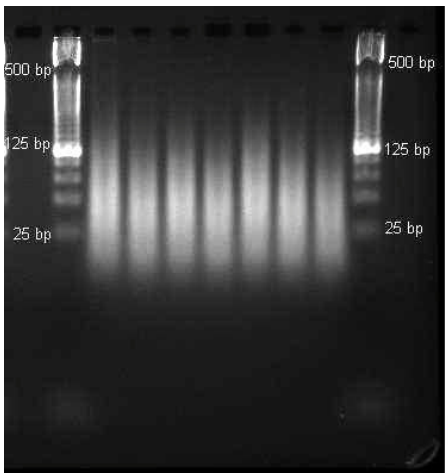
Fragment/Label Plate



UV Spec Plate for Quantitation



Fragmentation gel



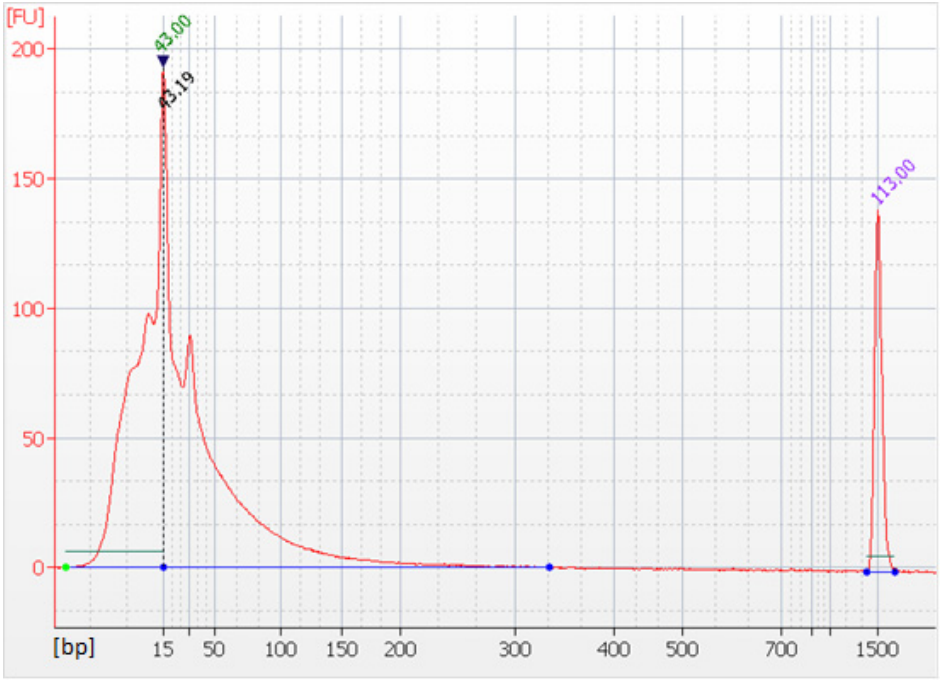
Quantitate, label and hyb samples onto arrays.

Analyzing Sample Fragmentation Using the Agilent 2100 Bioanalyzer

1. Thaw the fragmentation aliquot prepared in Step 8 of *Check the Fragmentation Reaction by Running a Gel* *on page 85* at room temperature. Mix sample by vortexing. Use 1µL per sample as input volume for the bioanalyzer.
2. Use the Agilent DNA 1000 Assay Kit (Cat # 5067-1504). Refer to the user guide for instructions on sample preparation and sample analysis on the bioanalyzer.
3. Evaluate the DNA fragmentation distribution using the sample elution profile shown in the electropherogram (Figure C.1).
4. To evaluate each profile, the 15bp control peak should be identified and assigned correctly (tallest peak, elution time around 43 seconds).

   Correctly fragmented DNA samples have profiles which stretch out below and above 15bp. Under- or over-fragmentation is indicated by profiles where the majority of the area under the curve lies either above or below 15 bp, respectively.

