Affymetrix® SNP 6.0 Cytogenetics Copy Number Assay User Guide

P/N 702607 Rev. 3

For research use only.
Not for use in diagnostic procedures.

CONTENTS

Chapter 1

BEFORE YOU START

Topics in this chapter include:

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

An assay protocol for processing 48 samples is described in the Affymetrix® *Genome-Wide Human SNP Nsp/Sty 6.0 User Guide*, P/N 702504.

About the Affymetrix Cytogenetics Solution

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.

The combination of Affymetrix SNP 6.0 arrays, the Cytogenetics Copy Number Assay, and Genotyping Console 2.1 software allows 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 Cytogenetics Solution a great tool for next generation cytogenetics studies.

Tips for Ensuring Successful Performance of the Protocol

Successful performance of the SNP 6.0 Cytogenetics Copy Number Assay requires accuracy and attention to detail. Many stages involve specific yet distinct enzymatic reactions. For example, in stage 1, genomic DNA is digested with the restriction enzymes NspI and StyI. In stage 2, it is ligated to a common adaptor with T4 DNA ligase. Following ligation, the template undergoes PCR using TITANIUM™ *Taq* DNA polymerase. Once the product has been purified, it is then fragmented and end-labeled using terminal deoxynucleotidyl transferase.

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

Equipment and Calibration

Keep dedicated equipment in each of the areas used for this protocol including pipettors, ice buckets, coolers, etc. To avoid contamination, do not move equipment from one area to another.

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

- All thermal cyclers
- GeneChip® Hybridization Oven
- GeneChip® Fluidics Station
- GeneChip® Scanner 3000 7G
- Plate spectrophotometer or NanoDrop
- All multi-channel pipets

Pipetting

Since the SNP 6.0 Cytogenetics Copy Number Assay involves a series of ordered stages, the output of one stage directly impacts the performance of the subsequent stage. For example, the quantity and purity of the DNA after purification can affect the kinetics of the Fragmentation Reagent (enzyme) during the subsequent fragmentation stage.

To efficiently process samples:

- Always use pipets that have been calibrated to ± 5%.
- It is essential that you be proficient with the use of single- and multi-channel pipets.

To familiarize yourself with the use of multi-channel pipets, we strongly recommend practicing several times before processing actual samples. You can use water to get a feel for aspirating and dispensing solutions to multiple wells simultaneously.

Reagent Handling and Storage

IMPORTANT: Always use the 30 reaction Genome-Wide Human SNP Nsp/Sty Assay Kit 5.0/6.0 (P/N 901013) for this protocol. This kit has been tested for multiple freeze/thaw cycles.

You can freeze/thaw the reagents in the 30 reaction kit ≤ 8 times.

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

- Use only fresh reagents from the recommended vendors to help eliminate changes in pH or the salt concentration of buffers.
- Properly store all enzyme reagents. Storage methods can profoundly impact activity.
- Store the reagents used for the digestion, ligation and PCR in the Pre-PCR Clean Area.
- Consult the appropriate MSDS for reagent storage and handling requirements.

When Using Reagents at the Lab Bench

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

Master Mix Preparation

Carefully follow each master mix recipe. Use pipets that have been calibrated to ± 5%. When molecular biology-grade water is specified, be sure to use the AccuGENE® water listed in Appendix C. Using in-house ddH_2O or other water can negatively affect your results. 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 pipets 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.

Preparing the Work Area for Each Stage

Many of the stages in the SNP 6.0 Cytogenetics Copy Number 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.

NOTE: The illustrations in this user guide depict the recommended setup for 8 samples: 6 genomic DNA samples plus 1 positive and 1 negative control.

Thermal Cyclers, 96-well Plate, and Adhesive Seals

The SNP 6.0 Cytogenetics Copy Number 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 1.1, and only the thermal cyclers listed in Table 1.2. Using other plates and seals that are incompatible with these thermal cyclers can result in loss of sample or poor results.

Table 1.1 96-well plate and adhesive seals optimized for use with this protocol

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

Table 1.2 Thermal cyclers optimized for use with this 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
	Bio-Rad units: • MJ Tetrad PTC-225 • DNA Engine Tetrad 2
Post-PCR Area Use one of these units. (if processing > 8 samples, you may want to use additional thermal cyclers for PCR.)	Applied Biosystems: • GeneAmp® PCR System 9700 (silver block or gold-plated silver block)
	Bio-Rad units: • MJ Tetrad PTC-225 • DNA Engine Tetrad 2

Program Your Thermal Cyclers

The thermal cycler programs listed in Table 1.3 and Table 1.4 are used during 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 D, *Thermal Cycler Programs*.

Table 1.3 Pre-PCR Clean Area

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

Table 1.4 Post-PCR Area

# of Thermal Cyclers Required	Program Name
1 (if routinely processing > 8 samples, you may want to use additional thermal cyclers for PCR.)	Cyto PCR
	Cyto Fragment
	Cyto Label
	Cyto Hyb

Chapter 2

LABORATORY SETUP AND RECOMMENDATIONS

This chapter provides an overview of two laboratory setups that can used when performing the Affymetrix® SNP 6.0 Cytogenetics Copy Number 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 is presented in Table 2.1.

Table 2.1 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 2.1.

Activities that take place in this room include:

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

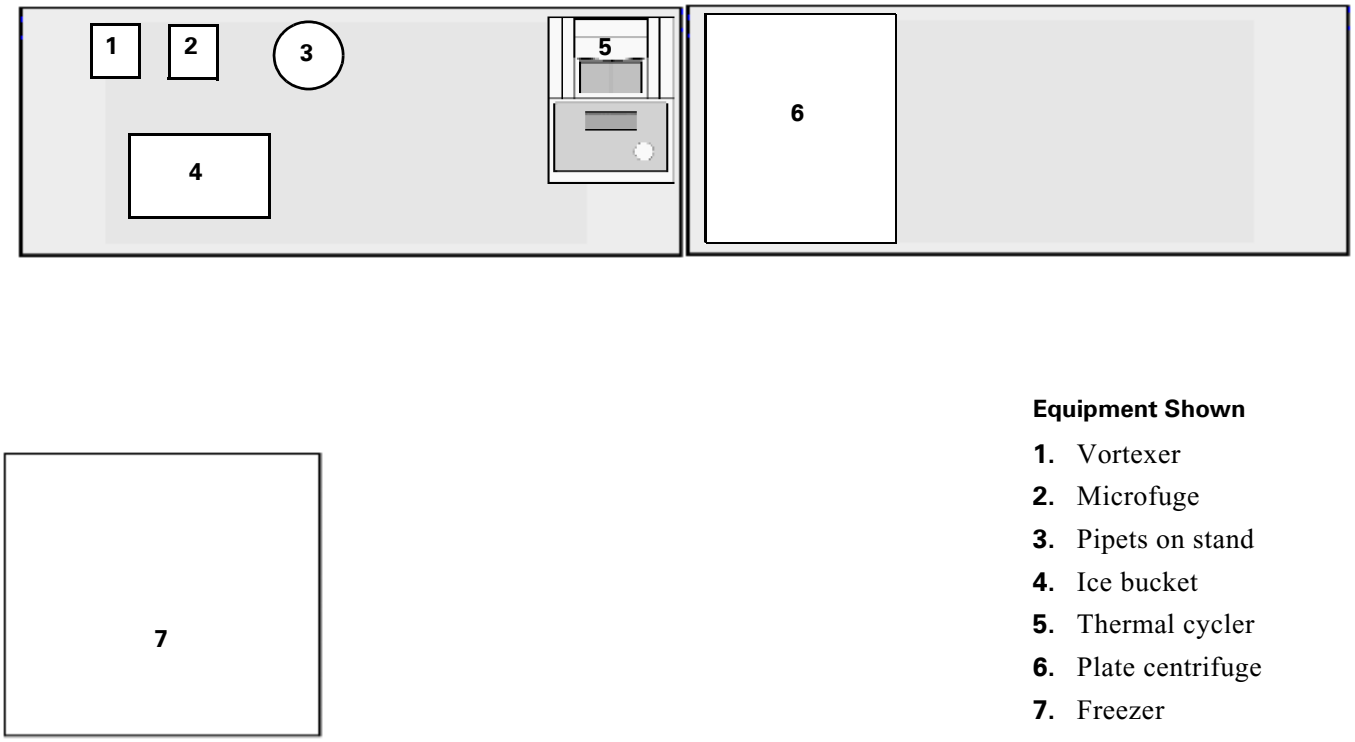


Figure 2.1 Pre-PCR Clean Room

To help prevent sample contamination:

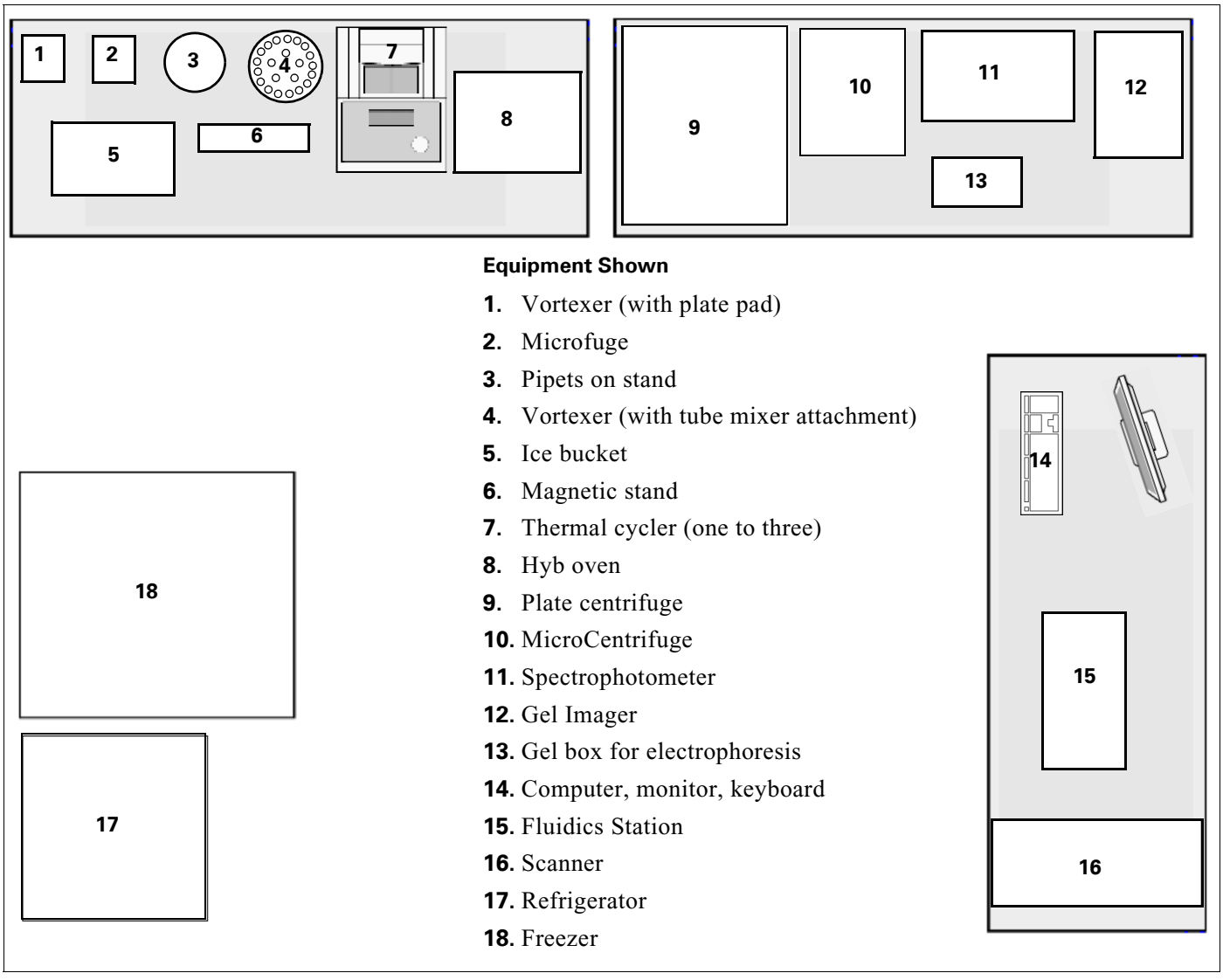
- All of the reagents and master stocks required for the steps performed in the Pre-PCR Clean Room should be stored in this room under the appropriate conditions.
- All of the equipment required for the steps performed in this room should be dedicated. Do not move any equipment including ice buckets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

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:

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



Equipment Shown

1. Vortexer (with plate pad)
2. Microfuge
3. Pipets on stand
4. Vortexer (with tube mixer attachment)
5. Ice bucket
6. Magnetic stand
7. Thermal cycler (one to three)
8. Hyb oven
9. Plate centrifuge
10. MicroCentrifuge
11. Spectrophotometer
12. Gel Imager
13. Gel box for electrophoresis
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 pipets 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.*

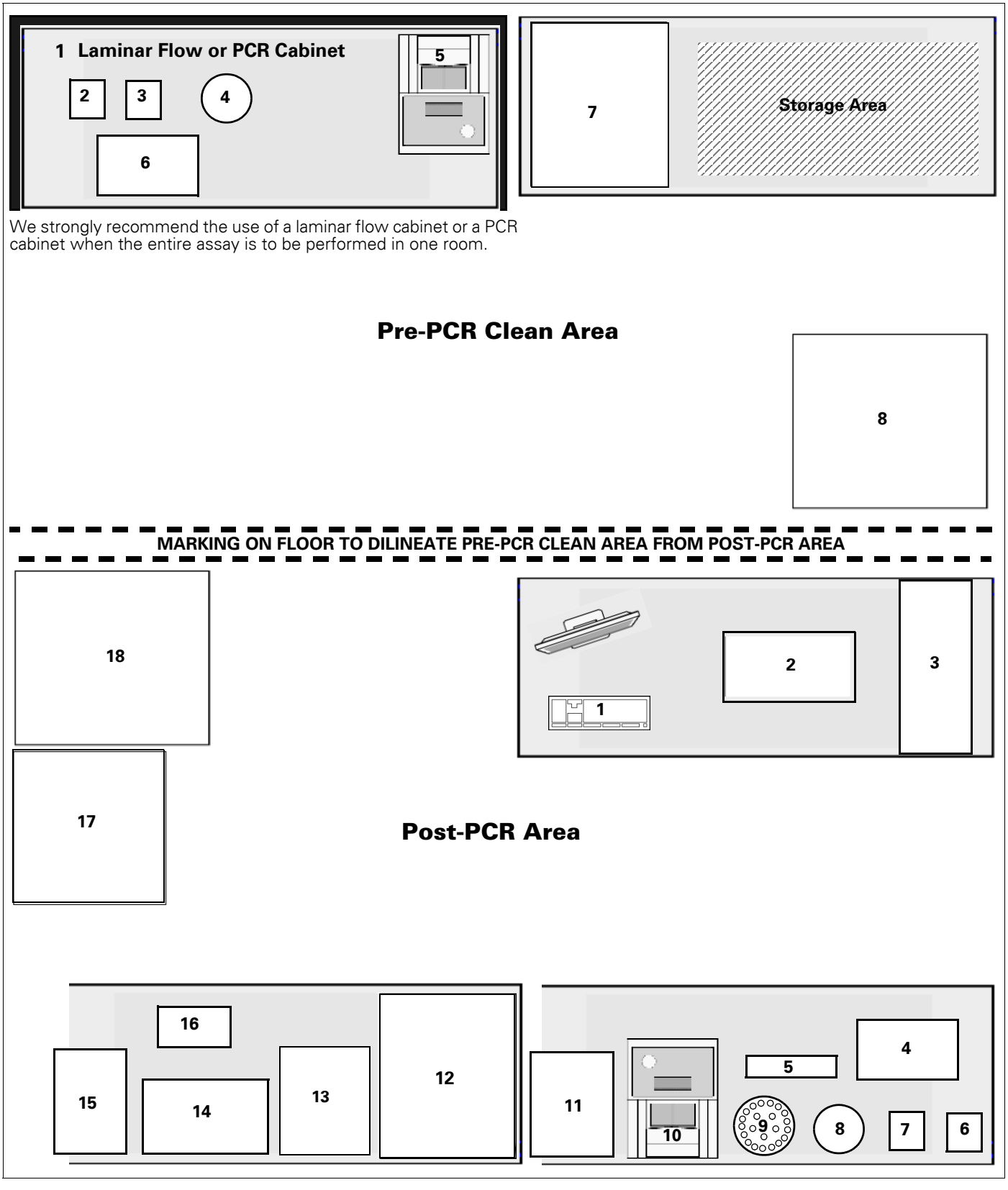


Figure 2.2 One room configuration

Pre-PCR Clean Area

For the best results, adhere to the following guidelines.

- Keep the Pre-PCR Clean Area
  - Free of DNA template and PCR amplicons.
  - Protected from contaminants by performing all steps inside a laminar flow cabinet or a PCR cabinet.
- If using a laminar flow cabinet, 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 2.2 on page 10 is listed below.

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

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. Store master stocks of PCR primer and adaptor 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 pipets 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 2.2 on page 10 consists of:

1. Computer, monitor and keyboard
2. Fluidics station
3. Scanner
4. Ice bucket
5. Magnetic stand
6. Vortexer with plate stand

7. Microfuge
8. Pipets on stand
9. Vortexer with tube mixer attachment
10. Thermal cycler (one to three)
11. Hybridization oven
12. Plate centrifuge
13. MicroCentrifuge
14. Spectrophotometer
15. Gel imager
16. Gel box for electrophoresis
17. Refrigerator
18. 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.

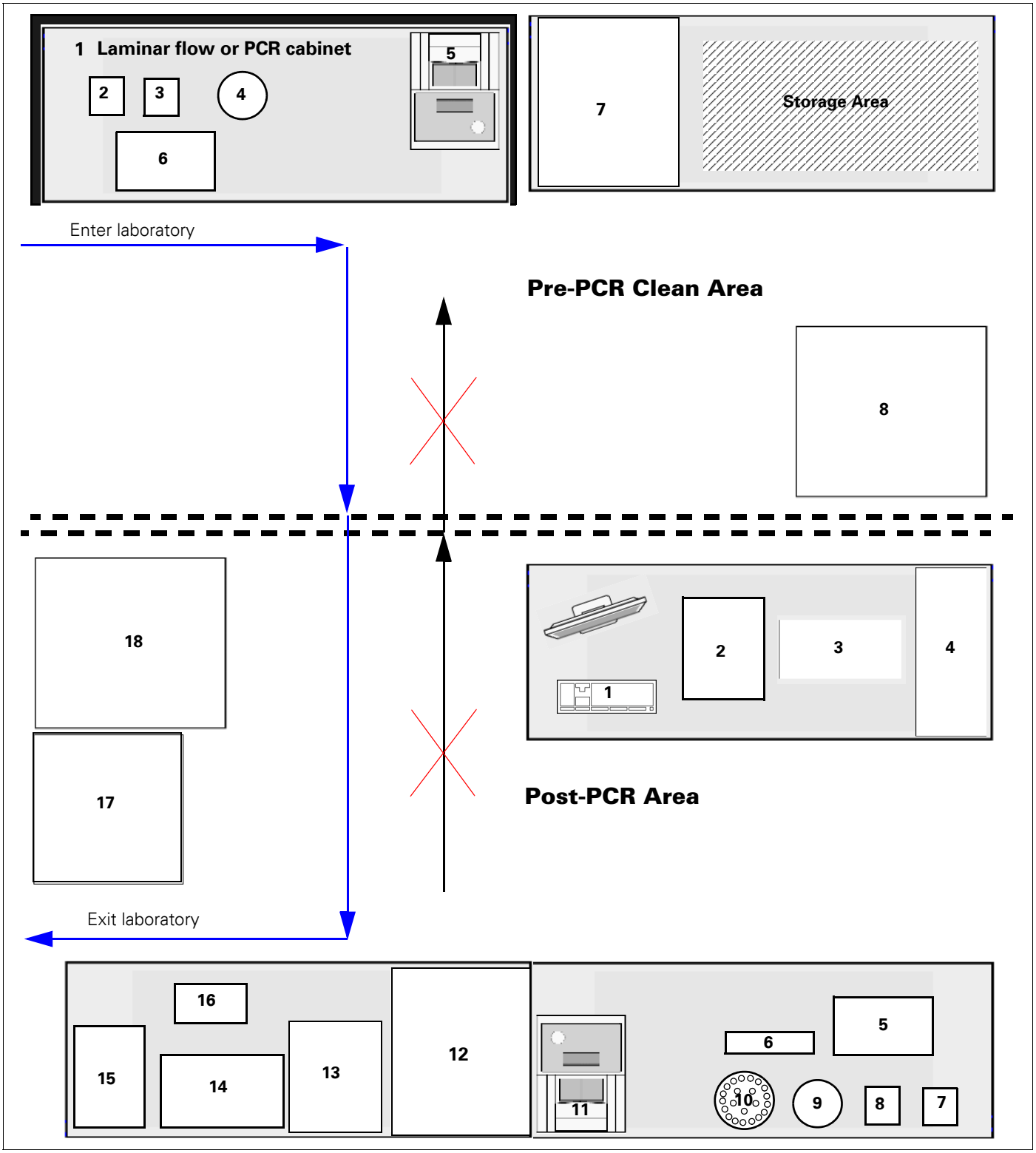


Figure 2.3 Single direction workflow

Contamination Prevention

Care should be taken to minimize possible sources of contamination that would reduce genotyping accuracy, call rate, and consequently, genetic power. To reduce the possibility of cross-contamination, Affymetrix 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*.