Figure C.1 Example of Correctly Fragmented DNA Sample



Running E-Gels

Equipment, E-Gels, and Reagents Required

Table D.1 Equipment, E-Gels, and Reagents Required

Item	Supplier	Part No.
Mother E-Base™ Device	Life Technologies	EB-M03
Daughter E-Base™ Device (optional for running multiple gels simultaneously)		EB-D03
E-Gel® 48 1% Agarose Gels		G8008-01
E-Gel® 48 2% Agarose Gels		G8008-02
E-Gel® 48 4% Agarose Gels		G8008-04
*Redi*Load™ Loading Buffer		750026
E-Gel® 96 High Range DNA Marker		12352-019
TrackIt™ 25 bp DNA Ladder (25 - 500 bp)		10488-022
TrackIt™ Cyan/Orange Loading Buffer		10482-028
PCR Marker (50 - 2000 bp)	Affymetrix	76710

NOTE: The E-Gel® contains ethidium bromide. Review the manufacturer's Material Safety Data Sheet for proper handling and disposal. Use good laboratory practices and always wear gloves when handling E-Gels. Dispose of the gel and gloves in accordance with national, state, and local regulations.

Genomic DNA on 1% E-Gel

Diluting Genomic DNA Samples

Loading a DNA mass of approximately 25 ng per well is recommended. If lower amounts are loaded, omission of the loading dye is recommended in order to improve visualization.

1. Dilute the *Redi*Load dye to 0.1X concentration and use 3 µL of the diluted dye (0.1X concentration) for each sample.

2. Bring each sample to a total volume of 20 µL using Nuclease-Free water.

   For example, if the volume of genomic DNA required for 25 ng is 5 µL, add 3 µL of 0.1× *Redi*Load and 12 µL of Nuclease-Free water for a total volume of 20 µL. Strip tubes or 96-well PCR plates can be used for diluting genomic DNA samples.
3. Briefly vortex and spin down the diluted DNA samples before loading onto the E-Gel.

Running the E-Gel

1. Turn on the power for the E-Base (red light).
2. Push the Power/Prg button to make sure the program is set to EG mode (not EP).
3. Remove the comb(s) from a 48-well 1% Agarose E-Gel and wipe away any buffer that comes out of the gel or is on the surface.
4. Insert the E-Gel into the slot (12-well E-Gels can also be used if running a smaller number of genomic DNA samples).
5. Load 20 µL of genomic DNA sample onto the 48-well 1% agarose E-Gel.
6. Dilute the High Range DNA Marker (1:3 dilution, 5 µL of Marker in 10 µL of Nuclease-Free water) and load all 15 µL into each of the marker wells (as needed).
7. Fill all empty wells with 20 µL water.
8. Set the run time to 27 minutes.
9. Push the Power/Prg button again (it will change from red to green).

   When the run time is reached, the system will automatically shut off (the dye should be near the end of the lane). The gel is now ready for imaging.

PCR Product on 2% E-Gel

Diluting the TrackIt Cyan/Orange Loading Buffer

The following instructions prepare a 1000-fold dilution of the TrackIt™ Cyan-Orange Loading Buffer.

1. Add 50 µL of TrackIt Cyan/Orange Loading Buffer to 49.95 mL Nuclease-Free water (total volume is 50 mL).
2. Mix well and store at room temperature.

Diluting PCR Product

Dilutions can be prepared in strip tubes or 96-well plates.

1. After the PCR step is complete, aliquot 3 µL from the first row of the PCR product to 17 µL of the 1:1000 fold diluted Loading Buffer to give a total volume of 20 µL.