IMPORTANT:

- **The most likely potential source of contamination for the SNP 6.0 Cytogenetics Copy Number Assay is previously amplified PCR product.**
- **Each area should contain dedicated equipment such as thermal cyclers, microfuges, pipets 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.**

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

- Store reagents in the proper area according to the box label and reagent kit insert.
- Use proper gowning procedures.
- Print separate copies of the protocol for each room.

Safety Precautions

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

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

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

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

Chapter 3

GENOMIC DNA GENERAL REQUIREMENTS

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

For this protocol, you will use the Affymetrix® Genome-Wide Human SNP Nsp/Sty Assay Kit 5.0/6.0 (30 reaction; P/N 901013). This kit contains the genomic DNA control Reference Genomic DNA 103 (Ref 103). This control meets the requirements outlined below. The size of the starting genomic DNA can be compared with Ref103 DNA to assess the quality. The control DNA should also be used as a routine experimental positive control and for troubleshooting.

Assay performance may vary for genomic DNA samples that do not meet the general requirements described below. However, the reliability of any given result should be assessed in the context of overall experimental design and goals.

General Requirements

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

  This requirement relates to the restriction enzyme digestion step in the protocol.
- DNA must be free of PCR inhibitors.

  Examples of inhibitors include high concentrations of heme (from blood) and high concentrations of chelating agents (i.e., EDTA). The genomic DNA extraction/purification method should render DNA that is generally salt-free because high concentrations of certain salts can also inhibit PCR and other enzyme reactions. DNA should be prepared as described in Chapter 4, *Affymetrix® SNP 6.0 Cytogenetics Copy Number Assay*.
- DNA must not be contaminated with other human genomic DNA sources, or with genomic DNA from other organisms.

  PCR amplification of the ligated genomic DNA is not human specific, so sufficient quantities of non-human DNA may also be amplified and could potentially result in compromised genotype calls. Contaminated or mixed DNA may manifest as high detection rates and low call rates.
- DNA must not be highly degraded.

  For any particular SNP, the genomic DNA fragment containing the SNP must have Nsp I (or Sty I) restriction sites intact so that ligation can occur on both ends of the fragment and PCR can be successful. The approximate average size of genomic DNA may be assessed on a 1% or 2% agarose gel using an appropriate size standard control. Ref 103 can be run on the same gel for side-by-side comparison. High quality genomic DNA will run as a major band at approximately 10-20 kb on the gel.

  Pre-amplification methods or pre-digestion with restriction enzymes other than Nsp I or Sty I have not been tested by Affymetrix. If other methods are desired, we recommend conducting experiments to evaluate their performance with 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* on page 15.

- blood
- cell line

Success with other types of samples such as saliva will depend on quality (degree of degradation, degree of inhibitors present, etc.), quantity of genomic DNA extracted, and purity of these types of samples, as described under *General Requirements* on page 15.

Genomic DNA Extraction/Purification Methods

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

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

DNA Cleanup

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

1. Add 0.5 volumes of 7.5 M NH_4OAc, 2.5 volumes of absolute ethanol (stored at –20°C), and 0.5 µL of glycogen (5 mg/mL) to 250 ng genomic DNA.
2. Vortex and incubate at –20°C for 1 hr.
3. Centrifuge at 12,000 × x g in a microcentrifuge at room temperature for 20 min.
4. Remove supernatant and wash pellet with 0.5 mL of 80% ethanol.
5. Centrifuge at 12,000 × g at room temperature for 5 min.
6. Remove the 80% ethanol and repeat the 80% ethanol wash one more time.
7. Resuspend the pellet in reduced EDTA TE buffer (10 mM Tris, pH 8.0, 0.1 mM EDTA, pH 8.0).

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Chapter 4

AFFYMETRIX® SNP 6.0 CYTOGENETICS COPY NUMBER ASSAY

About the Protocol

The Affymetrix® SNP 6.0 Cytogenetics Copy Number Assay is designed for processing as few as four samples (including controls). The protocol is presented in the following stages:

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

An assay protocol for processing 48 samples is described in the Affymetrix® *Genome-Wide Human SNP Nsp/Sty 6.0 User Guide*, P/N 702504.

About the Illustrations in this Chapter

This protocol has been optimized for processing 4 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 9 to 24 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.

About the Reagents, Equipment and Consumables Specified in this Chapter

Genome-Wide Human SNP Nsp/Sty Assay Kit 5.0/6.0 — 30 Reactions

IMPORTANT: **Always use the 30 reaction Genome-Wide Human SNP Nsp/Sty Assay Kit 5.0/6.0 for this protocol. This kit has been tested for multiple freeze/thaw cycles. You can freeze/thaw the reagents in the 30 reaction kit ≤ 8 times.**

Equipment, Consumables, and Other Reagents

IMPORTANT: **This protocol has been optimized using the equipment, consumables and reagents listed herein. For the best results, we strongly recommend that you adhere to the protocol as described (no deviations); do not substitute reagents.**

Workflows

Recommended 4-Day Workflow

Figure 4.1 shows the recommended 4-day workflow for one operator processing four to 24 samples including controls.

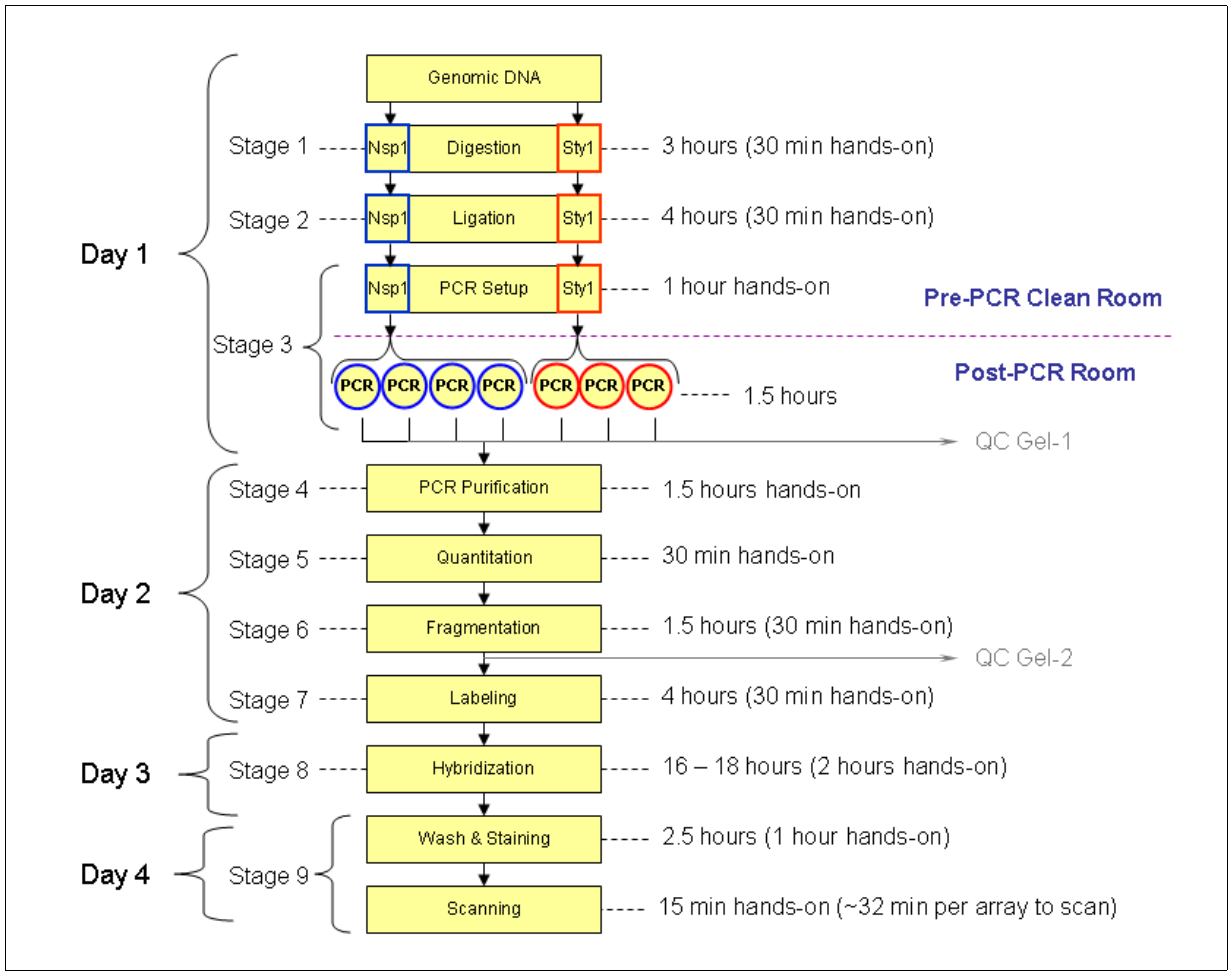


Figure 4.1 Workflow recommended for processing one to 24 samples

Optional 3-Day Workflow

Figure 4.2 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 (≤ 8 samples). If processing > 8 samples, the length of time required to complete all Day 2 activities will likely require more than an 8 hr.

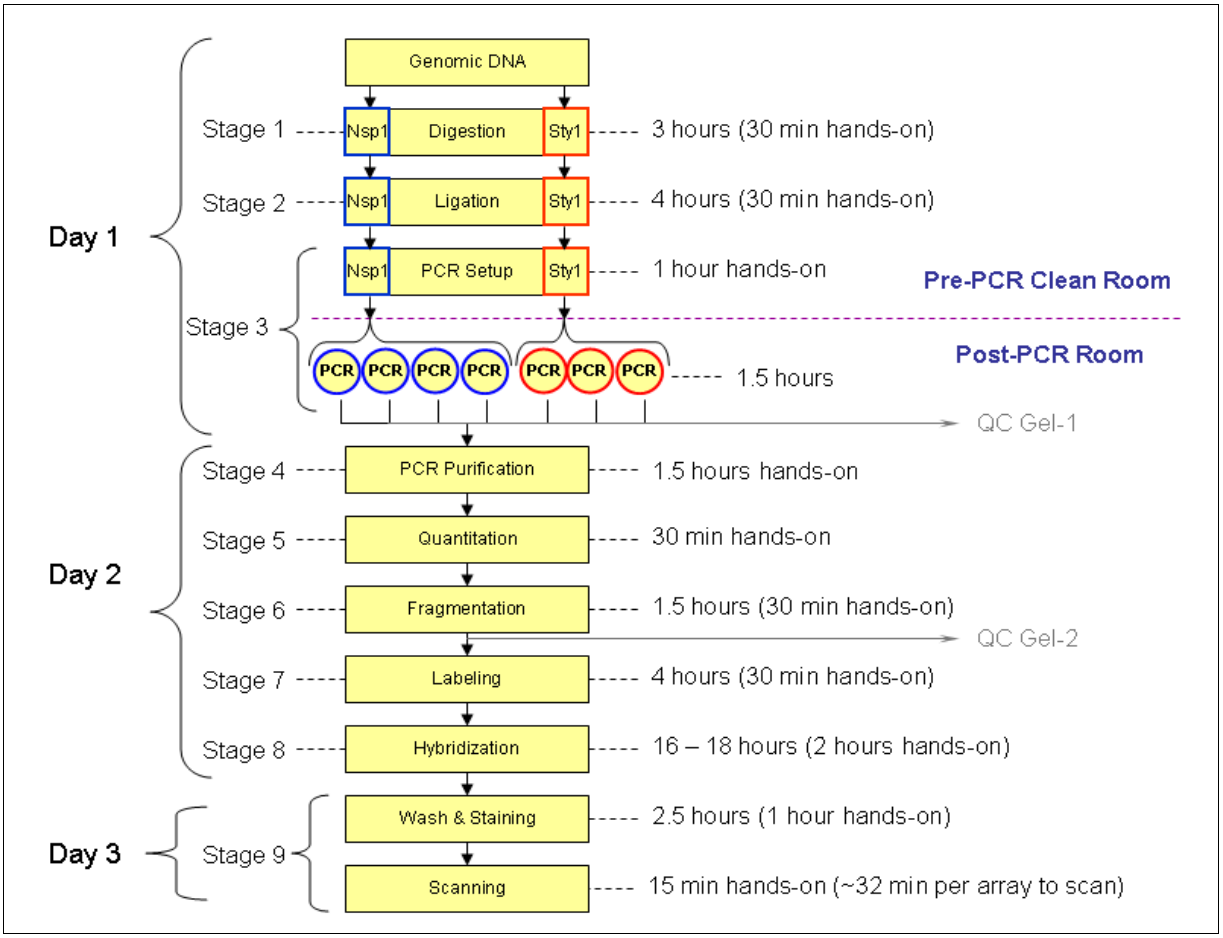


Figure 4.2 Optional 3-day workflow

Genomic DNA Preparation

About this Stage

The human genomic DNA you will process using the SNP 6.0 Cytogenetics Copy Number Assay should meet the general requirements listed in Chapter 3, *Genomic DNA General Requirements*. During this stage, you will prepare the genomic DNA by:

1. Determining the concentration of each sample (if required).
2. Diluting each sample to 50 ng/µL using reduced EDTA TE buffer.

Location and Duration

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

Input Required

The illustrations in this user guide depict the processing of eight samples: six genomic DNA samples, plus one positive and one negative control.

Table 4.1 Input Required for *Genomic DNA Preparation*

Quantity	Item
4 to 24	Genomic DNA samples that meet the requirements listed in Chapter 3, *Genomic DNA General Requirements*.

About Using Controls

We recommend including one positive and one negative control with every set of samples processed. For the positive control, use the Ref 103 included in the Genome-Wide Human SNP Nsp/Sty Assay Kit 5.0/6.0. For the negative control, use water (AccuGENE).

Equipment and Consumables Required

The equipment and consumables listed in Table 4.2 are required for this stage.

Table 4.2 Equipment and Consumables Required for *Genomic DNA Preparation*

Quantity	Item
As required	Adhesive seals for 96-well plates
1	Cooling chamber, double, chilled to 4°C on ice (do not freeze)
1	Ice bucket, filled with ice
2	Markers, red and blue, fine point, permanent
1	Mini microcentrifuge (microfuge)
1	Pipet, single channel P20
1	Pipet, single channel P100 or P200
As needed	Pipet tips
2	Plate, Bio-Rad 96-well unskirted
1	Plate centrifuge
1	Plate spectrophotometer or NanoDrop (required only if no OD measurements available for samples)
1	Vortexer
**** 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 4*.	

Reagents Required

The following reagents are required for this stage.

Table 4.3 Reagents Required for *Genomic DNA Preparation*

Reagent
Reduced EDTA TE Buffer (**0.1 mM EDTA**, 10 mM Tris HCL, pH 8.0)
Reference Genomic DNA 103 (positive control)
AccuGENE water (negative control)

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. Therefore, you should correlate readings from other methods to the equivalent UV absorbance reading.

Setup the Work Area

To setup the work area:

1. Place a double cooling chamber on ice (Figure 4.3).
2. Place a 96-well plate in the top half of the cooling chamber.

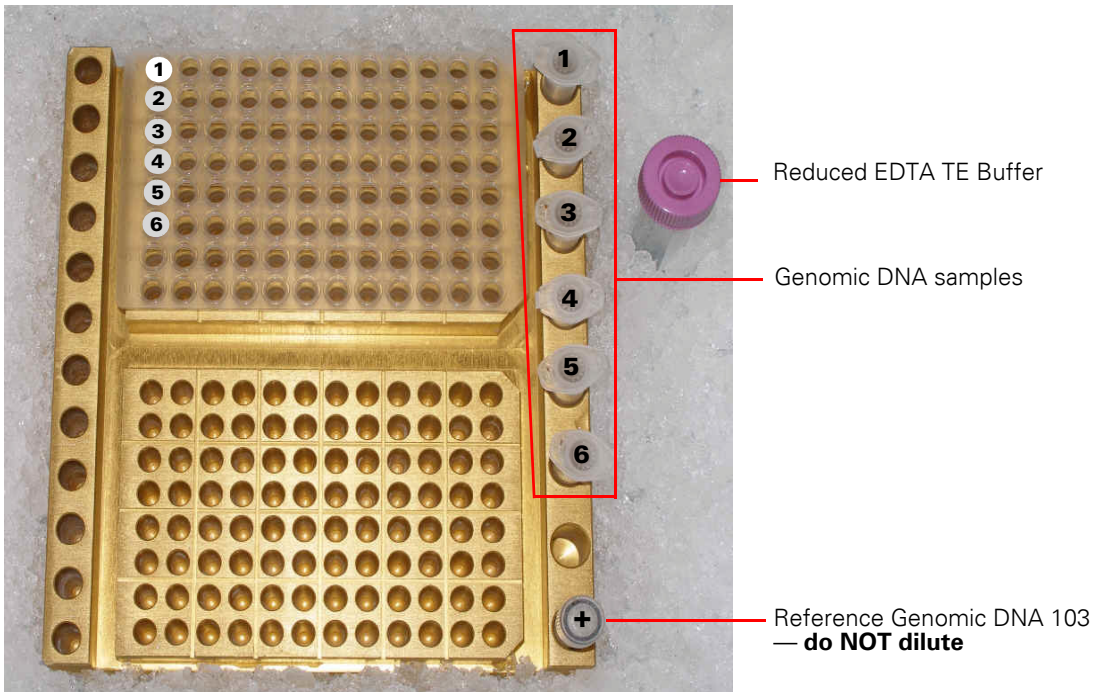


Figure 4.3 Diluting genomic DNA samples to 50 ng/µL

NOTE: The illustrations in this user guide 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) and Ref 103 as follows:
   A. Place on the bench top at room temperature until thawed.
   B. Once thawed, place in the cooling chamber on ice.
2. Vortex the gDNA samples at high speed for 3 sec.
3. Spin down for 30 sec; then place back in the cooling chamber.
4. If sample concentration is unknown, take an OD measurement of each sample now.

IMPORTANT: **To avoid contaminating samples with PCR product, take an aliquot of each sample to the plate spectrophotometer or NanoDrop.**

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

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

IMPORTANT: **Do NOT dilute Ref 103; it is already at a working concentration. An elevated EDTA level may interfere with subsequent reactions.**

6. Seal the plate, vortex at high speed for 3 sec; then spin down for 30 sec.
7. Place back on the cooling chamber.

Aliquoting the Prepared Genomic DNA and Controls

Setup the Work Area

To setup the work area:

1. Mark a 96-well plate as shown in Figure 4.4 [use a blue marker for Nsp (N) and a red marker for Sty (S)].

   The Nsp and Sty digestion and ligation reactions will be performed in this plate.
2. Place the plate on the bottom half of the cooling chamber (Figure 4.5 on page 27).
3. Place at least 5 µL of water on ice (negative control).

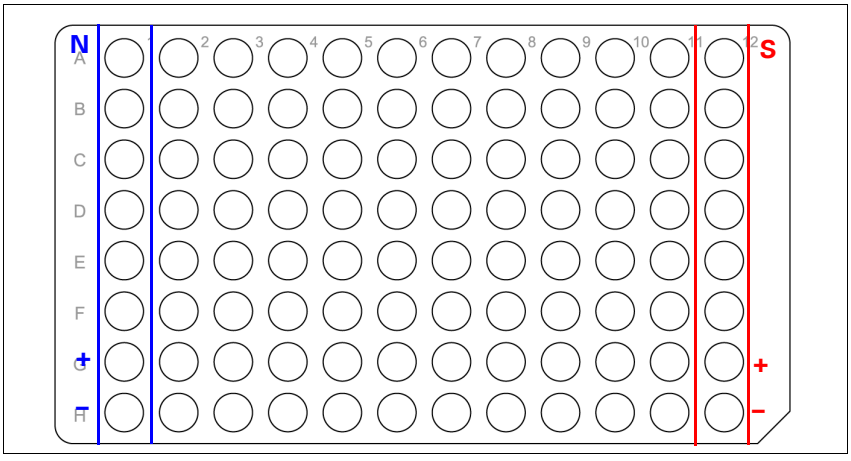


Figure 4.4 Marking a 96-well plate for Nsp and Sty digestion and ligation

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. Vortex the Ref 103 for 3 sec; then spin down for 30 sec.
2. Transfer two 5 µL aliquots of the first sample to wells A1 and A12 of the digest/ligate plate (Figure 4.5 on page 27).
3. Transfer two 5 µL aliquots of each remaining gDNA sample in the same manner.
4. For the controls, aliquot 5 µL of:
   A. Ref 103 (+) to wells G1 and G12.
   B. Water (–) to wells H1 and H12.
5. Tightly seal the digest/ligate plate.

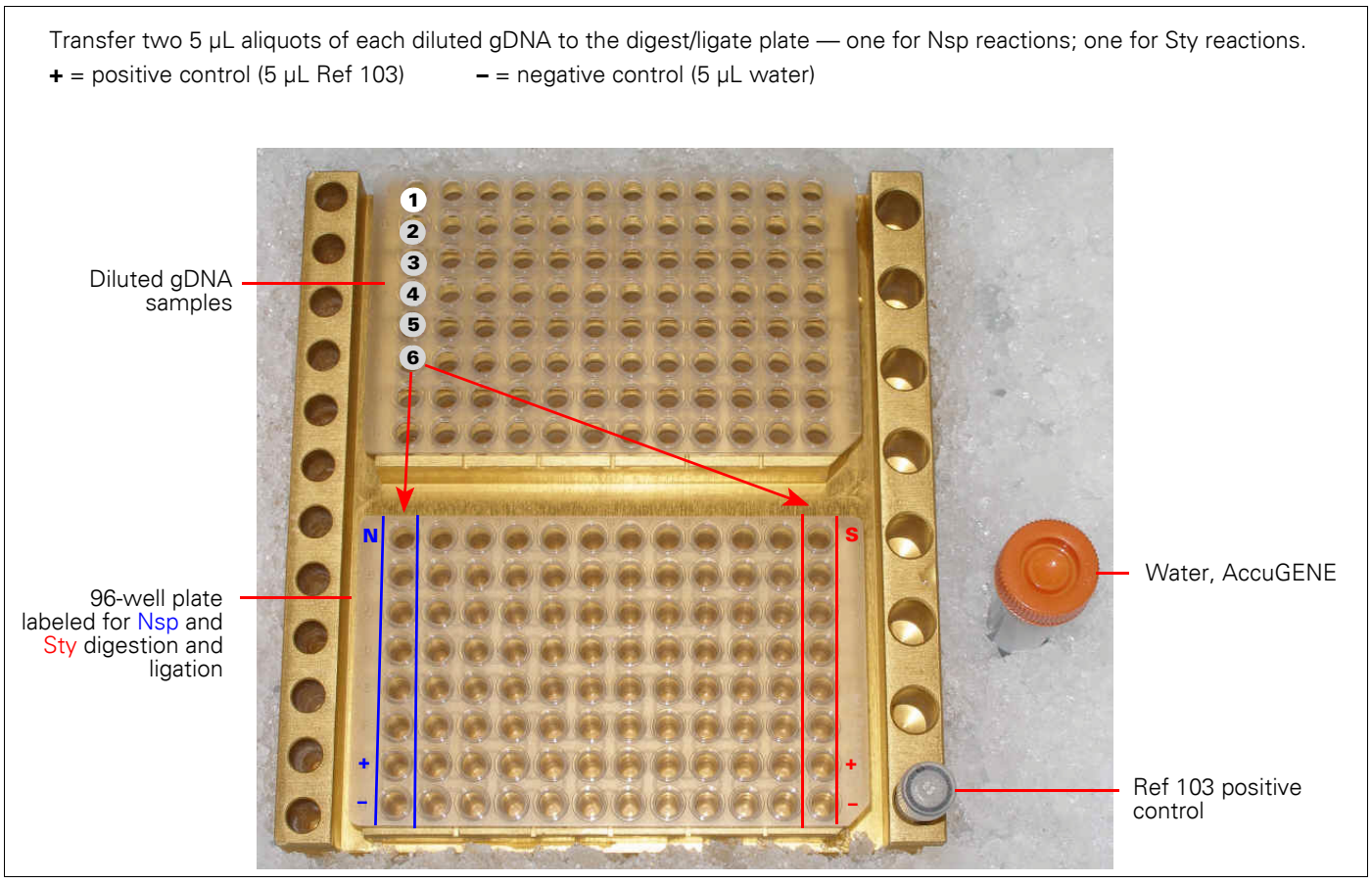


Figure 4.5 Setup for aliquoting diluted gDNA and controls to a 96-well plate labeled for Nsp and Sty digest/ligation

What To Do Next

Do one of the following:

- Proceed to *Stage 1: Nsp and Sty Restriction Enzyme Digest* on page 28.
- Store the prepared digest/ligate plate at –20°C.

Stage 1: Nsp and Sty Restriction Enzyme Digest

About this Stage

During this stage, one aliquot of each sample is digested by the NspI restriction enzyme; the other aliquot by the StyI restriction enzyme. You will:

1. Prepare a Nsp Digest Master Mix and add it to the samples in column 1.
2. Prepare a Sty Digest Master Mix and add it to the samples in column 12.
3. Place the samples onto a thermal cycler and run the Cyto Digest program.

Location and Duration

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

Input Required From Previous Stage

The input required is shown below.

Item
Plate containing two equal aliquots of each genomic DNA and each control prepared as instructed under *Genomic DNA Preparation* on page 23 (5 µL at 50 ng/µL in each well).

Equipment and Consumables Required

The following equipment and consumables are required for this stage.

Table 4.4 Equipment and Consumables Required for *Stage 1: Nsp and Sty Restriction Enzyme Digest*

Quantity	Item
As required	Adhesive seals for 96-well plates
1	Centrifuge, plate
1	Cooler, chilled to –20°C
1	Cooling chamber, double, chilled to 4°C on ice (do not freeze)
1	Ice bucket, filled with ice
1	Markers, blue and red, fine point, permanent
1	Mini centrifuge (microfuge)
1	Pipet, single channel P10
1	Pipet, single channel P100 or P200
As required	Pipet tips for pipets listed above
1	Thermal cycler
2	Tubes, Eppendorf 1.5 mL
1	Vortexer
**** 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 4*.	

Reagents Required

The following reagents are required for this stage.

Table 4.5 Reagents Required for *Stage 1: Nsp and Sty Restriction Enzyme Digest*

Reagent
BSA (100X; 10 mg/mL)
NE Buffer 2 (10X)
NE Buffer 3 (10X)
NspI (10 U/µL; NEB)
StyI (10 U/µL; NEB)
AccuGENE® Water, molecular biology-grade

Prepare the Reagents, Equipment and Consumables

Thaw Reagents and Genomic DNA

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

> **IMPORTANT:** **Leave the NspI and StyI enzymes at –20°C until ready to use.**

Setup the Work Area

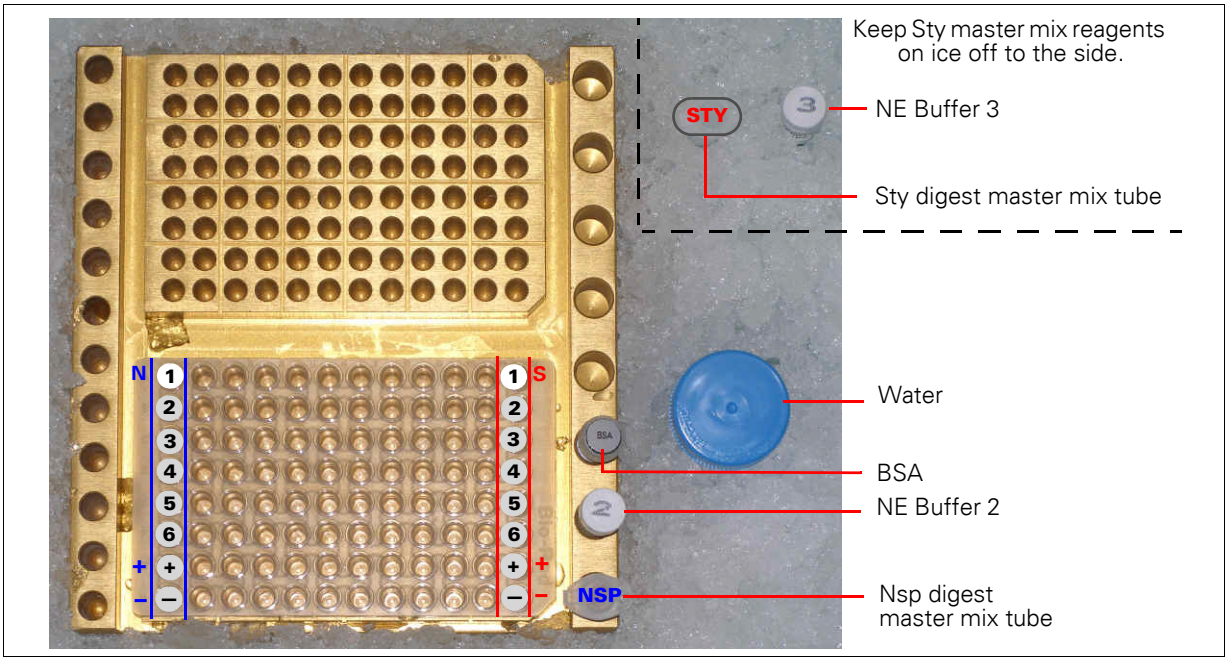


Figure 4.6 Setup for Nsp and Sty Digest (NspI and StyI enzymes not pictured; still at –20°C)

To setup the work area (Figure 4.6):

1. Place a double cooling chamber and the water on ice.
2. Label the 1.5 mL Eppendorf tubes as follows:
   - Using a blue marker, label one tube *NSP* and place in the cooling chamber.
   - Using a red marker, label one tube *STY* and set aside.
3. Prepare the genomic DNA and controls as follows:
   A. Vortex at high speed for 3 sec.
   B. Spin down at 2000 rpm for 30 sec.
   C. Place in the cooling chamber.
4. Prepare the NE Buffer 2 and BSA as follows:
   A. Vortex 3 times, 1 sec each time.
   B. Pulse spin for 3 sec.
   C. Place in the cooling chamber on ice.

Preheat the Thermal Cycler Lid

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

Prepare the Nsp Digest Master Mix

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

1. To the 1.5 mL Eppendorf tube labeled *NSP*, add the appropriate volumes of the following reagents (see Table 4.6):
   - Water (AccuGENE)
   - NE Buffer 2
   - BSA
2. Place the master mix in the cooling chamber.
3. Remove the NspI enzyme from the freezer and immediately place in a cooler.
4. Pulse spin the enzyme for 3 sec.
5. Immediately add the enzyme to the master mix.
6. Return the enzyme to the cooler.
7. Vortex the master mix at high speed 3 times, 1 sec each time.
8. Pulse spin for 3 sec.
9. Place in the cooling chamber.
10. Proceed immediately to *Add Nsp Digest Master Mix to Samples* on page 31.

Table 4.6 NspI Digest Master Mix

Reagent	1 Sample	4 Samples (25% extra**)	8 Samples (15% extra)	12 Samples (15% extra)	24 Samples (15% extra)
AccuGENE® Water	11.55 µL	57.8 µL	106.3 µL	159.4 µL	318.8 µL
NE Buffer 2 (10X)	2 µL	10 µL	18.4 µL	27.6 µL	55.2 µL
BSA (100X; 10 mg/mL)	0.2 µL	1 µL	1.8 µL	2.8 µL	5.5 µL
NspI (10 U/µL)	1 µL	5 µL	9.2 µL	13.8 µL	27.6 µL
Total	**14.75 µL**	**73.8 µL**	**135.7 µL**	**203.6 µL**	**407.1 µL**
** To avoid pipetting < 1 µL of BSA, prepare 25% extra when processing ≤ 4 samples.					

Add Nsp Digest Master Mix to Samples

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

To add Nsp Digest Master Mix to samples:

1. Aliquot 14.75 µL of Nsp Digest Master Mix to each sample and controls in column 1.
2. Return remaining NE Buffer 2 and NspI enzyme to the freezer.
3. Discard remaining Nsp Digest Master Mix.

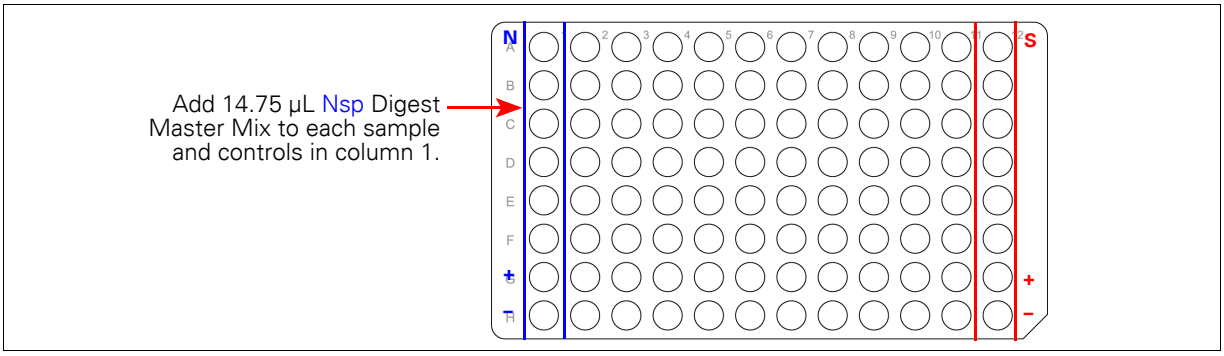


Figure 4.7 Adding Nsp Digest Master Mix to gDNA samples and controls

Prepare the Sty Digest Master Mix

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

1. To the 1.5 mL Eppendorf tube labeled *STY*, add the appropriate volumes of the following reagents as shown in Table 4.7:
   - Water (AccuGENE)
   - NE Buffer 3
   - BSA
2. Place the master mix in the cooling chamber.
3. Remove the StyI enzyme from the freezer and immediately place in a cooler.
4. Pulse spin the enzyme for 3 sec.
5. Immediately add the enzyme to the master mix.
6. Return remaining enzyme to the cooler.
7. Vortex the master mix at high speed 3 times, 1 sec each time.
8. Pulse spin for 3 sec.
9. Place in the cooling chamber.
10. Proceed immediately to *Add Sty Digest Master Mix to Samples* on page 33.

Table 4.7 StyI Digest Master Mix

Reagent	1 Sample	4 Samples (25% extra**)	8 Samples (15% extra)	12 Samples (15% extra)	24 Samples (15% extra)
AccuGENE® Water	11.55 µL	57.8 µL	106.3 µL	159.4 µL	318.8 µL
NE Buffer 3 (10X)	2 µL	10 µL	18.4 µL	27.6 µL	55.2 µL
BSA (100X; 10 mg/mL)	0.2 µL	1 µL	1.8 µL	2.8 µL	5.5 µL
StyI (10 U/µL)	1 µL	5 µL	9.2 µL	13.8 µL	27.6 µL
Total	**14.75 µL**	**73.8 µL**	**135.7 µL**	**203.6 µL**	**407.1 µL**
** 25% extra is required for 4 samples only. If processing 8 samples, 15% extra is sufficient.					

Add Sty Digest Master Mix to Samples

To add the Sty Digest Master Mix to samples:

1. Aliquot 14.75 µL of Sty Digest Master Mix to each sample and control in column 12. The total volume in each well is now 19.75 µL.
2. Tightly seal the plate.

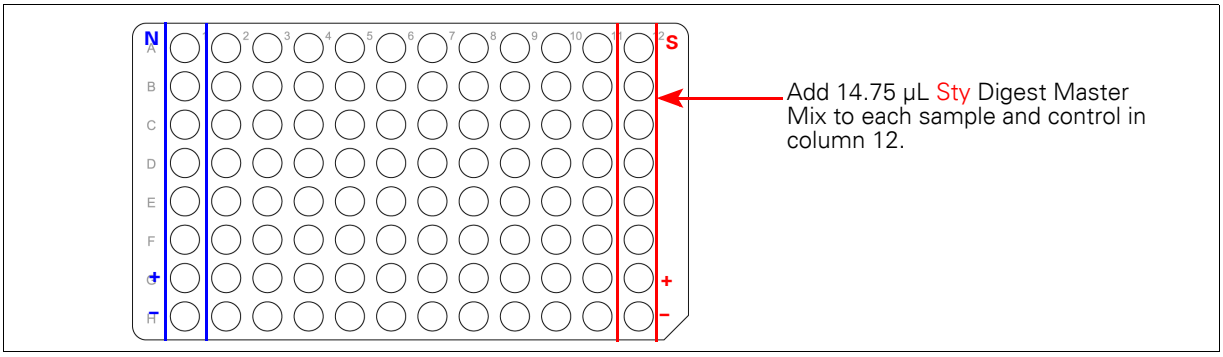


Figure 4.8 Adding Sty Digest Master Mix

Load Nsp and Sty Samples onto the Thermal Cycler

1. Vortex the plate at high speed for 3 sec; then spin down at 2000 rpm for 30 sec.
2. Ensure that the lid of thermal cycler is preheated.
3. Load the plate onto the thermal cycler and run the Cyto Digest program (Table 4.8).

> **IMPORTANT:** **Ensure that the seal is not pulled off the wells when you close the thermal cycler lid.**

4. Return any remaining reagents to the freezer.
5. When the program is finished, remove the plate and spin down at 2000 rpm for 30 sec.

Table 4.8 Cyto Digest Program

Cyto Digest Program	
Temperature	**Time**
37°C	120 min
65°C	20 min
4°C	Hold

What To Do Next

Do one of the following:

- If following the recommended workflow (Figure 4.1 on page 21), place the plate in a cooling chamber on ice and proceed immediately to *Stage 2: Nsp and Sty Ligation* on page 34.
- If not proceeding directly to the next step, store the plate at –20°C.

Stage 2: Nsp and Sty Ligation

About this Stage

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

1. Prepare a Nsp Ligation Master Mix and add it to the Nsp digested samples.
2. Prepare a Sty Ligation Master Mix and add it to the Sty digested samples.
3. Place samples onto a thermal cycler and run the Cyto Ligate program.
4. Dilute the ligated samples with water.

Location and Duration

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

Input Required From Previous Stage

The input required from *Stage 1: Nsp and Sty Restriction Enzyme Digest* is:

Item
Plate of Nsp and Sty digested samples

Equipment and Consumables Required

The following equipment and consumables are required for this stage.

Table 4.9 Equipment and Consumables Required for *Stage 2: Nsp and Sty Ligation*

Quantity	Item
1	Adhesive seals for 96-well plates
1	Centrifuge, plate
1	Cooler, chilled to –20°C
1	Cooling chamber, double, chilled to 4°C on ice (do not freeze)
1	Ice bucket, filled with ice
1	Marker, blue and red, fine point, permanent
1	Mini centrifuge (microfuge)
1	Pipet, single channel P10
1	Pipet, single channel P20
1	Pipet, single channel P100 or P200
As needed	Pipet tips for pipets listed above
1	Thermal cycler
3	Tubes, Eppendorf 1.5 mL
1	Vortexer
**** 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 4*.	

Reagents Required

The following reagents are required for this stage.