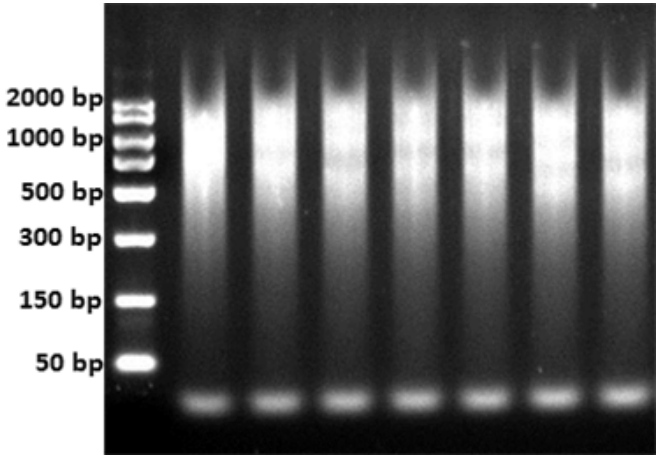
2. Briefly vortex and spin down the diluted samples before loading onto the E-Gel.

Running the E-Gel

1. Turn on the power for the E-Base (red light).
2. Push the Power/Prg button to make sure the program is set to EG mode (not EP).
3. Remove the comb(s) from the E-Gel and wipe away any buffer that comes out of the gel or is on the surface.
4. Insert the 48-well 2% Agarose E-Gel into the slot (12-well E-Gels can also be used if running a smaller number of samples).
5. Load all 20 µL of the diluted PCR product from above onto the 48-well 2% agarose E-Gel.
6. Dilute the PCR marker (1:3 dilution, 5 µL in 10 µL of Nuclease-Free water) and load all 15 µL into each of the marker wells (as needed). Fill empty wells with 20 µL water.
7. Set the run time to 21 minutes.
8. Push the Power/Prg button again (it will change from red to green).

   When the run time is reached, the system will automatically shut off (the dye should be near the end of the lane). The gel is then ready for imaging.

Figure D.1 Gel Image of the PCR Product (from Ref103 Genomic DNA) on 2% E-Gel



Fragmented Product on 4% E-Gel

Diluting the TrackIt Cyan/Orange Loading Buffer

The following instructions prepare a 1000-fold dilution of the TrackIt™ Cyan-Orange Loading Buffer.

1. Add 50 µL of TrackIt Cyan/Orange Loading Buffer to 49.95 mL Nuclease-Free water (total volume is 50 mL).
2. Mix well and store at room temperature.

Diluting Fragmented Product

Dilutions can be prepared in strip tubes or 96-well plates.

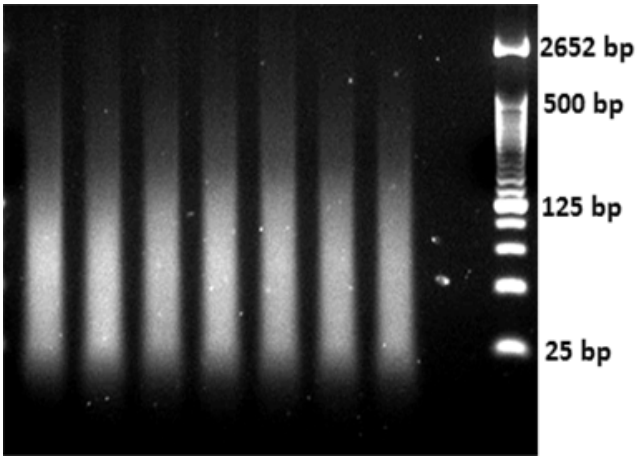
1. After the Fragmentation step is complete, aliquot 4 µL and make 1:8 dilution by adding 28 µL water.
2. Add 8 µL of the above diluted fragmented product to 12 µL of the 1:1000 fold diluted Loading Buffer to give a total volume of 20 µL.
3. Briefly vortex and spin down the diluted samples before loading onto the E-Gel.