Table 4.10 Reagents Required for *Stage 2: Nsp and Sty Ligation*

Reagent
T4 DNA Ligase (400 U/µL; NEB)
T4 DNA Ligase Buffer (10X)
Adaptor, Nsp (50 µM)
Adaptor, Sty (50 µM)
Water, AccuGENE molecular biology-grade

Prepare the Reagents, Consumables and Other Components

Thaw the Reagents and Digested Samples

To thaw the reagents and digested samples:

1. Allow the following reagents to thaw on ice:
   - Adaptor Nsp
   - Adaptor Sty
   - T4 DNA Ligase Buffer (10X; requires approximately 20 min to thaw)
2. If the digested samples were frozen, allow them to thaw in a cooling chamber on ice.

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

Setup the Work Area

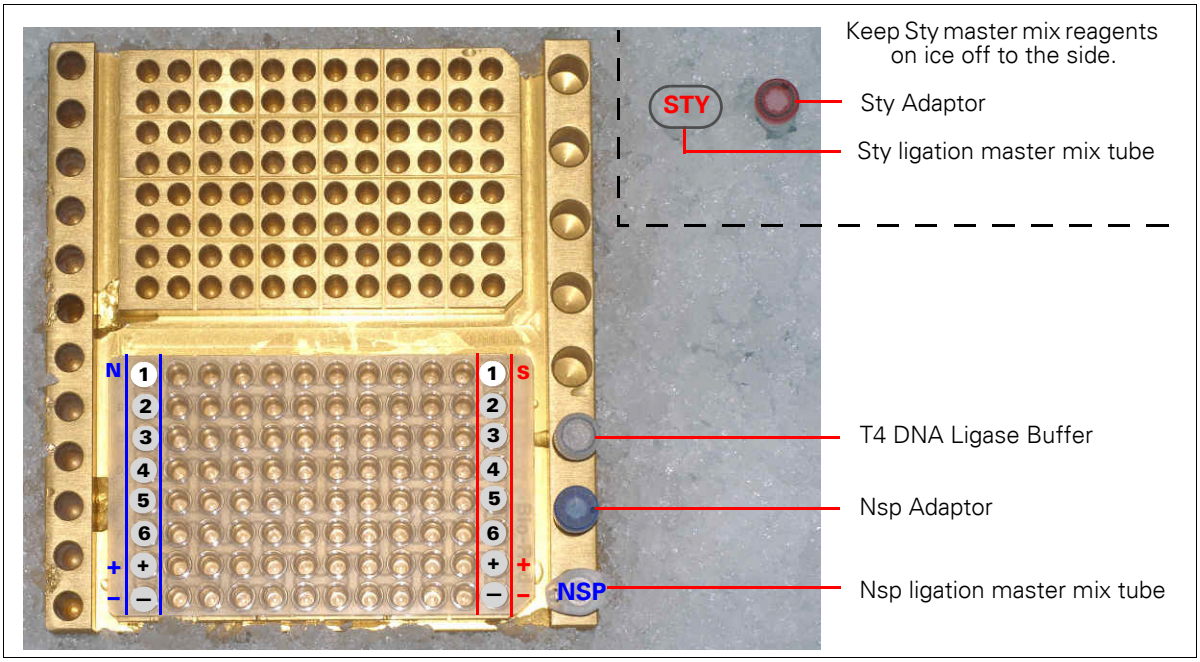


Figure 4.9 Setup for Nsp and Sty Ligation (T4 DNA Ligase enzyme not pictured; still at –20°C)

To setup the work area (Figure 4.9):

1. Place a double cooling chamber on ice.
2. Label the 1.5 mL Eppendorf tubes as follows:
   - Label one tube *NSP* and place in the cooling chamber.
   - Label one tube *STY* and set aside.
3. Prepare the digested samples as follows:
   A. Vortex at high speed for 3 sec; then spin down at 2000 rpm for 30 sec.
   B. Place in the cooling chamber on ice.
4. To prepare the reagents:
   A. Vortex at high speed 3 times, 1 sec each time (except for the enzyme).
   B. Pulse spin for 3 sec.
   C. Place in the cooling chamber.

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

Preheat the Thermal Cycler Lid

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

The lid must be preheated before samples are loaded.

Prepare the Nsp Ligation Master Mix

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

1. To the 1.5 mL Eppendorf tube labeled *NSP*, add the following reagents based on the volumes shown in Table 4.11:
   - T4 DNA Ligase Buffer (10X)
   - Adaptor Nsp
2. Remove the T4 DNA Ligase from the freezer and immediately place in the cooler.
3. Pulse spin the T4 DNA Ligase for 3 sec.
4. Immediately add the T4 DNA Ligase to the master mix; then place back in the cooler.
5. Vortex the master mix at high speed 3 times, 1 sec each time.
6. Pulse spin for 3 sec.
7. Place the master mix on ice.
8. Proceed immediately to *Add Nsp Ligation Master Mix to Reactions*.

Table 4.11 NspI Ligation Master Mix

Reagent	1 Sample	4 Samples (15% extra)	8 Samples (15% extra)	12 Samples (15% extra)	24 Samples (15% extra)
T4 DNA Ligase Buffer (10X)	2.5 µL	11.5 µL	23.0 µL	34.5 µL	69 µL
Adaptor, Nsp (50 µM)	0.75 µL	3.45 µL	6.90 µL	10.35 µL	20.7 µL
T4 DNA Ligase (400 U/µL)	2 µL	9.2 µL	18.4 µL	27.6 µL	55.2 µL
Total	**5.25 µL**	**24.15 µL**	**48.30 µL**	**72.45 µL**	**144.90 µL**

Add Nsp Ligation Master Mix to Reactions

To add Nsp Ligation Master Mix to samples:

1. Using a P20 pipet, aliquot 5.25 µL of Nsp Ligation Master Mix to each Nsp digested sample and control (Figure 4.10).
2. Discard any remaining Nsp Ligation Master Mix.

Nsp Digested DNA	19.75 µL
Nsp Ligation Master Mix*	5.25 µL
Total	**25.00 µL**
* Contains ATP and DTT. Keep on ice.	

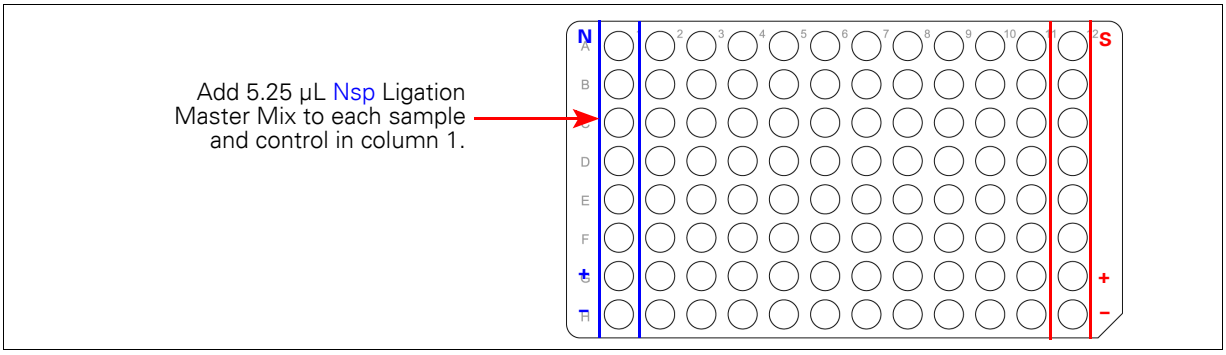


Figure 4.10 Adding Nsp ligate master mix to Nsp digested samples and controls

Prepare the Sty Ligation Master Mix

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

1. To the 1.5 mL Eppendorf tube labeled *STY*, add the following reagents based on the volumes shown in Table 4.12 on page 39:
   - T4 DNA Ligase Buffer (10X)
   - Adaptor, Sty
2. Immediately add the T4 DNA Ligase to the master mix; then place back in the cooler.
3. Vortex the master mix at high speed 3 times, 1 sec each time.
4. Pulse spin for 3 sec.
5. Place the master mix on ice.
6. Proceed immediately to *Add Sty Ligation Master Mix to Reactions*.

Table 4.12 Sty Ligation Master Mix

Reagent	1 Sample	4 Samples (15% extra)	8 Samples (15% extra)	12 Samples (15% extra)	24 Samples (15% extra)
T4 DNA Ligase Buffer (10X)	2.5 µL	11.5 µL	23.0 µL	34.5 µL	69 µL
Adaptor, Sty (50 µM)	0.75 µL	3.45 µL	6.90 µL	10.35 µL	20.7 µL
T4 DNA Ligase (400 U/µL)	2 µL	9.2 µL	18.4 µL	27.6 µL	55.2 µL
Total	**5.25 µL**	**24.15 µL**	**48.30 µL**	**72.45 µL**	**144.90 µL**

Add Sty Ligation Master Mix to Reactions

To add Sty Ligation Master Mix to samples:

1. Using a P20 pipet, aliquot 5.25 µL of Sty Ligation Master Mix to each Sty digested sample and control. (Figure 4.11).

   The total volume in each well is now 25 µL.

2. Tightly seal the plate.

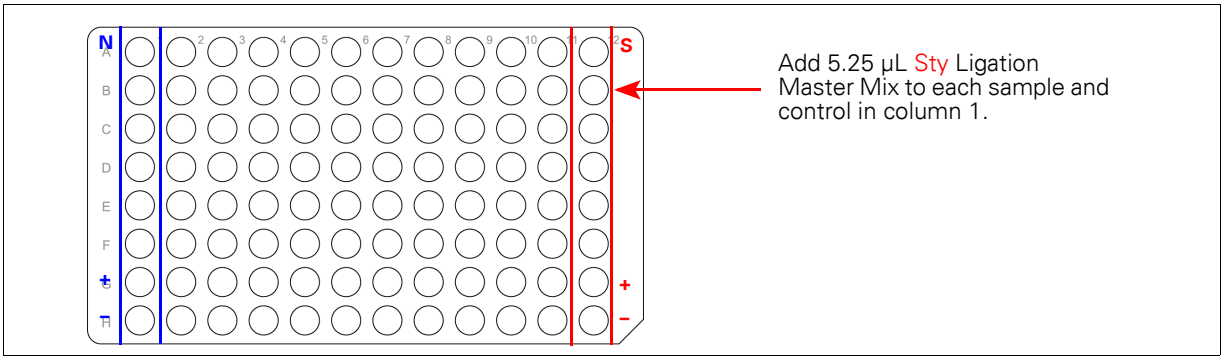


Figure 4.11 Adding Sty Ligation Master Mix to Sty digested samples and controls

Load the Nsp and Sty Samples Onto the Thermal Cycler

1. Vortex the plate at high speed for 3 sec; then spin down at 2000 rpm for 30 sec.
2. Ensure that the thermal cycler lid is preheated.
3. Load the plate onto the thermal cycler and run the Cyto Ligate program.

> **IMPORTANT:** **Ensure that the seal is not pulled off the wells when you close the thermal cycler lid.**

4. Return remaining reagents to the freezer and discard remaining master mix.

Table 4.13 Cyto Ligate Thermal Cycler Program

Cyto Ligate Program	
Temperature	**Time**
16°C	180 min
70°C	20 min
4°C	Hold

Dilute the Ligated Samples

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

To dilute the samples:

1. Place the water on ice 20 min prior to use.
2. When the Cyto Ligate program is finished, remove the plate and spin down at 2000 rpm for 30 sec.
3. Place in a cooling chamber on ice.
4. Using a P200 pipet, add 75 µL of water to each reaction.

Nsp and Sty Ligated DNA	25 µL
Water, AccuGENE	75 µL
Total	**100 µL**

5. Tightly seal the plate.
6. Vortex at high speed for 3 sec; then spin at 2000 rpm for 30 sec.

What To Do Next

Do one of the following:

- If following the recommended workflow (Figure 4.1 on page 21), proceed immediately to *Stage 3: Nsp and Sty PCR* on page 41.

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

Stage 3: Nsp and Sty PCR

About this Stage

During this stage, you will:

1. Transfer equal aliquots of each:
   - Nsp diluted ligated sample into *four* wells of a 96-well plate.
   - Sty diluted ligated sample into *three* wells of the same 96-well plate.
2. Prepare a PCR Master Mix and add it to each ligated sample.
3. Place the samples onto a thermal cycler and run the Cyto PCR program.
4. Confirm each PCR reaction by running 3 µL of each PCR product on a gel.

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
- Cyto PCR thermal cycler program time: 1.5 hr
  Samples can be held overnight at 4°C.

Input Required from Previous Stage

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

Item
Diluted ligated Nsp and Sty samples

Equipment and Materials Required

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

Table 4.14 Equipment and Consumables Required for *Stage 3: Nsp and Sty PCR*

Quantity	Item
As required	Adhesive seals for 96-well plates
1	Centrifuge, plate
1	Cooler, chilled to –20°C
1	Cooling chamber, double, chilled to 4°C on ice (do not freeze)
1	Ice bucket, filled with ice
2	Markers, blue and red, fine point, permanent
1	Mini centrifuge (microfuge)
1	Pipet, single channel P20
1	Pipet, single channel P100
1	Pipet, single channel P200
1	Pipet, single channel P1000
1	Optional: Pipet, 8-channel P20
1	Optional: Pipet, 8-channel P200
As required	Pipet tips for pipets listed above
1 to 3	Plate, Bio-Rad 96-well PCR • 1 to 8 samples: 1 plate • 9 to 16 samples: 2 plates • 17 to 24 samples: 3 plates
As required	Plate holder, 96-well PCR
1	Solution basin, 55 mL
1	Thermal cycler (If routinely processing > 8 samples, you may want to use more than one thermal cycler.)
1	Tube, centrifuge 50 mL
1	Vortexer
**** 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 4*.	

Reagents Required

The following reagents are required for this stage.

Table 4.15 Reagents Required for *Stage 3: Nsp and Sty PCR*

Reagent
AccuGENE water, molecular biology-grade
PCR Primer 002 (100 µM)
From the Clontech TITANIUM™ DNA Amplification Kit:
• dNTPs (2.5 mM each)
• GC-Melt (5M)
• TITANIUM™ *Taq* DNA Polymerase (50X)
• TITANIUM™ *Taq* PCR Buffer (10X)

Gels and Related Materials Required

Verifying the PCR reaction is required for this stage.

Table 4.16 Gels and Related Materials Required for *Stage 3: Nsp and Sty PCR*

Reagent
DNA Marker (BioNexus All Purpose Hi-Lo Ladder)
Gels, 2% TBE (precast or house-made)
Gel loading solution
Plates, 96-well reaction

Important Information About This Stage

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

! **IMPORTANT:**

- **Ensure that the Nsp and Sty ligated samples were diluted to 100 µL with AccuGENE water.**
- **Prepare the PCR Master Mix immediately prior to use in Pre-PCR Clean Area (ideally in a laminar flow or PCR cabinet). To help ensure the correct distribution of fragments, be sure to add the correct amount of primer. Mix well to ensure the even distribution of primers.**
- **Ensure that the PCR product distribution is between ~250 bp to 1100 bp by running 3 µL aliquots of each PCR reaction on a gel.**

About Controls

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

Prepare the Reagents, Consumables and Other Components

Thaw Reagents and Samples

To thaw the reagents and samples:

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

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

2. If the diluted ligated samples are frozen, thaw them in a cooling chamber on ice.

Setup Your Work Area (Pre-PCR Clean Area)

To setup your work area (Figure 4.13 on page 45):

1. Place a double cooling chamber on ice.
2. Place the plate of diluted ligated samples in the top half of the cooling chamber.
3. Label a fresh 96-well plate as shown in Figure 4.12 (blue for Nsp; red for Sty).
4. Place the plate in the lower half of the cooling chamber.

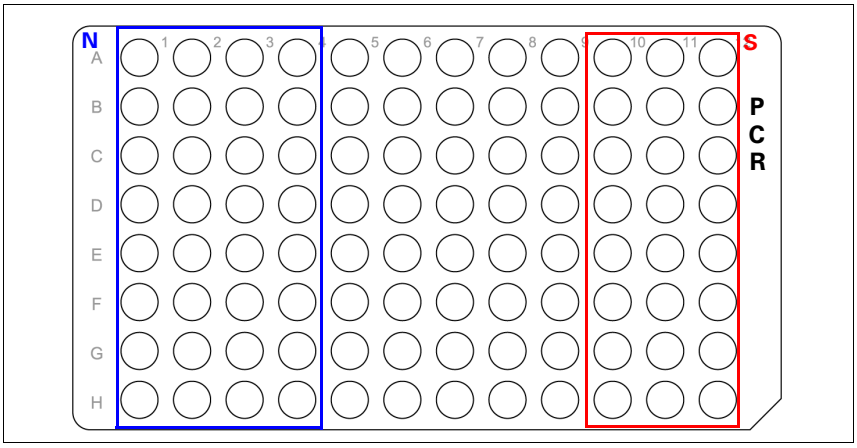


Figure 4.12 Labeling the 96-well plate for PCR.

Prepare the Ligated Samples and Reagents

To prepare the ligated samples, and reagents:

1. Label the 50 mL centrifuge tubes *PCR*.
2. Place on ice:
   - Water (AccuGENE)
   - GC-Melt
   - Solution basin
3. Prepare the diluted ligated samples as follows:
   A. Vortex at high speed for 3 sec; then spin down at 2000 rpm for 30 sec.
   B. Place in the top half of the chamber as shown in Figure 4.13 on page 45.

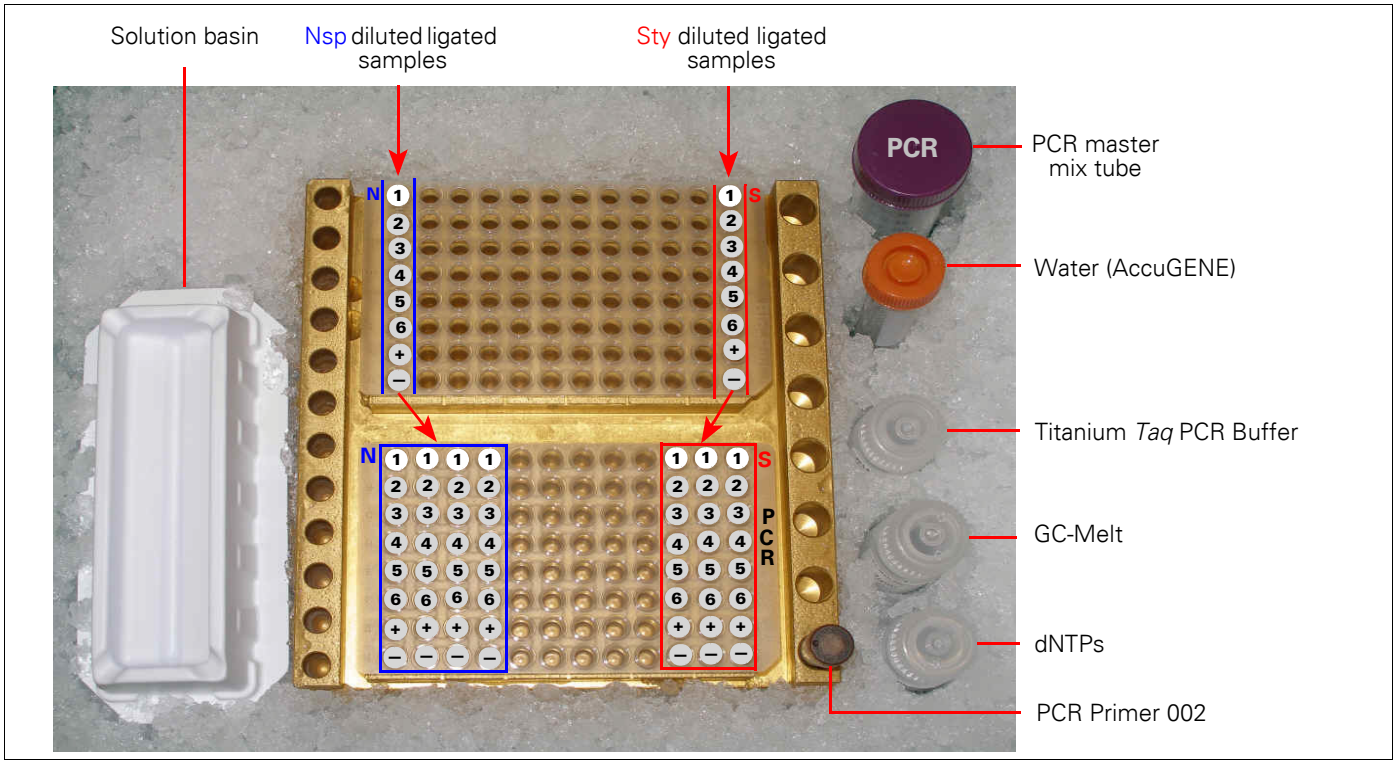


Figure 4.13 Setup for PCR (Titanium *Taq* DNA Polymerase not shown; still at –20°C)

4. To prepare the reagents:
   - **A.** Vortex at high speed 3 times, 1 sec each time (except for the enzyme).
   - **B.** Pulse spin for 3 sec.
   - **C.** Place on ice or in the cooling chamber.

Preheat the Thermal Cycler Lids (Post-PCR Area)

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

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

Transfer Diluted Ligated Samples to the PCR Plate

To transfer the diluted ligated samples to the PCR plate:

1. Using a P20 pipet (single or multichannel):
   - **A.** Transfer 10 µL of each Nsp ligated sample to the corresponding **four** wells of the PCR plate Figure 4.13 on page 45).
   - **B.** Transfer 10 µL of each Sty ligated sample to the corresponding **three** wells of the PCR plate.
2. Seal, label and store the plate with the remaining ligated Nsp and Sty samples at –20°C.

   Discard this plate once you have successfully processed these samples.

Prepare the PCR Master Mix

The same PCR master mix is used for both Nsp and Sty ligated samples.

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

Check the PCR reactions on a gel to ensure that the distribution is correct.

To prepare the PCR Master Mix:

1. Keeping the 50 mL centrifuge tube in the cooling chamber, add the reagents in the order shown in Table 4.17 on page 46 (**except for the *Taq* DNA polymerase**).
2. Remove the TITANIUM *Taq* DNA Polymerase from the freezer and immediately place in a cooler.
3. Pulse spin the *Taq* DNA polymerase for 3 sec.
4. Immediately add the *Taq* DNA polymerase to the master mix; then return the tube to the cooler.
5. Vortex the master mix at high speed 3 times, 1 sec each time.
6. Pour the master mix into the solution basin, keeping the basin on ice.

Table 4.17 PCR Master Mix — Volumes sufficient for processing both Nsp and Sty ligated samples

Reagent	1 Sample	4 Samples (15% extra)	8 Samples (15% extra)	12 Samples (15% extra)	24 Samples (15% extra)
AccuGENE water	39.5 µL	1272 µL	2544 µL	3816 µL	7632 µL
TITANIUM *Taq* PCR Buffer (10X)	10 µL	322 µL	644 µL	966 µL	1932 µL
GC-Melt (5M)	20 µL	644 µL	1288 µL	1932 µL	3864 µL
dNTP (2.5 mM each)	14 µL	451 µL	902 µL	1352 µL	2704 µL
PCR Primer 002 (100 µM)	4.5 µL	145 µL	290 µL	435 µL	870 µL
TITANIUM *Taq* DNA Polymerase (50X) (*do not add until ready to aliquot master mix to ligated samples*)	2 µL	64.4 µL	129 µL	193 µL	386 µL
Total	**90 µL**	**2898 µL**	**5796 µL**	**8694 µL**	**17.4 mL**

Add PCR Master Mix to Each Sample

To add the PCR Master Mix to samples:

1. Aliquot 90 µL PCR Master Mix to each sample and control on the PCR plate.

   To avoid contamination, change pipet tips after each dispense.

   For four samples, you may have to tilt the solution basin for the last pickup/dispense to ensure 90 µL picked up in each pipet tip.

   Total volume in each well is 100 µL.
2. Tightly seal the plate.
3. Vortex at high speed for 3 sec; then spin down at 2000 rpm for 30 sec.
4. Keep in the cooling chamber on ice until ready to load onto a thermal cycler.

Load PCR Plate onto a Thermal Cycler

Location

Post-PCR Area

Procedure

To load the plate and run the Cyto PCR program:

1. Transfer the plate 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.

> **IMPORTANT:** **Ensure that the seal is not pulled off any wells when you close the thermal cycler lid.**

4. Run the Cyto PCR program.

> **IMPORTANT:** **PCR protocols for the MJ Tetrad PTC-225 and Applied Biosystems thermal cyclers are different. See Table 4.18 and Table 4.19 on page 48.**
>
> **If using GeneAmp® PCR System 9700 thermal cyclers, be sure the blocks are silver or gold-plated silver. Do NOT use thermal cyclers with aluminum blocks. It is not easy to visually distinguish between silver and aluminum blocks.**

Table 4.18 Cyto PCR Thermal Cycler Program for the GeneAmp® PCR System 9700 (silver or gold-plated silver blocks)

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

Table 4.19 Cyto PCR Thermal Cycler Program for the MJ Tetrad PTC-225

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

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

Run the Gels

When the Cyto PCR program is finished:

1. Remove the plate from the thermal cycler.
2. Spin down at 2000 rpm for 30 sec.
3. Place in a cooling chamber on ice or keep at 4°C.
4. Label a fresh 96-well plate as shown in Figure 4.14 on page 49.
   This plate is referred to as the *gel plate*.
5. Aliquot 3 µL of 2X Gel Loading Dye to each well to be used.
6. Load 10 µL BioNexus Hi-Lo Ladder to the first and last lanes of the gel.
7. Transfer 3 µL of Nsp PCR product from each well in one column only to the corresponding wells of the gel plate.
8. Transfer 3 µL of Sty PCR product from each well in one column only to the corresponding wells of the gel plate.

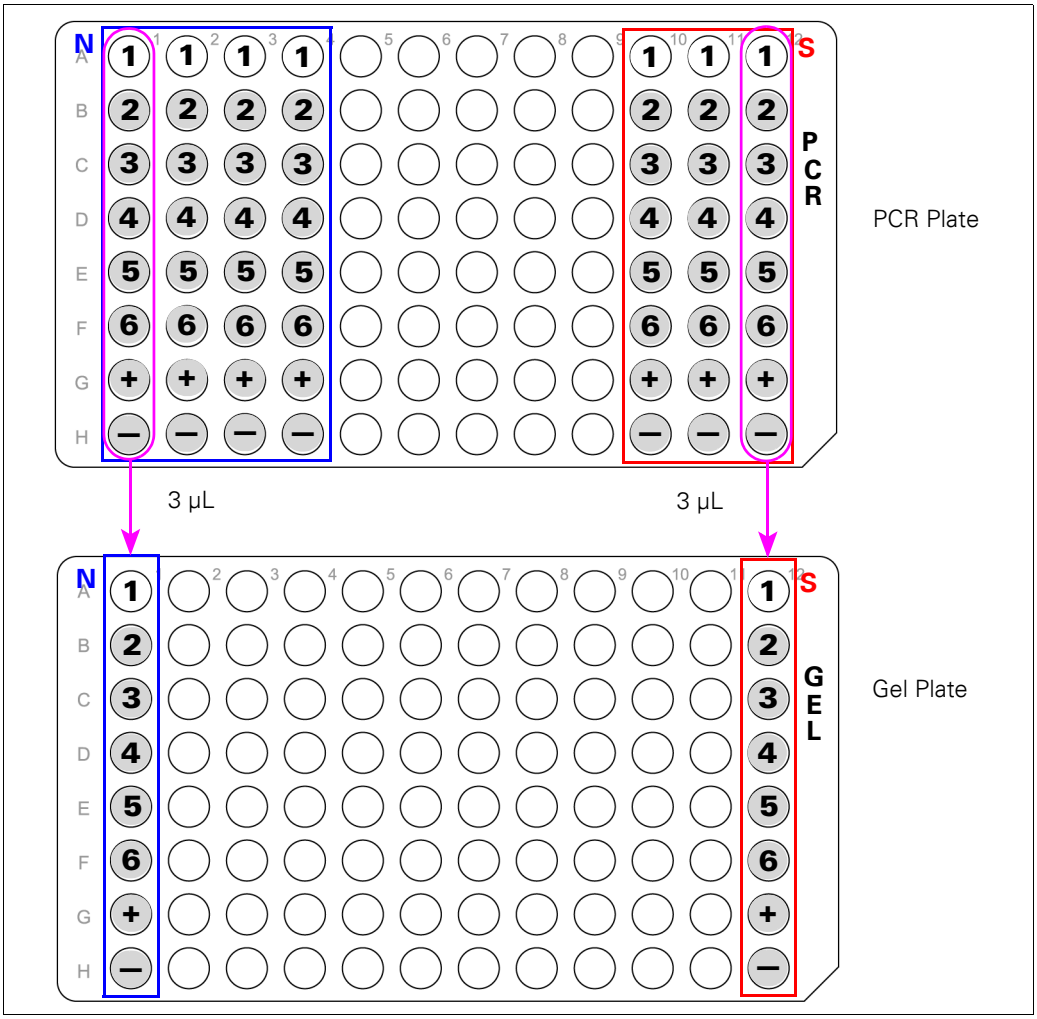


Figure 4.14 Transferring aliquots of each Nsp and Sty PCR product to the gel plate

9. Seal both plates.

10. Vortex the gel plate, then spin them down at 2000 rpm for 30 sec.

11. Load the total volume from each well of the gel plate onto a 2% TBE gel.

12. Run the gel at 120V for 40 min to 1 hr.

13. Verify that the PCR product distribution is between ~250 bp to 1100 bp (Figure 4.15 on page 50).

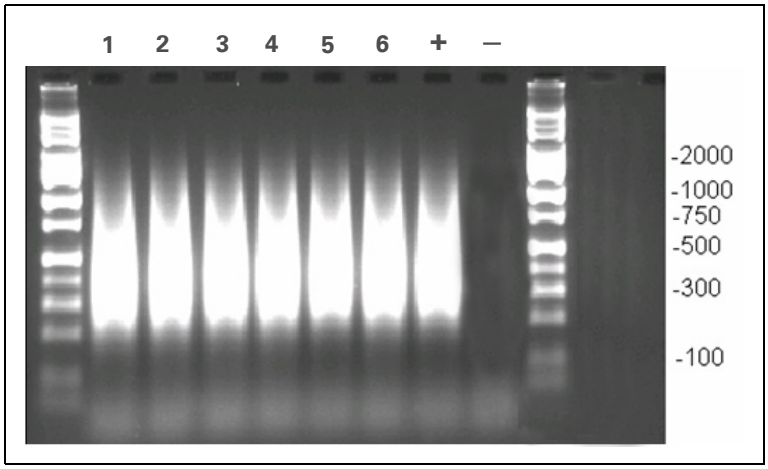


Figure 4.15 Example of PCR products run on 2% TBE agarose gel at 120V for 1 hr. Average product distribution is between ~200to 1100 bp.

What To Do Next

Do one of the following:

- If the PCR has been confirmed, proceed to *Stage 4: PCR Product Purification* on page 51.
- If not proceeding directly to the next stage, seal the plate with PCR product and store at –20°C.

Stage 4: PCR Product Purification

About this Stage

During this stage, you will purify the PCR products by:

- Pooling the Nsp and Sty PCR reactions.
- Adding magnetic beads (Agencourt AMPure®) to each pooled reaction and incubating the mix.
- Adding 75% EtOH to wash DNA.
- Adding Buffer EB to resuspend the beads and elute the DNA.

Location and Duration

- Post-PCR Area
- Hands-on time: 1 hr
- DNA binding to magnetic bead: 15 to 20 min
- EtOH wash: approximately 10 to 20 min
- Elution: 15 to 30 min
- Total time for this stage: approximately 1.5 hr

Input Required from Previous Stage

The input required from *Stage 3: Nsp and Sty PCR* is:

Item
Plate of Nsp and Sty PCR products

Equipment and Consumables Required

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

Table 4.20 Equipment and Consumables Required for *Stage 4: PCR Product Purification*

Quantity	Item
1	Adhesive seals for 96-well plates
1	Microcentrifuge, Eppendorf 5415D with rotor for 24 – tubes, 2.0 mL
1	MagnaRack magnetic stand
1	Marker, fine point, permanent
1	Microtube Foam Insert (for vortexing 2.0 mL tubes)
1	Pipet, single channel P20
1	Pipet, single channel P200
1	Pipet, single channel P1000
As needed	Pipet tips for pipets listed above
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)	Tubes, 2.0 mL Microcentrifuge Safe-Lock Must be round bottom. Do NOT use conical tubes.
1	Tube holder
1	Vortexer (with foam tube adaptor attached)
**** 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 4*.	

Reagents Required

The following reagents are required for this stage.

Table 4.21 Reagents Required for *Stage 4: PCR Product Purification*

Reagent
Elution Buffer (Buffer EB)
75% EtOH (ACS-grade ethanol diluted to 75% using AccuGENE water)
Agencourt AMPure® magnetic beads

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 storage temperature for Agencourt AMPure is 4° C (refrigerator). The pH should be 5.5. If not pH 5.5, discard and use fresh reagent.**
- **To avoid cross-contamination, pipet very carefully when pooling the PCR reactions.**

Prepare the 75% EtOH

Dilute ACS-grade ethanol to 75% using water (AccuGENE).

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. Vortex the plate at high speed for 3 sec; then spin down at 2000 rpm for 30 sec.
3. Mark each 2.0 mL microcentrifuge tube with a sample number such as *1, 2, 3*, *4,* etc.
4. Using a P200 single-channel pipet, transfer all 7 aliquots of each sample to the appropriately marked 2.0 mL tube (Figure 4.16 on page 54).

   Do not pool the negative control. Discard.

   IMPORTANT: Use round-bottom tubes only. Do NOT use conical tubes. Change pipet tips after pooling each sample.

Sty PCR wells (3):	100 µL from each well	= 300 µL
Nsp PCR wells (4):	100 µL from each well	= 400 µL
Total Volume in Each 2.0 mL Microcentrifuge Tube		**= 700 µL/tube**

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

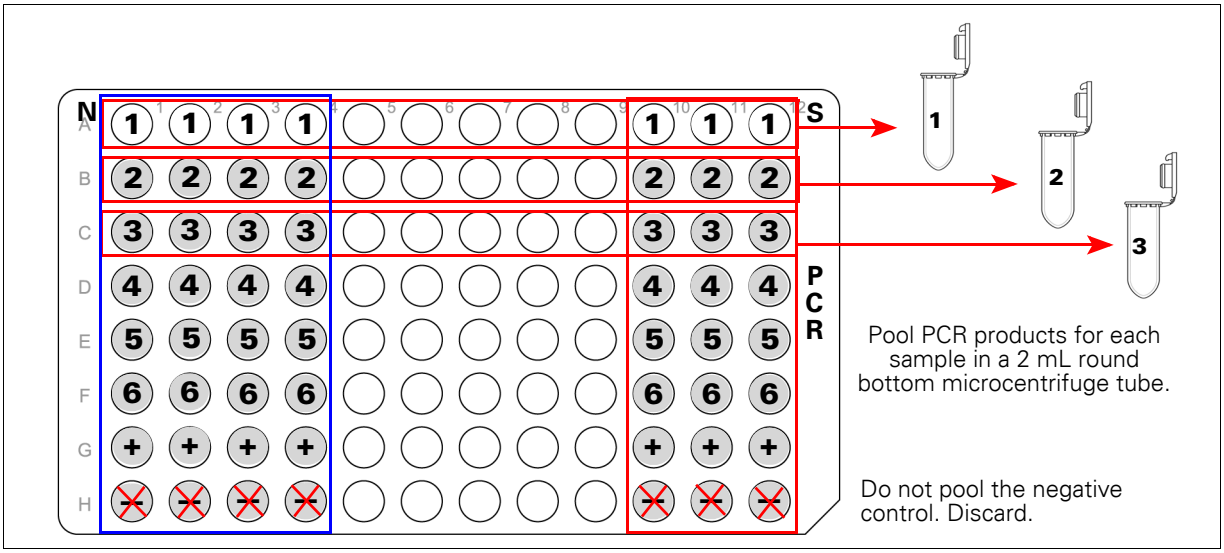


Figure 4.16 Pooling PCR products

Purify the Pooled PCR Products

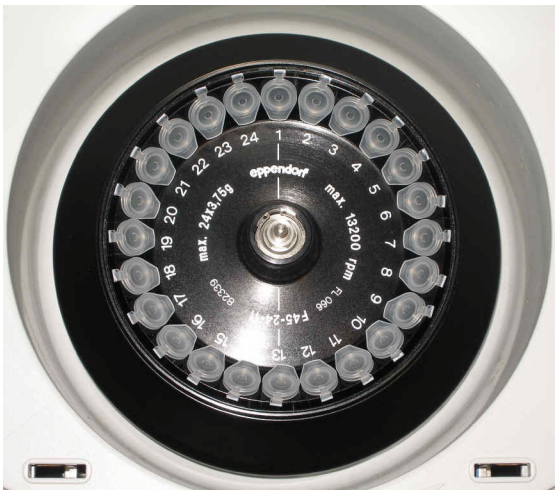
Add Agencourt AMPure Magnetic Beads and Incubate

To add magnetic beads and incubate:

1. Thoroughly mix the magnetic bead stock by vigorously shaking the bottle.
   Examine the bottom of the bottle and ensure that the solution appears homogenous.
2. Optional: Pour magnetic beads into a 50 mL conical tube.
3. Aliquot 1 mL of magnetic beads to each pooled sample.

> **IMPORTANT: The solution is viscous and sticky. Pipet carefully to ensure that you aspirate and dispense 1 mL.**
>
> **Thorough mixing is critical to ensure that the PCR products bind to the beads.**

4. Securely cap each tube and mix well by inverting 10✕.
5. Incubate at room temperature for 10 min.
   During incubation, the DNA binds to the magnetic beads.
6. Load the tubes – cap hinge facing out – onto the microcentrifuge and spin for 3 min at maximum speed (16,100 rcf; Figure 4.17 on page 55).
7. Place the tubes on the magnetic stand (Figure 4.18 on page 55).
8. Leaving the tubes in the rack, pipet off the supernatant without disturbing the bead pellet and discard.



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

Figure 4.17 Position tubes with cap hinges out

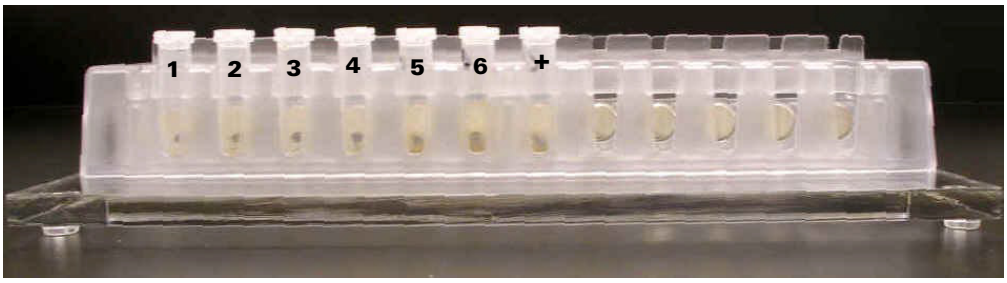
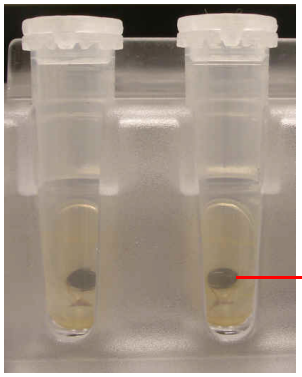


Figure 4.18 Samples with magnetic beads on MagnaRack



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

Figure 4.19 Bead pulled to back and side of tube in magnetic stand

Add Ethanol

To add ethanol:

1. Using a P1000 pipet, add 1.5 mL of 75% EtOH to each tube.
2. Cap the tubes and load them into the foam tube adaptor (Figure 4.20).
   Fully insert tubes into the foam to ensure they are secure. Space tubes adequately to balance.
3. Vortex at 75% power for 2 min.
4. Centrifuge the tubes for 3 min at maximum speed (hinges facing out; 16,100 rcf).
5. Place the tubes on the magnetic stand.