Running the E-Gel

1. Turn on the power for the E-Base (red light).
2. Push the Power/Prg button to make sure the program is set to EG mode (not EP).
3. Remove the comb(s) from the E-Gel and wipe away any buffer that comes out of the gel or is on the surface.
4. Insert the 48-well 4% Agarose E-Gel into the slot (12-well E-gels can also be used if running a smaller number of samples).
5. Load all 20 µL of the diluted fragmented product from above onto the 48-well 4% agarose E-Gel.
6. Dilute the TrackIt 25 bp DNA Marker (1:15 dilution, 2 µL in 28 µL of Nuclease-Free water) and load 15 µL into each of the marker wells (as needed). Fill empty wells with 20 µL water.
7. Set the run time to 19 minutes.
8. Push the Power/Prg button again (it will change from red to green).

   When the run time is reached, the system will automatically shut off (the dye should be near the end of the lane). The gel is then ready for imaging.

Figure D.2 Gel Image of Fragmented Product (from Ref103 Genomic DNA) on 4% E-Gel



Thermal Cycler Programs

This appendix includes the thermal cycler programs required for the Affymetrix® CytoScan™ Assay.

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

CytoScan Digest

Table E.1 CytoScan Digest Program

Temperature	Time
37°C	2 hours
65°C	20 minutes
4°C	Hold

CytoScan Ligate

Table E.2 CytoScan Ligate Program

Temperature	Time
16°C	3 hours
70°C	20 minutes
4°C	Hold

CytoScan 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

Table E.3 CytoScan PCR Program for GeneAmp® PCR System 9700

Temperature	Time	Cycles
94°C	3 minutes	1X
94°C	30 seconds	30X
60°C	45 seconds	
68°C	15 seconds	
68°C	7 minutes	1X
4°C	HOLD (Can be held overnight)	

CytoScan Fragment

Table E.4 CytoScan Fragment Program

Temperature	Time
37°C	35 minutes
95°C	15 minutes
4°C	Hold

CytoScan Label

Table E.5 CytoScan Label Program

Temperature	Time
37°C	4 hr
95°C	15 minutes
4°C	Hold (Samples can remain at 4°C overnight.)

CytoScan Hyb

Table E.6 CytoScan Hyb Program

Temperature	Time
95°C	10 minutes
49°C	Hold

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® CytoScan™ Assay.

IMPORTANT: **This protocol has been optimized using the equipment, consumables and reagents listed in this user guide. For the best results, we strongly recommend that you adhere to the protocol as described. Do not deviate from the protocol; do not substitute reagents.**

Affymetrix Equipment Required

Table F.1 Affymetrix® Equipment Required

✓	Item	Part Number
❒	GeneChip® Fluidics Station 450	00-0079
❒	Tubing, Silicone peristaltic for GeneChip® Fluidics Station 450	400110
❒	GeneChip® Hybridization Oven 645	00-0331
❒	GeneChip® 3000 Scanner with 7G upgrade	Contact Affymetrix

Affymetrix Software Required

Table F.2 Affymetrix® Software Required

✓	Item	Part Number
❒	GeneChip® Command Console	Version 3.2.2 or higher

Affymetrix® Arrays Required

Table F.3 Affymetrix® CytoScan™ HD Reagents and Array Kits

✓	Item	Part Number
❒	CytoScan™ Reagent Kit (24 rxns per kit)	901808
❒	CytoScan™ HD Array Kit, 6 pack	901833
❒	CytoScan™ HD Array and Reagent Kit Bundle (24 arrays/rxns)	901835
❒	CytoScan™ HD Training Kit	901834

Table F.4 Affymetrix® CytoScan™ 750K Reagents and Array Kits

✓	Item	Part Number
❒	CytoScan™ Reagent Kit (24 rxns per kit)	901808
❒	CytoScan™ 750K Array Kit, 6 pack	901858
❒	CytoScan™ 750K Array and Reagent Kit Bundle (24 arrays/rxns)	901859
❒	CytoScan™ 750K Training Kit	901860