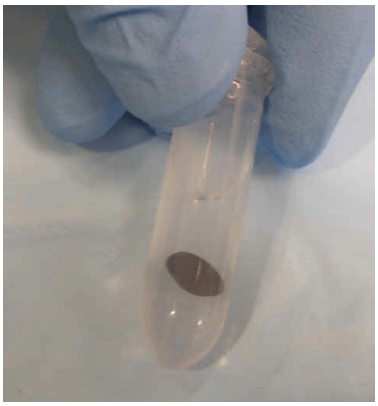



Figure 4.20 Resuspended bead clump and vortexer with foam tube adaptor

6. Leaving the tubes in the rack, pipet off the supernatant without disturbing the bead pellet and discard.
7. Spin the tubes for 30 sec at maximum speed (hinges facing out; 16,100 rcf).
8. Place the tubes back on the magnetic stand.
9. Using a P20 pipet, remove remaining drops of EtOH from the bottom of each tube.

> **IMPORTANT:** **Be careful not to disturb or break off any of the bead pellet.**

10. Allow the remaining EtOH to evaporate by leaving the tubes uncapped at room temperature for 15 min.

Add Buffer EB

To add Buffer EB to each sample:

1. Using a P200 pipet, add 55 µL of Buffer EB to each tube.
2. Cap the tubes and load them into the foam tube adaptor.
3. Vortex at 75% power for 10 min.
   Vortexing will resuspend the magnetic beads.
4. Examine each tube to ensure that the beads are resuspended in a homogeneous slurry.

> **IMPORTANT:** **If the beads are not fully resuspended, flick the tube to dislodge the pellet, and vortex an additional 2 min. Re-examine.**

5. Centrifuge the tubes for 5 min at maximum speed (hinges facing out; 16,100 rcf).
6. Place the tubes on the magnetic stand for 5 min.
   The magnetic beads are pulled to the side of the tube (Figure 4.19).

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

   If all of the beads have not been pulled to the side of the tubes, leave the tubes on the stand an additional 3 min.

> **NOTE: The eluate will appear yellowish. If you open the cap and look directly into the tube, you will see that the eluate is clear.**

8. Transfer 47 µL of eluted sample to the appropriate well on a fresh 96-well plate (Figure 4.21 on page 57).

   Brown residue on pipet tips is OK.

9. Tightly seal the plate.

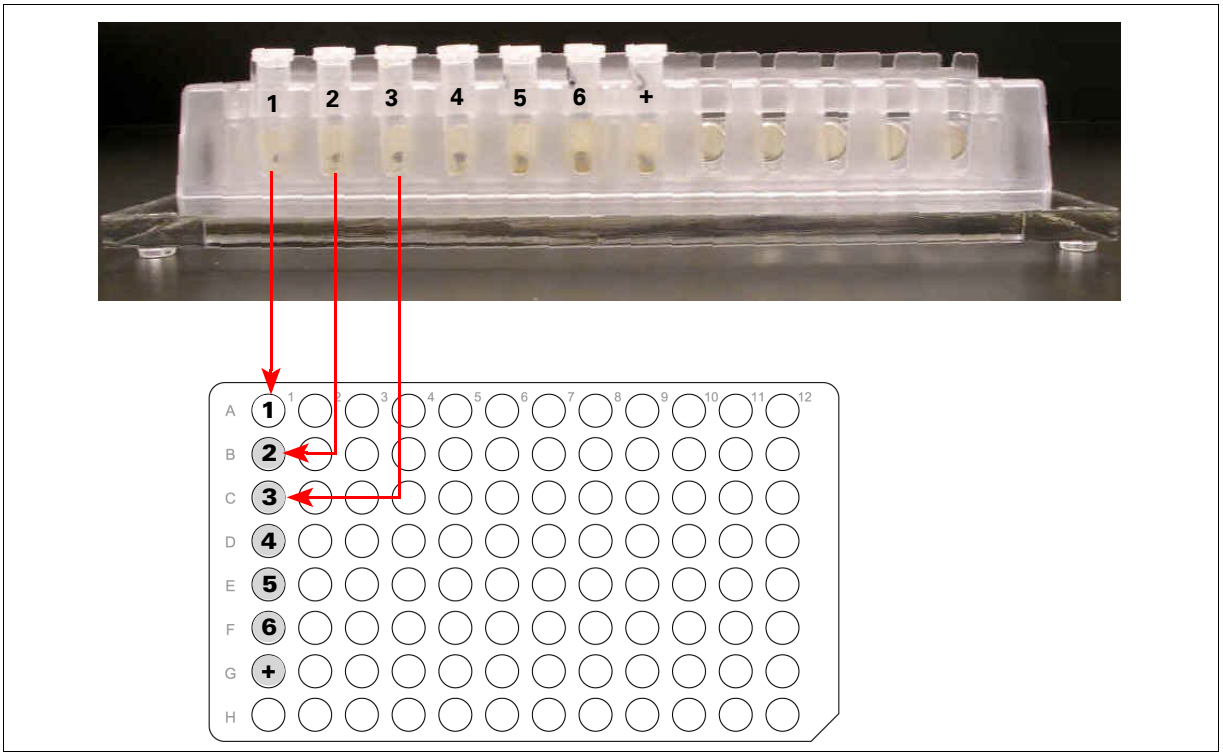


Figure 4.21 Transferring each purified sample to a fresh 96-well plate.

What To Do Next

Proceed to *Stage 5: Quantitation* on page 58. Here you will remove 2 µL from each sample for an OD measurement.

Stage 5: Quantitation

About this Stage

During this stage, you will quantitate each sample.

Location and Duration

- Post-PCR Room
- Hands-on time: 20 min

Input Required from Previous Stage

Input required from *Stage 4: PCR Product Purification* is:

Item
Pooled, purified PCR products (47 µL each sample)

Equipment and Consumables Required

The following equipment and consumables are required for this stage.

Table 4.22 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
As needed	Pipette tips for pipets 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	Optional: Tube, 50 mL conical or solution basin
** **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 4*.	

Reagents Required

The following reagents are required for this stage.

Table 4.23 Reagents Required for *Stage 5: Quantitation*

Reagent
Water, AccuGENE molecular biology-grade

Important Information About This Stage

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

IMPORTANT:

- **The accuracy of the OD measurement is critical. Carefully follow this procedure and be sure the OD measurement is within the quantitative linear range of the instrument.**
- **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 10 min before use.

Prepare Your Work Area

To prepare the work area:

1. Place the following on the bench top:
   - Optional: conical tube or solution basin
   - Water
   - UV or 96-well plate
2. Spin down the purified samples at 2000 rpm for 30 sec, and put in a plate holder.

Procedure if Using a Microplate Spectrophotometer

Prepare Diluted Aliquots of Purified Sample

IMPORTANT: The P20 pipet must be accurate to within ± 5%.

To prepare diluted aliquots of the purified samples:

1. Using a P200 pipet, 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 column (Figure 4.22 below).
3. Using a P20 pipet:
   A. Transfer 2 µL of each purified sample to the corresponding well of the UV plate.
   B. Pipette up and down 2 times to ensure that all of the sample is dispensed.
   The result is a 100-fold dilution.
4. Do one of the following to mix the samples:
   - Set a P200 pipet to 170 µL and pipet up and down 5 times.
   - Seal the plate, vortex, and spin down at 2000 rpm for 30 sec.

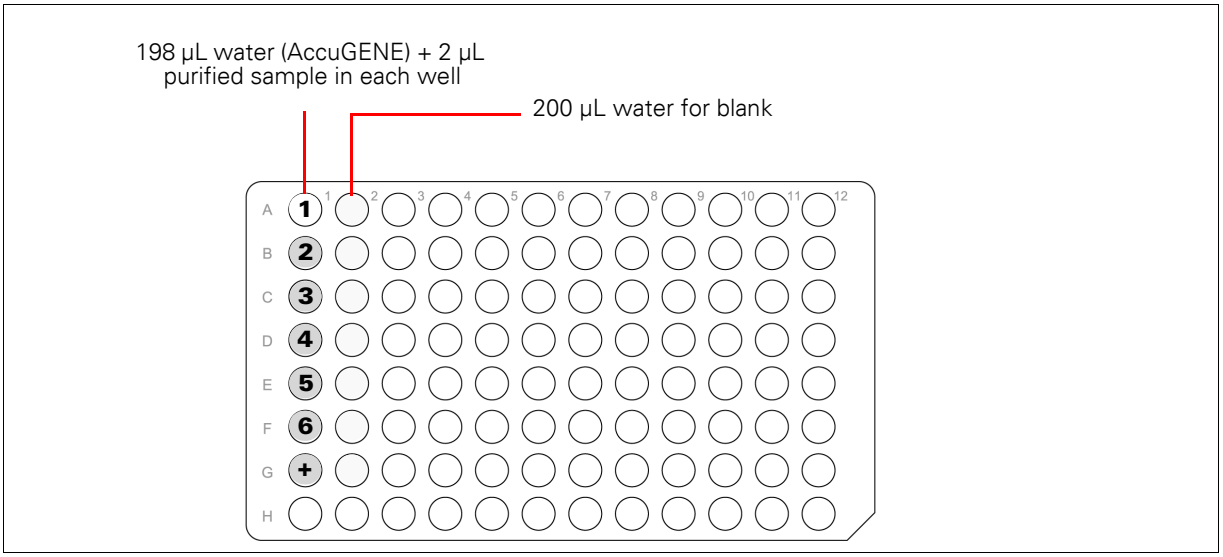


Figure 4.22 UV plate layout.

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 pipet must be accurate to within ± 5%.**

To prepare diluted aliquots of the purified samples:

1. Using a P20 pipet, aliquot 18 µL of water to the corresponding wells of a 96-well plate.
2. Using a P20 pipet:
   A. Transfer 2 µL of each purified sample to the corresponding well of the 96-well plate.
   B. Pipette 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 pipet to 17 µL and pipet up and down 5 times.
   - Seal the plate, vortex, and spin down at 2000 rpm for 30 sec.

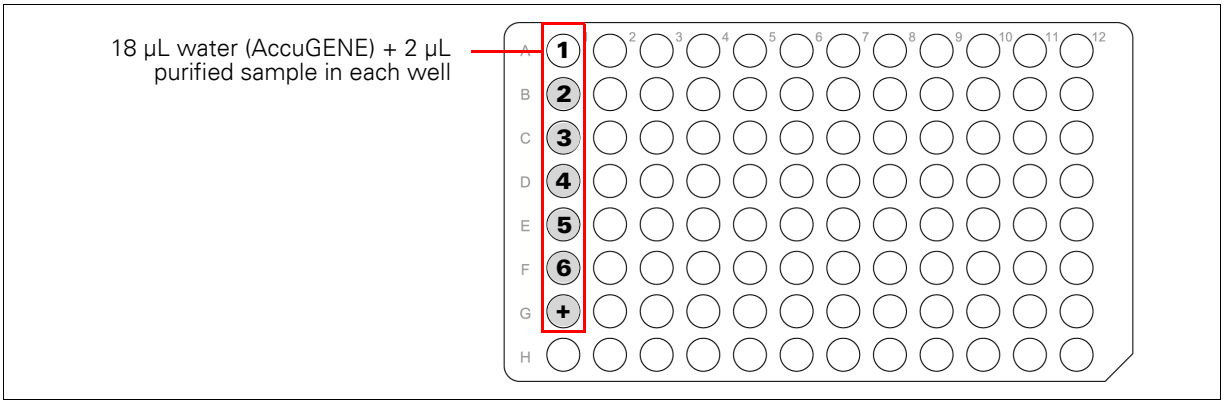


Figure 4.23 96-well plate layout for NanoDrop

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 = OD X 10

Assess the OD Readings

Acceptable OD Range and DNA Yield

- An acceptable OD should fall within this range: 1.1 to 1.4.

  DNA yield equivalent = 5.5 to 7.0 µg/µL

  This OD range is based on the use of a conventional UV spectrophotometer plate reader and assumes 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 > 0.2:

    A. Centrifuge the sample for 5 min.

    B. Place on the MagnaRack and pipet off the eluate.

    C. Requantitate the sample.

    D. If the OD320 reading is now ≤ 0.1, proceed.

If your OD readings are not within the acceptable range, refer to *OD Troubleshooting Guidelines on page 99*.

What To Do Next

Do one of the following:

- Proceed immediately to *Stage 6: Fragmentation* on page 63.
- If not proceeding immediately to the next step, seal the plate of purified samples, and store at –20°C.

Stage 6: Fragmentation

About this Stage

During this stage the purified samples are fragmented using Fragmentation Reagent (enzyme) by:

- Preparing a Fragmentation Master Mix.
- Quickly adding the mix to each sample.
- Placing the samples onto a thermal cycler and running the Cyto Fragment program.
- Checking each reaction on a gel.

Location and Duration

- Post-PCR Area
- Hands-on time: 30 min
- Cyto Fragment thermal cycler program time: 1 hr

Input Required from Previous Stage

The input required from *Stage 5: Quantitation* is:

Item
Plate of purified samples that have been quantitated.

Equipment and Consumables Required

The following equipment and consumables are required for this stage.

Table 4.24 Equipment and Consumables Required for *Stage 6: Fragmentation*

Quantity	Item
As required	Adhesive seals for 96-well plates
1	Centrifuge, plate
1	Cooler, chilled to –20°C
1	Cooling chamber, single, chilled to 4°C on ice (do not freeze)
1	Ice bucket, filled with ice
1	Marker, fine point, permanent
1	Mini centrifuge (microfuge)
1	Pipet, single channel P20
1	Pipet, single channel P100
1	Pipet, single channel P1000
1	Pipet, 8-channel P20 (accurate to within ± 5%)
As needed	Pipet tips for pipets listed above
1	Plate, Bio-Rad 96-well
1	Thermal cycler
1	Tube, Eppendorf 1.5 mL
1	Tubes, strip of 8 with caps, 0.2 mL
1	Vortexer
**** 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 4*.	

Reagents Required

The following reagents are required for this stage.

Table 4.25 Reagents Required for *Stage 6: Fragmentation*

Reagent
Fragmentation Buffer (10X)
Fragmentation Reagent (enzyme; DNase I)
AccuGENE® water, molecular biology-grade

Gels and Related Materials Required

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

Table 4.26 Gels and Related Materials Required

Item/Reagent
4% TBE Gel (precast or house-made)
DNA Markers, 5 µL per well
Gel loading solution

Important Information About This Stage

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

! IMPORTANT:

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

About the Fragmentation Reagent (Enzyme)

- 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.
  - Dilute immediately prior to use.
  - Keep at –20°C until ready to use. Transport and hold in a –20°C cooler. Return to the cooler immediately after use.
  - Spin down so that the contents of the tube are uniform.
  - Perform all steps rapidly and without interruption.
- This enzyme is **sticky**, and may adhere to the walls of some microfuge tubes and 96-well plates.
- 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 pipet tip.
  - Avoid excess solution on the outside of the pipet tip.

Prepare the Reagents, Consumables and Other Components

Thaw Reagents

Thaw the Fragmentation Buffer (10X) on ice.

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

Setup Your Work Area

To setup your work area (Figure 4.24):

1. Place a cooling chamber and the water on ice.
2. Place the plate of purified, quantitated samples in the cooling chamber.
3. Prepare the Fragmentation Buffer as follows:
   A. Vortex 3 times, 1 sec each time.
   B. Pulse spin for 3 sec.
   C. Place in the cooling chamber.
4. Label the 1.5 mL Eppendorf tube *Frag* and place in the cooling chamber.

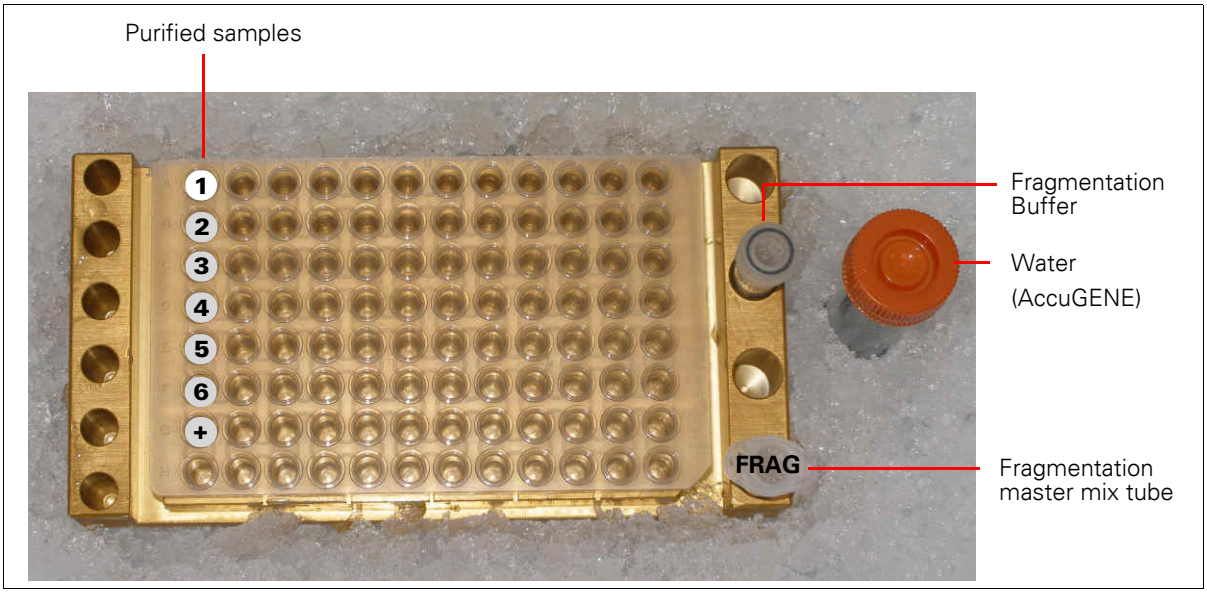


Figure 4.24 Setup for fragmentation.

Preheat the Thermal Cycler Block

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

To preheat the thermal cycler:

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

Prepare the Samples for Fragmentation

Add Fragmentation Buffer to Samples

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

To prepare the samples for Fragmentation:

1. Add 5 µL of Fragmentation Buffer to each sample.
2. Check your pipet tips each time to ensure that all of the buffer has been dispensed.
   The total volume in each well is now 50 µL.

Prepare the Fragmentation Master Mix

IMPORTANT: **The concentration of stock Fragmentation Reagent (U/µL) may vary from lot-to-lot. Therefore, read the label on the tube and record the stock concentration before diluting this reagent.**

To prepare the Fragmentation Master Mix:

The Fragmentation Reagent must be diluted to 0.1 U/µL in the master mix.

1. Read the Fragmentation Reagent tube label and record the concentration.
2. Dilute the Fragmentation Reagent to 0.1U/µL as described below using the appropriate recipe from Table 4.27.
3. Add the appropriate volume of water and Fragmentation Buffer to the *Frag* tube on ice.
4. Allow to cool on ice for 5 min.

Table 4.27 Fragmentation Master Mix

Reagent	Fragmentation Reagent Concentration				
	2 U/µL	2.25 U/µL	2.5 U/µL	2.75 U/µL	3 U/µL
AccuGENE water	136.00 µL	154.00 µL	172.00 µL	190.00 µL	208.00 µL
10X Fragmentation Buffer	16.00 µL	18.00 µL	20.00 µL	22.00 µL	24.00 µL
Fragmentation Reagent (enzyme)	8.00 µL	8.00 µL	8.00 µL	8.00 µL	8.00 µL
Total	**160 µL**	**180 µL**	**200 µL**	**220 µL**	**240 µL**

5. Remove the Fragmentation Reagent (enzyme) from the freezer and:
   A. Immediately pulse spin for 3 sec.
      Spinning is required because the reagent tends to cling to the top of the tube, making it warm quicker.
   B. Immediately place in a cooler.
6. Add the appropriate volume of Fragmentation Reagent.
7. Vortex the master mix at high speed 3 times, 1 sec each time.
8. Pulse spin for 3 sec and immediately place on ice.
9. Proceed immediately to the next set of steps, *Add Fragmentation Master Mix to the Samples*.