Affymetrix Reagents Required

Table F.5 Affymetrix® CytoScan™ Assay Kit – 24 Reaction Kit Components

✓	Item	Qty	Part Number
❒	**Affymetrix® GeneChip® Restriction and Ligation Reagents**	**1**	**901803**
	▫ Nsp I	1	901718
	▫ 10X Nsp 1 Buffer	1	901719
	▫ 100X BSA	1	901720
	▫ Low EDTA TE Buffer	1	901697
	▫ 10X T4 DNA Ligase Buffer	1	901722
	▫ T4 DNA Ligase	1	901723
	▫ Water, Nuclease-Free	1	901781
❒	**Affymetrix® GeneChip® Ligation Adaptors and Fragmentation Reagents**	**1**	**901749**
	Pouch 1: Store in the Pre-PCR Room		
	▫ Genomic DNA	1	900421
	▫ 50 µM Adaptor, Nsp I	1	900697
	▫ PCR Primer, 002	1	901016
	Pouch 2: Store in the Post-PCR Room		
	▫ GeneChip® Fragmentation Reagent	1	901010
	▫ 10X Fragmentation Buffer	1	900422
	▫ TdT	1	901154
	▫ 5X TdT Buffer	1	900696
	▫ 30 mM DNA Labeling Reagent	1	900699
	▫ Oligo Control Reagent 0100	1	900541
❒	**Affymetrix® GeneChip® Hybridization Reagents**	**1**	**901804**
	▫ Hyb Buffer Part 1	1	901725
	▫ Hyb Buffer Part 2	1	901726
	▫ Hyb Buffer Part 3	1	901727
	▫ Hyb Buffer Part 4	1	901728
❒	**Affymetrix® GeneChip® Stain Reagents**	**1**	**901805**
	▫ Stain Buffer 1	1	901751
	▫ Stain Buffer 2	1	901752
	▫ Affymetrix® GeneChip® Array Holding Buffer	1	901733
	▫ Purification Beads	1	901807
❒	**Affymetrix® GeneChip® Purification Reagents**	**1**	**901826**
	▫ Elution Buffer	1	901738
	▫ Water, Nuclease-Free	1	901781
	▫ Purification Wash Buffer		901372
❒	**Affymetrix® GeneChip® Wash A**	**2**	**901680**
❒	**Affymetrix® GeneChip® Wash B**	**2**	**901681**

Table F.6 Other Affymetrix® Reagents Required

✓	Item	Part Number
❒	DNA Marker, PCR Markers 50-2000 bp	USB 76710
❒	TBE Buffer, 5X Solution	USB 75891
❒	1 mL, RapidRun™ Loading Dye 5 mL, RapidRun™ Loading Dye	USB 77524 1 ML USB 77524 5 ML
❒	Ethidium Bromide Drops	USB, 75816

Reagents Required from Other Suppliers

Table F.7 Reagents Required from Other Suppliers

✓	Item	Vendor	Part Number
❒	TITANIUM™ DNA Amplification Kit (300 rxn)	Clontech	639240
❒	TITANIUM™ DNA Amplification Kit (400 rxn)	Clontech	639243
❒	Absolute Ethanol	Sigma-Aldrich	459844
❒	Bleach (6.15% Sodium Hypochlorite)	VWR	21899-504 (or equivalent)

Optional Affymetrix Equipment

Table F.8 Optional Affymetrix® Equipment

✓	Item	Part Number
❒	GeneChip® System 3000Dx v.2 with Data Transfer Server	00-0349

Equipment Required from Other Suppliers

Pre-PCR Clean Area Equipment Required

When performing the pre-PCR stages of the CytoScan™ Assay, great care should be taken to avoid sample contamination with PCR products. If the assay is to be run in a single room, we strongly recommend that the pre-PCR stages be performed in a laminar flow or PCR cabinet.