Add Fragmentation Master Mix to the Samples

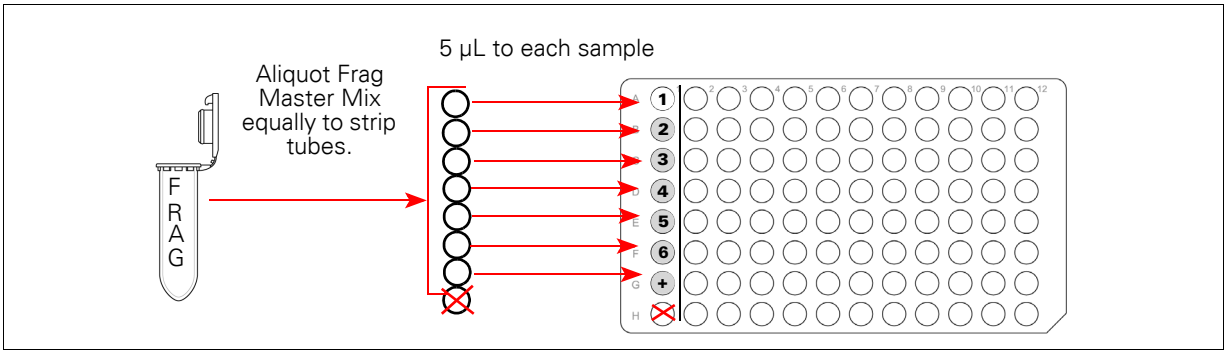


Figure 4.25 Adding Fragmentation Master Mix to samples

To add Fragmentation Master Mix to the samples:

1. Quickly and on ice, aliquot out the Fragmentation Master Mix equally to the strip tubes (Figure 4.25).
2. Spin down the strip tubes.
3. Using an 8-channel P20 pipet, transfer 5 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 5 µL to each sample.

> **IMPORTANT:** **To help ensure the same amount of fragmentation for each sample, add the master mix to the samples as quickly as possible.**

Sample with Fragmentation Buffer	50 µL
Fragmentation Master Mix	5 µL
Total	**55 µL**

4. Remove and discard any remaining Fragmentation Master Mix.

   Never re-use Fragmentation Master Mix.
5. Tightly seal the plate.
6. Vortex at high speed for 3 sec; then spin down for 30 sec.
7. Immediately load the samples onto the pre-heated block of the thermal cycler (37°C) and run the Cyto Fragment program (Table 4.28).

> **IMPORTANT:** **Ensure that the seal is not pulled off any wells when you close the thermal cycler lid.**

Table 4.28 Cyto Fragment Thermal Cycler Program

Cyto Fragment Program	
Temperature	**Time**
37°C	35 min
95°C	15 min
4°C	Hold

What To Do Next

Proceed directly to the next stage. While the labeling reaction is taking place, check the fragmentation reaction by running gels as described under *Check the Fragmentation Reaction by Running a Gel* on page 70.

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 Cyto Fragment program is finished:
   A. Remove the samples from the thermal cycler.
   B. Spin down for 30 sec, and place in a cooling chamber on ice.
2. Remove 2.0 µL of each sample and place in a 96-well plate.
3. Add 4 µL gel loading dye to each sample.
4. Load the samples onto the gel.
5. Load 10 µL BioNexus All Purpose Hi-Lo Ladder to the first and last lanes.
6. Run the samples on a 4% TBE gel at 120V for 30 min to 1 hr.
7. Inspect the gel and compare it against the example shown in Figure 4.26 below.

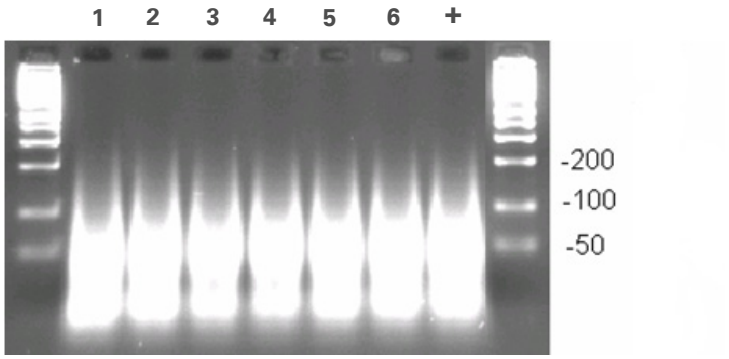


Figure 4.26 Typical example of fragmented PCR products run on 4% TBE agarose gel at 120V for 30 min to 1 hr. Average fragment size is < 180 bp.

Stage 7: Labeling

About this Stage

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

- Preparing a Labeling Master Mix.
- Adding the mix to each sample.
- Placing the samples onto a thermal cycler and running the Cyto Label program.

Location and Duration

- Post-PCR Area
- Hands-on time: 30 min
- Cyto Label thermal cycler program time: 4.25 hr

Input Required from Previous Stage

The input required from *Stage 6: Fragmentation* is:

Item
Fragmented samples

Equipment and Consumables Required

The following equipment and consumables are required for this stage.

Table 4.29 Equipment and Consumables Required for *Stage 7: Labeling*

Quantity	Item
As required	Adhesive seals for 96-well plates
1	Centrifuge, plate
1	Cooler, chilled to –20°C
1	Cooling chamber, single, chilled to 4°C on ice (do not freeze)
1	Ice bucket, filled with ice
1	Marker, fine point, permanent
1	Mini centrifuge (microfuge)
1	Pipet, single channel P200
1	Pipet, single channel P1000
1	Pipet, 8-channel P20 (accurate to within ± 5%)
As needed	Pipet tips for pipets listed above
1	Thermal cycler
1	Tube, centrifuge 1.5 mL
1	Vortexer
**** 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 4*.	

Reagents Required

The following reagents are required for this stage.

Table 4.30 Reagents Required for *Stage 7: Labeling*

Reagent
DNA Labeling Reagent (30 mM)
Terminal Deoxynucleotidyl Transferase (TdT; 30 U/µL)
Terminal Deoxynucleotidyl Transferase Buffer (TdT Buffer; 5X)

Prepare the Reagents, Consumables and Other Components

Thaw Reagents

Thaw the following reagents on ice:

- 5X TdT Buffer
- DNA Labeling Reagent

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

Prepare Your Work Area

To prepare the work area:

1. Place a cooling chamber on ice (Figure 4.27 on page 72).
2. Prepare the reagents as follows:
   A. Vortex each reagent at high speed 3 times, 1 sec each time.
   B. Pulse spin for 3 sec; then place in the cooling chamber.
3. Label the 1.5 mL centrifuge tube *LBL*, and place in the cooling chamber.

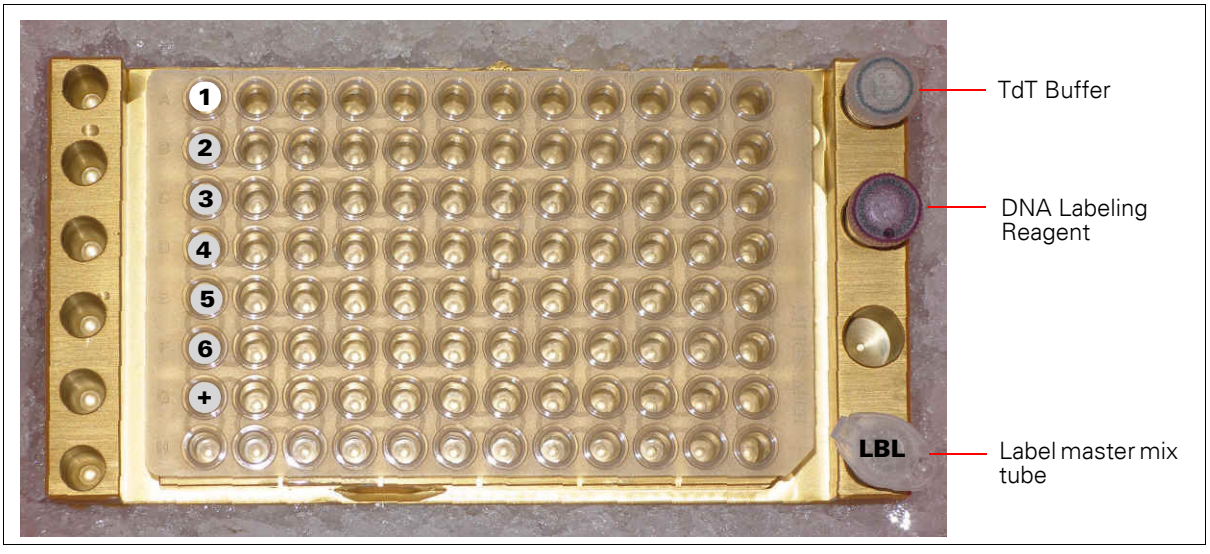


Figure 4.27 Setup for labeling

Preheat the Thermal Cycler Block

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

To preheat the thermal cycler block:

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

Prepare the Labeling Master Mix

Preparation

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

To prepare the Labeling Master Mix:

1. Add the following to the 1.5 mL centrifuge tube on ice using the volumes shown in Table 4.31 on page 73:
   - 5X TdT Buffer
   - DNA Labeling Reagent
2. Remove the TdT enzyme from the freezer and immediately place in the cooler.
3. Pulse spin the enzyme for 3 sec; then immediately place back in the cooler.
4. Add the TdT enzyme to the master mix.
5. Vortex the master mix at high speed 3 times, 1 sec each time.
6. Pulse spin for 3 sec.
7. Immediately proceed to the next set of steps, *Add the Labeling Master Mix to the Samples*.

Table 4.31 Labeling Master Mix

Reagent	1 Sample	4 Samples (15% extra)	8 Samples (15% extra)	12 Samples (15% extra)	24 Samples (15% extra)
TdT Buffer (5X)	14.0 µL	64.4 µL	128.8 µL	193.2 µL	386.4 µL
DNA Labeling Reagent (30 mM)	2.0 µL	9.2 µL	18.4 µL	27.6 µL	55.2 µL
TdT enzyme (30 U/µL)	3.5 µL	16.1 µL	32.2 µL	48.3 µL	96.6 µL
Total	**19.5 µL**	**89.5 µL**	**179.4 µL**	**269.1 µL**	**538.2 µL**

Add the Labeling Master Mix to the Samples

To add the Labeling Master Mix to the samples:

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

1. Optional: If processing 16 or more samples, aliquot the Labeling Master Mix equally into strip tubes.
2. Using a P20 single or 8-channel pipet:
   A. Aliquot 20 µL of Labeling Master Mix to each sample.
   B. Pipet up and down one time to ensure that all of the mix is added to the samples.

Fragmented DNA (less 2.0 µL for gel analysis)	53.0 µL
Labeling Mix	19.5 µL
Total	**72.5 µL**

3. Tightly seal the plate.
4. Vortex at high speed for 3 sec; then spin down for 30 sec.
5. Place on the pre-heated thermal cycler block and run the Cyto Label program.

> **IMPORTANT:** **Ensure that the seal is not pulled off any wells when you close the thermal cycler lid.**

6. When the Cyto Label program is finished, remove the plate from the thermal cycler and spin down for 30 sec.

Table 4.32 Cyto Thermal Cycler Program

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

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

About this Stage

During this stage, each sample will be hybridized onto a Genome-Wide Human SNP Array 6.0 by:

- Preparing a Hybridization Master Mix and adding it to each sample.
- Denaturing the samples on a thermal cycler.
- Loading each sample onto a Genome-Wide Human SNP Array 6.0.
- Placing the arrays into a hybridization oven at 50°C for 16 to 18 hr.

Location and Duration

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

Input Required from Previous Stage

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

Item
Labeled samples

Equipment and Consumables Required

The following equipment and consumables are required for this stage.

IMPORTANT: **Increased variability in SNP 6.0 Cytogenetics Copy Number Assay performance has been observed in GeneChip® Hybridization Oven 640 models (P/N 800138 or 800189) manufactured prior to 2001. Check the serial number of your hybridization oven(s). If the serial numbers are 11214 or lower, contact Affymetrix for an upgrade.**

The following table lists the equipment and consumables required.

Table 4.33 Equipment and Consumables Required for *Stage 8: Target Hybridization*

Quantity	Item
1	Adhesive seals for 96-well plates
1	Cooling chamber, chilled to 4°C on ice (do not freeze)
One array per sample	Genome-Wide Human SNP Array 6.0
1	GeneChip® Hybridization Oven 640 or 645
1	Ice bucket, filled with ice
1	Pipet, single channel P200
1	Pipet, single channel P1000
As needed	Pipet tips for pipets listed above
1	Solution basin, 55 mL
1	Thermal cycler
2 per array	Tough-Spots®
1	Tube, centrifuge 50 mL
1	Vortexer
**** 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 4*.	

Reagents Required

The following reagents are required for this stage.

Table 4.34 Reagents Required for *Stage 8: Target Hybridization*

Reagent
Denhardt's Solution (50X)
DMSO (100%)
EDTA (0.5 M)
Herring Sperm DNA (HSDNA; 10 mg/mL)
Human Cot-1 DNA® (1 mg/mL)
MES Hydrate SigmaUltra
MES Sodium Salt
Tetramethyl Ammonium Chloride (TMACL; 5M)
Tween-20, 10%
Oligo Control Reagent (OCR), 0100

Important Information About This Stage

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

IMPORTANT:

- **It is critical that the samples remain on the thermal cycler at 49°C after denaturation and while being loaded onto arrays.**
- **About DMSO:**

  When adding to the Hybridization Master Mix, pipet DMSO into the middle of the tube. Do not touch the sides of the tube as the DMSO can leach particles out of the plastic which, in turn, may cause high background.

  DMSO is light sensitive and must be stored in a dark glass bottle. Do not store in a plastic container.
- **Be sure to equilibrate the arrays to room temperature; otherwise, the rubber septa may crack and the array may leak.**
- **An accurate hybridization temperature is critical for this assay. Therefore, we recommend that your hybridization ovens be serviced at least once per year to ensure that they are operating within specifications.**
- **Gloves, safety glasses, and lab coats must be worn when preparing the Hybridization Master Mix.**
- **Consult the appropriate MSDS for reagent storage and handling requirements.**

Prepare the Reagents, Consumables and Other Components

Prepare a 12X MES Stock Solution

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

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

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

To prepare 500 mL of 12X MES Stock Solution (1.25 M MES, 0.89 M [Na^+]):

1. Combine:
   - 35.2 g MES hydrate
   - 96.65 g MES sodium salt
   - 400 mL AccuGENE® water
2. Mix and adjust volume to 475 mL.
3. Test the pH.

   The pH should be between 6.5 and 6.7.
4. Adjust the pH so it falls between 6.5 and 6.7.
5. Adjust the volume to 500 mL.
6. Filter the solution through a 0.2 µm filter.
7. Protect from light using aluminum foil and store at 4°C.

Preheat the Hybridization Ovens

To preheat the hybridization ovens:

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

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

Prepare the Samples

To 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.
2. Vortex at high speed for 3 sec; then spin down for 30 sec.
3. Place in a cooling chamber on ice.

Preheat the Thermal Cycler Lid

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

Prepare the Arrays

To prepare the arrays:

1. Unwrap the arrays and place on the bench top, septa-side up.
2. Mark the front or back of each array with a designation that will identify which sample is loaded onto each array (Figure 4.28).
3. Allow the arrays to warm to room temperature on the bench top 10 to 15 min.
4. Insert a 200 µL pipet tip into the upper right septum of each array.

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

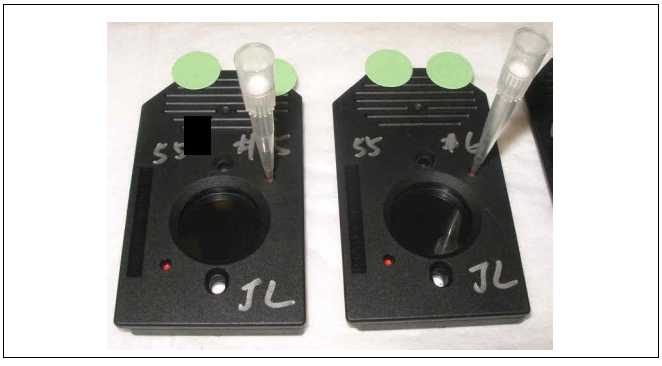


Figure 4.28 Arrays prepared for sample loading

Prepare the Hybridization Master Mix

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

Preparing Fresh Hybridization Master Mix

To prepare the Hybridization Master Mix:

1. To the 50 mL centrifuge tube, add the appropriate volume of each reagent in the order shown in Table 4.35.

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

Table 4.35 Hybridization Master Mix

Reagent	1 Sample	4 Samples (15% extra)	8 Samples (15% extra)	12 Samples (15% extra)	24 Samples (15% extra)
MES (12X; 1.25 M)	12.0 µL	55.2 µL	110.4 µL	165.6 µL	331.2 µL
Denhardt's Solution (50X)	13.0 µL	59.8 µL	119.6 µL	179.4 µL	358.8 µL
EDTA (0.5 M)	3.0 µL	13.8 µL	27.6 µL	41.4 µL	82.8 µL
HSDNA (10 mg/mL)	3.0 µL	13.8 µL	27.6 µL	41.4 µL	82.8 µL
OCR, 0100	2.0 µL	9.2 µL	18.4 µL	27.6 µL	55.2 µL
Human Cot-1 DNA® (1 mg/mL)	3.0 µL	13.8 µL	27.6 µL	41.4 µL	82.8 µL
Tween-20 (3%)	1.0 µL	4.6 µL	9.2 µL	13.8 µL	27.6 µL
DMSO (100%)	13.0 µL	59.8 µL	119.6 µL	179.4 µL	358.8 µL
TMACL (5 M)	140.0 µL	644.0 µL	1288.0 µL	1932.0 µL	3864.0 µL
Total	**190 µL**	**874 µL**	**1748 µL**	**2622 µL**	**5244 µL**

Using Premixed Hybridization Master Mix

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

To prepare stored Hybridization Master Mix:

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

Add Hybridization Master Mix and Denature

To add Hybridization Master Mix and denature the samples:

1. Optional: pour the Hybridization Master Mix into a solution basin.
2. Using a P200 pipet, add 190 µL of Hybridization Master Mix to each sample.
   Total volume in each well is 262.5 µL.
3. Tightly seal the plate.
4. Vortex the plate for 30 sec; then spin down for 30 sec.
5. Place the plate onto the thermal cycler and run the Cyto Hyb program.

> **IMPORTANT:** **Ensure that the seal is not pulled off any wells when you close the thermal cycler lid.**

Table 4.36 Cyto Hyb Thermal Cycler Program

Cyto Hyb Program	
Temperature	**Time**
95°C	10 min
49°C	Hold

Load the Samples onto Arrays

To load the samples onto arrays:

1. When the thermal cycler reaches 49°C, open the lid.
2. If > 7 samples, cut and remove the film from one column of samples.
   Leave the remaining wells covered. Keeping these wells covered will help to prevent cross-contamination and evaporation.
3. Using a P200 pipet, remove 200 µL of the first sample and immediately inject it into an array.
4. Cover the septa on the array with a Tough-Spot (Figure 4.29).
   Press firmly to ensure a tight seal to prevent evaporation and leakage.

Figure 4.29 Loading samples onto arrays

5. When 4 arrays are loaded and the septa are covered:
   A. Load the arrays into an oven tray evenly spaced.
   B. Immediately place the tray into the hybridization oven.

      Do not allow loaded arrays to sit at room temperature for more than approximately 1 min. 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 a hybridization oven.

   All samples should be loaded within 30 min.
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 ovens for 16 to 18 hr at 50°C and 60 rpm. This temperature is optimized for this product, and should be stringently followed.**

Chapter 5

WASHING, STAINING AND SCANNING ARRAYS

This chapter describes how to wash, stain and scan the Affymetrix® Genome-Wide Human SNP Array 6.0. The instruments that you will use include the:

- Fluidics Station 450 to wash and stain arrays
- GeneChip® Scanner 3000 7G to scan arrays

Once the arrays are scanned, the array image (.dat file) is ready for analysis.

Equipment and Consumables Required

The following equipment and consumables are required for washing, staining and scanning arrays.

Table 5.1 Equipment and Consumables Required for Washing, Staining and Scanning Arrays

Item	Vendor	Part Number
GeneChip® Scanner 3000 7G	Affymetrix	—
GeneChip® Fluidics Station 450	Affymetrix	—
One of the following instrument control applications: • Affymetrix GeneChip® Operating Software • Affymetrix GeneChip® Command Console	Affymetrix	—
Sterile, RNase-free, microcentrifuge vials, 1.5 mL	USA Scientific	1415-2600 (or equivalent)
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	USA Scientific	9185-0000

Reagents Required

The following reagents are required for washing and staining arrays. These reagents are recommendations, and have been tested and evaluated by Affymetrix scientists. Information and part numbers listed are based on U.S. catalog information.