Table F.9 Pre-PCR Clean Area Equipment Required

✓	Item	Vendor	Part Number
❒	Recommended if protocol is to be performed in one room only: ▪ Laminar Flow Cabinet, 6 ft (ESCO, SVE-6A) ▪ PCR Cabinet	Laminar Cabinet: ESCO SVE-6A or equivalent PCR Cabinet: C.B.S. Scientific P-048-02 or equivalent	
❒	Benchtop Cooler, –20°C	Agilent Technologies	401349
❒	Biocooler aluminum block, 96-well Required if processing > 8 samples. (1 for 9 to 16 samples, 2 for 17 to 24 samples)	Bio-Smith	81001
❒	Cooling Chamber, double block	Diversified Biotech	CHAM-1020
❒	Freezer, –20°C; deep freeze; manual defrost; 17 cu ft	Any vendor	—
❒	Rectangular Ice Tray Large – 9L (16 x 13 in, 41 x 33cm)	LabScientific	RECB1202
❒	Microfuge (for tubes and strip tubes)	Any vendor	—
❒	96-well Tube Storage Racks	GeneMate	R-7909-2
❒	Pipette, single-channel, 2–20 µL	Rainin	L-20
❒	Pipette, single-channel, 20–200 µL	Rainin	L-200
❒	Pipette, single-channel, 100–1000 µL	Rainin	L-1000
❒	Pipette, 12-channel, 2–20 µL	Rainin	L12-20
❒	Pipette, 12-channel, 20–200 µL	Rainin	L12-200
❒	Plate centrifuge, multipurpose	Eppendorf	5804 or 5810
❒	Vortexer	Any vendor	—
❒	Select one of these thermal cyclers:		
	▫ GeneAmp PCR System 9700 (gold/silver block)	Applied Biosystems	N8050200
	▫ 2720 Thermal Cycler	Applied Biosystems	4359659

Post-PCR Area Equipment Required

Table F.10 Post-PCR Area Equipment Required

✓	Item	Vendor	Part Number
❒	Benchtop Cooler, –20°C	Agilent Technologies	401349
❒	Cooling Chamber, double block	Diversified Biotech	CHAM-1020
❒	Freezer, –20°C; deep freeze; manual defrost; 17 cu ft	Any vendor	—
❒	Electrophoresis gel box	Any vendor	—
❒	Electrophoresis power supply	VWR	VWR105
❒	Gel imager	Any vendor	—
❒	Rectangular ice tray, large - 9L (16 x 13in; 41 x 33cm)	LabScientific	RECB1202
❒	96-well tube storage racks	GeneMate	R-7909-2
❒	MicroAmp® Adhesive Film Applicator	Applied Biosystems	4333183
❒	Magnetic stand – select one of the following:		
	▪ MagnaRack	Life Technologies	CS15000
	▪ DynaMag-2 Magnet	Life Technologies	123-21D
	▪ PureProteome Magnetic Stand	Millipore	LSKMAGS08
❒	Microcentrifuge 5415D or R	Eppendorf	022621408
❒	Microcentrifuge Standard Rotor F-45-24-11, 24 bores	Eppendorf	22636502
❒	Microfuge (for tubes and strip tubes)	Any vendor	—
❒	Microtube Foam Insert	Scientific Industries	504-0234-00
❒	6" Platform Head for the Microtube Foam Insert	Scientific Industries	146-6005-00
❒	Pipette, single-channel, 2–20 µL	Rainin	L-20
❒	Pipette, single-channel, 20–200 µL	Rainin	L-200
❒	Pipette, single-channel, 100–1000 µL	Rainin	L-1000

Table F.10 Post-PCR Area Equipment Required (Continued)

✓	Item	Vendor	Part Number
❒	Pipette, 12-channel, 2–20 µL	Rainin	L12-20
❒	Pipette, 12-channel, 20–200 µL	Rainin	L12-200
❒	Pipette, 12-channel, 100–1200 µL	Rainin	L12-1200
❒	Plate centrifuge, multipurpose	Eppendorf	5804R or 5810R
❒	Refrigerator, 4°C, 6 cu ft	Any vendor	—
❒	Spectrophotometer – select one of the following:		
	▪ SpectraMax Plate Spectrophotometer	Molecular Devices	Spectramax Plus384
	▪ NanoDrop	NanoDrop	ND-1000
❒	GeneAmp PCR System 9700 (gold/silver block) (If routinely processing > 8 samples, you may to use additional thermal cyclers for PCR.)	Applied Biosystems	N8050200
❒	Vortex Genie 2	USA Scientific	7404-5600
❒	Richter Anti-vibration Pad The microtube foam insert listed above will be attached to one of the vortexers. The vortex used with the foam insert may migrate across the bench top during operation. We recommend the use of a pad such as the one listed here to prevent movement.	ISC BioExpress	S-7350-25

Consumables Required from Other Suppliers

Table F.11 Consumables Required From Other Suppliers

✓	Item	Vendor	Part Number
❒	MicroAmp Clear Adhesive Film for 96-well plates	Applied Biosystems	4306311
❒	Pipette tips, 20 µL filter tips	Rainin	GP-L10F
❒	Pipette tips, 200 µL filter tips	Rainin	GP-L200F
❒	Pipette tips, 1000 µL filter tips	Rainin	GP-L1000F
❒	Plates, 96-well unskirted PCR	Bio-Rad	MLP-9601
❒	Plate, OD for UV spec, 96-well (required only if using microplate spectrophotometer)	E & K Scientific	EK-25801
❒	Reagent Reservoir, 25 mL	Diversified Biotech	RESE-3000
❒	TBE Gel, 4%, BMA Reliant precast	Lonza Group LTD	54929
❒	TBE Gel, 2%, BMA Reliant precast	Lonza Group LTD	54939
❒	TBE for electrophoresis	Any vendor or house made	
❒	TrackIt™ 25 bp DNA Ladder	Life Technologies	10488-022
❒	Tough-Spots, 1/2"	Diversified Biotech	Spot 2200
❒	Tough-Spots, 3/8"	USA Scientific	9185-0000
❒	Tube, Safe-Lock Tube 1.5 mL, Amber	Eppendorf	022363221
❒	Tube, Safe-Lock Tube 1.5 mL, Blue	Eppendorf	022363247
❒	Tube, Safe-Lock Tube 1.5 mL, Natural	Eppendorf	022363204
❒	Tube, centrifuge 50 mL	VWR	93000-036
❒	Tube, centrifuge 15 mL	VWR	21008-103
❒	Tube strips, 8-well, 0.2 mL	VWR	20170-004

Supplier Contact List

Table F.12 Supplier Contact List

Supplier	Web Site Address
Affymetrix	www.affymetrix.com
Agilent Technologies	www.genomics.agilent.com
Applied Biosystems	www.appliedbiosystems.com
Bio-Rad	www.bio-rad.com
Bio-Smith	www.biosmith.com
Clontech	www.clontech.com
Diversified Biotech	www.divbio.com
E&K Scientific	www.eandkscientific.com
Eppendorf	www.eppendorf.com
ESCO	www.escoglobal.com
Fisher Scientific	www.fishersci.com
Life Technologies	www.lifetechnologies.com
ISC BioExpress	www.bioexpress.com
Lonza	www.lonza.com
Molecular Devices	www.moleculardevices.com
Molecular Probes	www.molecularprobes.com
NanoDrop	www.nanodrop.com
Neptune Scientific	www.neptunescientific.com/
New England Biolabs	www.neb.com
Pierce Biotechnology (part of Thermo Fisher Scientific)	www.piercenet.com
Promega	www.promega.com
Rainin	www.rainin.com

Table F.12 Supplier Contact List (Continued)

Supplier	Web Site Address
Scientific Industries	www.scientificindustries.com
Sigma-Aldrich	www.sigma-aldrich.com
USB	www.usb.affymetrix.com
Teknova	www.teknova.com
VWR	www.vwr.com