Table 5.2 Reagents Required for Washing and Staining Arrays

Reagent	Vendor	Part Number
AccuGENE® Molecular Biology-Grade Water, 1 L	Lonza	51200
Distilled water	Invitrogen	15230147
20X SSPE (3 M NaCl, 0.2 M NaH2PO4, 0.02 M EDTA)	Lonza	51214
Anti-streptavidin antibody (goat), biotinylated (reconstitute according to product instructions)	Vector Laboratories	BA-0500
R-Phycoerythrin Streptavidin	Molecular Probes	S-866
10% Surfact-Amps® 20 (Tween-20)	Pierce Chemical	28320
Bleach (5.25% Sodium Hypochlorite)	VWR Scientific	21899-504 (or equivalent)
Denhardt's Solution, 50X concentrate	Sigma-Aldrich	D2532
MES hydrate	Sigma-Aldrich	M5287
MES Sodium Salt	Sigma-Aldrich	M5057
5 M NaCl, RNase-free, DNase-free	Ambion	9760G

Reagent Preparation

Prepare the following buffers and antibody:

- Wash A (Non-Stringent Wash Buffer)
- Wash B (Stringent Wash Buffer)
- Anti-streptavidin Antibody (0.5 mg/mL)
- MES Stock Buffer
- Array Holding Buffer

Wash A: Non-Stringent Wash Buffer

(6X SSPE, 0.01% Tween 20)

For 1000 mL:

- 300 mL of 20X SSPE
- 1.0 mL of 10% Tween-20
- 699 mL of water

Filter through a 0.2 µm filter.

Store at room temperature.

Wash B: Stringent Wash Buffer

(0.6X SSPE, 0.01% Tween 20)

For 1000 mL:

- 30 mL of 20X SSPE
- 1.0 mL of 10% Tween-20
- 969 mL of water

Filter through a 0.2 µm filter.

Store at room temperature.

The pH should be 8.

IMPORTANT: Prepare Wash B in smaller quantities to avoid long term storage. Tightly seal the container to avoid changes in salt concentration due to evaporation.

0.5 mg/mL Anti-Streptavidin Antibody

Resuspend 0.5 mg in 1 mL of water.

Store at 4°C.

12X MES Stock Buffer

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

For 1000 mL:

- 70.4g of MES hydrate
- 193.3g of MES Sodium Salt
- 800 mL of Molecular Biology Grade Water

Mix and adjust volume to 1000 mL.

The pH should be between 6.5 and 6.7.

Filter through a 0.2 µm filter.

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

1X Array Holding Buffer

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

For 100 mL:

- 8.3 mL of 12X MES Stock Buffer
- 18.5 mL of 5 M NaCl
- 0.1 mL of 10% Tween-20
- 73.1 mL of water

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

Fluidics Station and Scanner Control Software

You will use one of the instrument control applications listed below to operate the fluidics station and the scanner. For more information on these applications, refer to the appropriate user's guide.

- Affymetrix GeneChip® Operating Software (GCOS)
  Affymetrix *GeneChip® Operating Software User's Guide*
- Affymetrix GeneChip® Command Console (AGCC)
  Affymetrix GeneChip® Command Console™ User's Guide

Register a New Experiment or Sample

To register a new experiment or sample:

- If using GCOS, register a new Experiment.
- If using AGCC, register a new Sample.

Prime the Fluidics Station

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

To prime the Fluidics Station:

1. Turn on the Fluidics Station.
2. Prime the Fluidics Station.
   - Select protocol **Prime_450** for each module
   - Intake buffer reservoir: use **Non-Stringent Wash Buffer**
   - Intake buffer reservoir B: use **Stringent Wash Buffer**

About Priming the Fluidics Station

Priming ensures the lines of the fluidics station are filled with the appropriate buffers and the fluidics station is ready to run fluidics station protocols.

Priming should be done:

- When the fluidics station is first started
- When wash solutions are changed
- Before washing, if a shutdown has been performed
- If the LCD window instructs the user to prime

Wash and Stain Arrays

The staining protocol for mapping arrays is a three stage process:

1. A Streptavidin Phycoerythin (SAPE) stain.
2. An antibody amplification step.
3. A final stain with SAPE.

Once stained, each array is filled with Array Holding Buffer prior to scanning.

Prepare Arrays for Washing and Staining

To prepare the arrays for washing and staining:

1. After 16 to 18 hr of hybridization, remove the arrays from the oven.
2. Extract the hybridization cocktail from each array and transfer it to the corresponding well of a 96-well plate.

   Store on ice during the procedure, or at –80°C for long-term storage.
3. Fill each array completely with 270 µL of Array Holding Buffer.

   See *Array Holding Buffer* on page 88 for buffer recipe.
4. Allow the arrays to equilibrate to room temperature before washing and staining.

NOTE: Arrays can be stored in the Array Holding Buffer at 4°C for up to 3 hr before proceeding with washing and staining. Equilibrate arrays to room temperature before washing and staining.

Prepare Buffers and Solutions

Prepare the following buffers and solutions (recipes follow). Mix well.

- Stain Buffer
- SAPE Stain Solution
- Antibody Stain Solution
- Array Holding Buffer

Stain Buffer

Mix well.

Table 5.3 Stain Buffer

Reagent	1 Array	4 Arrays (15% extra)	8 Arrays (15% extra)	12 Arrays (15% extra)	24 Arrays (15% extra)
H_2O	800.04 µL	3680 µL	7360 µL	11040 µL	22.08 mL
SSPE (20X)	360 µL	1656 µL	3312 µL	4968 µL	9.94 mL
Tween-20 (3%)	3.96 µL	18.2 µL	36.4 µL	54.6 µL	109.3 µL
Denhardt's (50X)	24 µL	110.4 µL	220.8 µL	331.2 µL	662.4 µL
Total	1188 µL	5465 µL	10929 µL	16394 µL	32.79 mL

SAPE Stain Solution

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

A vial containing SAPE Stain Solution must be placed in position 1 for each module used.

Table 5.4 SAPE Stain Solution

Reagent	1 Array	4 Arrays (10% extra)	8 Arrays (10% extra)	12 Arrays (10 extra)	24 Arrays (10% extra)
Stain Buffer	594 µL	2614 µL	5227 µL	7841 µL	15.68 mL
1 mg/mL Streptavidin Phycoerythrin (SAPE)	6 µL	26 µL	53 µL	79 µL	158.4 µL
Total	600 µL	2640 µL	5280 µL	7920 µL	15.84 mL

Antibody Stain Solution

Mix well. A vial containing Antibody Stain Solution must be placed in position 2 for each module used.

Table 5.5 Antibody Solution

Reagent	1 Array	4 Arrays (10% extra)	8 Arrays (10% extra)	12 Arrays (10 extra)	24 Arrays (10% extra)
Stain Buffer	594 µL	2614 µL	5227 µL	7841 µL	15.68 mL
0.5 mg/mL biotinylated antibody	6 µL	26 µL	53 µL	79 µL	158.4 µL
Total	600 µL	2640 µL	5280 µL	7920 µL	15.84 mL

Array Holding Buffer

Mix well. A vial containing Array Holding Buffer must be placed in position 3 for each module used.

Table 5.6 Array Holding Buffer

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

Washing and Staining Arrays

Wash and Stain Protocol

The GenomeWideSNP6_450 protocol is an antibody amplification protocol for mapping targets (described in Table 5.7). Use it to wash and stain the Genome-Wide Human SNP Array 6.0.

Table 5.7 GenomeWideSNP6_450 protocol for the Fluidics Station 450

GenomeWideSNP6_450 Protocol for 49 Format (Standard) Arrays	
Post Hyb Wash #1	6 cycles of 5 mixes/cycle with Wash Buffer A at 25°C.
Post Hyb Wash #2	24 cycles of 5 mixes/cycle with Wash Buffer B at 45°C.
Stain	Stain the array for 10 min in SAPE solution at 25°C.
Post Stain Wash	6 cycles of 5 mixes/cycle with Wash Buffer A at 25°C.
2nd Stain	Stain the array for 10 min in Antibody Stain Solution at 25°C.
3rd Stain	Stain the array for 10 min in SAPE solution at 25°C.
Final Wash	10 cycles of 6 mixes/cycle with Wash Buffer A at 30°C. The final holding temperature is 25°C.
Filling Array	Fill the array with Array Holding Buffer.

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

IMPORTANT: **These wash and stain buffers differ from the GeneChip® expression buffers.**

Washing and Staining Arrays

To wash and stain the arrays:

1. Select your experiment (GCOS) or sample (AGCC) name.
   The Probe Array Type appears automatically.
2. Select the protocol **GenomeWideSNP6_450**.
3. Start the protocol and follow the instructions in the LCD on the fluidics station.
   If you are unfamiliar with inserting and removing arrays from the fluidics station modules, refer to the appropriate Fluidics Station User's Guide, or Quick Reference Card (P/N 08-0093 for the Fluidics Station 450).
4. Insert an array into the designated module of the fluidics station while the cartridge lever is in the Down or Eject position.
5. When finished, verify that the cartridge lever is returned to the Up or Engaged position.
6. Remove any vials remaining in the positions of the fluidics station module(s) being used.
7. When prompted to "Load Vials 1-2-3," place the three vials into positions 1, 2 and 3 on the fluidics station.
   A. Place one vial containing 600 µL Streptavidin Phycoerythrin (SAPE) stain solution mix in position 1.
   B. Place one vial containing 600 µL anti-streptavidin biotinylated antibody stain solution in position 2.
   C. Place one vial containing 1 mL Array Holding Buffer in position 3.

D. Press down on the needle lever to snap needles into position and to start the run.

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

8. When staining is finished, remove the microcentrifuge vials containing stain and replace with three empty vials as prompted.

9. Remove the arrays from the fluidics station by first pressing down the cartridge lever to the eject position.

10. Check the array window for large bubbles or air pockets.

If bubbles are present, 1) use a pipette to manually fill the array with Array Holding Buffer, 2) remove one-half of the solution, then 3) manually fill the array with Array Holding Buffer.

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

11. If the array has no large bubbles, it is ready for scanning. Pull up on the cartridge lever to engage wash block and proceed to *Scanning Arrays* on page 91.

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.

12. When finished washing and staining, shut down the fluidics station following the procedure listed under *Shutting Down the Fluidics Station* on page 92.

Scanning Arrays

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

Prepare the Scanner

Turn on the scanner at least 10 min before use.

WARNING: The scanner uses a laser and is equipped with a safety interlock system. Defeating the interlock system may result in exposure to hazardous laser light.

Read and be familiar with the operation of the scanner before attempting to scan an array. Refer to the *GeneChip® Scanner 3000 Quick Reference Card* (P/N 08-0075).

Prepare Arrays for Scanning

To prepare arrays for scanning:

1. If the arrays were stored at 4°C, allow them to warm to room temperature before scanning.
2. If necessary, clean the glass surface of the array with a non-abrasive towel or tissue before scanning.

   Do not use alcohol to clean the glass.
3. On the back of the array cartridge, clean excess fluid from around the septa.
4. Carefully cover both septa with Tough Spots (See Figure 5.1 on page 91).

   Press to ensure the spots remain flat. If the spots do not apply smoothly (e.g. if you see bumps, bubbles, tears or curled edges) do not attempt to smooth out the spot. Remove the spot and apply a new spot.
5. Insert an array into the scanner and test the autofocus to ensure the spots do not interfere with the focus.

   If a focus error message is observed, remove the spot and apply a new spot. Ensure that the spots lie flat.

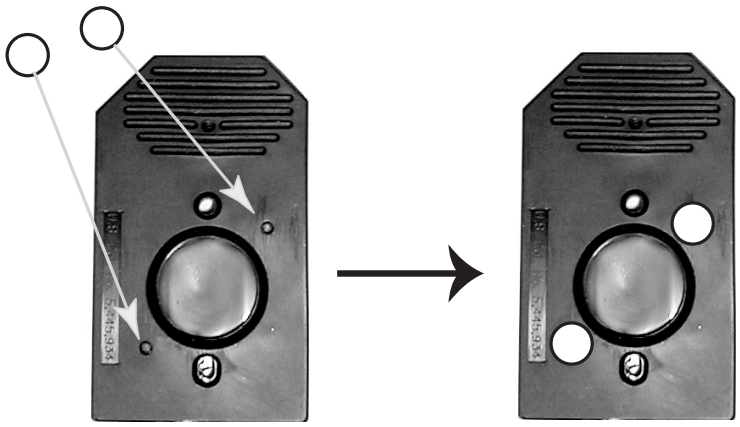


Figure 5.1 Applying Tough-Spots® to Arrays

Scanning the Array

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

To scan arrays:

1. Select the experiment name (GCOS) or sample name (AGCC) that corresponds to the array being scanned.
2. Following the GCOS or AGCC instructions as appropriate, load the array into the scanner and begin the scan.

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

WARNING: The scanner door will open and close automatically. Do not attempt to manually open or close the scanner door as this may damage the instrument.

Do not force the array into the holder.

Shutting Down the Fluidics Station

To shut down the Fluidics Station:

1. Gently lift up the cartridge lever to engage (close) the washblock.

   After removing an array from the holder, the LCD window displays the message **ENGAGE WASHBLOCK**. The instrument automatically performs a Cleanout procedure. The LCD window indicates the progress of this procedure.
2. When **REMOVE VIALS is displayed in the LCD**, remove the vials.

   The REMOVE VIALS message indicates the Cleanout procedure is complete.
3. If no other processing is to be performed, place the wash lines into a bottle filled with deionized water.
4. Using GCOS or AGCC, choose the **Shutdown_450** protocol for all modules.
5. Run the protocol for all modules.

   The Shutdown protocol is critical to instrument reliability. Refer to the instrument User's Guide for more information.
6. When the protocol is complete, turn the instrument off.
7. Place the wash lines in a different bottle of deionized water than the one used for the shutdown protocol.

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

Data Analysis

To analyze the data collected by the scanner, use Affymetrix Genotyping Console™ version 2.1 or later.

Genotyping Console includes copy number and LOH algorithms for the SNP Array 6.0. It also includes the Genotyping Console Browser and Segment Reporting Tool.

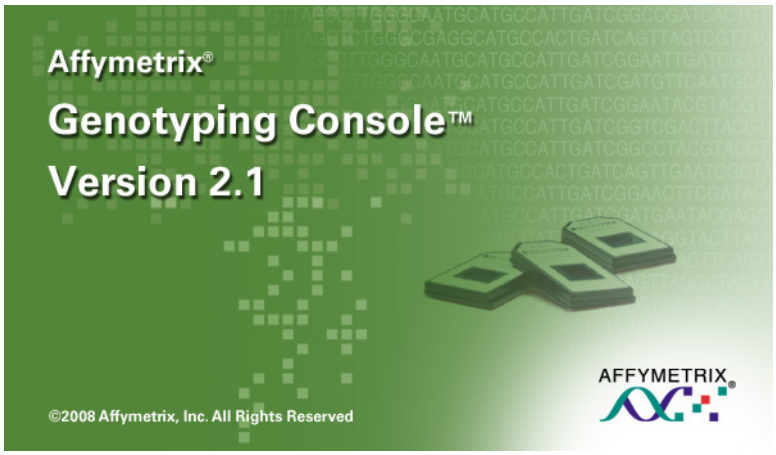


Figure 5.2 Use Genotyping Console version 2.1 or later to analyze your data

Chapter 6

TROUBLESHOOTING

General Assay Performance Recommendations

As with any assay using PCR, the SNP 6.0 Cytogenetics Copy Number Assay has an inherent risk of contamination with PCR product from previous reactions. In Chapter 2 *Laboratory Setup and Recommendations*, 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 only be carried out in the Post-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 SNP 6.0 Cytogenetics Copy Number 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 AccuGENE® water from Cambrex, and ligase and restriction enzymes from New England Biolabs.

Additional recommendations are as follows:

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

  To prevent loss of enzyme activity:

  - Immediately place enzymes in a cooler chilled to –20°C when removed from the freezer. Immediately return the enzyme to –20°C after use.
  - Take care when pipetting enzymes stored in glycerol, which is viscous. Do not store at –80°C.
  - Because Fragmentation Reagent activity can decline over time after dilution on ice, add it to the samples as quickly as possible.
- Preparing master mixes with a 15% excess ensures consistency in reagent preparation by minimizing pipetting errors and reducing handling time of temperature sensitive reagents. The success of this assay depends on the accurate pipetting and subsequent thorough mixing of small volumes of reagents.
- The PCR reaction for this assay has been validated using the specified thermal cyclers. These thermal cyclers were chosen because of their ramping times. 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 200to 1100 bp size range. See *Check the PCR Reaction by Running a Gel* on page 48 for more information.

Following fragmentation, run your samples on a gel. Successful fragmentation is confirmed by the presence of a smear of < 180 bp in size. See *Check the Fragmentation Reaction by Running a Gel* on page 70 for more information.

- Run controls in parallel with each group of samples.

  Substitute water for DNA as a negative control. The absence of bands on your PCR gel for this control confirms no previously amplified PCR product has contaminated your samples. Use Reference Genomic DNA 103 as a positive control (included in the reagent kit). These controls are effective troubleshooting tools that will help you confirm the successful completion of each stage of the assay.
- Oligonucleotide controls are included in the reagent kit. These controls are added to the target samples prior to hybridization and act to confirm successful hybridization, washing, staining, and sensitivity of the array. The oligonucleotide control reagents contain oligo B2 which is used for grid alignment.
- Regularly calibrate all multichannel pipettes.
- Check that your spectrophotometer is accurately calibrated, and be sure the OD measurement is within the quantitative linear range of the instrument (0.2 to 2.0 OD).
- Hybridization ovens should be serviced at least once per year to ensure that they are operating within the manufacturer's specifications.

Troubleshooting the SNP 6.0 Cytogenetics Copy Number Assay

Problem	Likely Cause	Solution
Faint/absent bands on PCR gel		
Both samples & positive control affected.	Problem with master mixes or individual reagents.	Ensure all reagents added to master mixes and enzymes are stored at –20°C. Work quickly with enzymes and return to –20°C directly after use to prevent loss of activity.
	Failed restriction digest.	Use restriction enzyme to digest a known good DNA sample. Run gel to confirm restriction enzyme activity. Use the correct concentration of BSA.
	Failed adaptor ligation reaction.	Confirm enzyme activity.
		Ligase buffer contains ATP and should be defrosted/ held at 4°C. Vortex ligase buffer thoroughly before use to ensure precipitate is re-suspended. Avoid multiple freeze-thaw cycles. Try a fresh tube of buffer.
	Reduced adaptor ligation efficiency due to adaptor self-ligation, DNA re-ligation.	To prevent self-ligation of adaptor, work rapidly and add DNA ligase last.
	Failed PCR reaction.	Check PCR reagents. Take care with preparation of master mixes and ensure accurate pipetting and thorough mixing.
	Reduced PCR reaction yield – non optimal PCR conditions.	Use a calibrated thermal cycler, and check PCR programs. Use the recommended 96-well PCR plates.
		Thoroughly mix PCR reaction.
	Ligation mix not diluted prior to PCR reaction.	Ligation mixture diluted 1:4 with molecular biology grade water to remove potential inhibitors and maintain optimal pH and salt concentration.
	Incorrect concentration of nucleotides.	Check dNTP stock concentration and vendor.
	Used Nsp adaptor for Sty digest, or vice versa.	Repeat ligation step with correct adaptors.
Samples affected (but positive controls OK).	Non-optimal reaction conditions.	Prepare master mixes as described and include a positive control to eliminate reagents and assay problems as detailed above.
	Insufficient starting material.	250 ng genomic DNA should be used. Confirm concentration using calibrated spectrophotometer.
	Sample DNA contains enzymatic or chemical inhibitors.	Ensure genomic DNA is purified and diluted in low EDTA (0.1mM) TE buffer.
		Use the DNA cleanup procedure on page 16 to remove inhibitors.
	Degraded sample DNA.	Confirm quality of genomic DNA sample.
Low PCR yield		
DNA lost during purification. Gel images show PCR product, but low OD.	Beads not fully resuspended during DNA elution step.	Ensure that beads are fully resuspended and mixed during the elution step (before pelleting and transfer of the eluate).

Problem	Likely Cause	Solution
Insufficient purified PCR product for fragmentation		
Volume of eluate for particular samples is < 47 µL.		Do the following in this order: 1.Measure the actual volume using a pipettor. 2.Add Buffer EB to a final volume of 47 µL. 3.Mix by pipetting up and down. 4.Transfer 2 µL to the corresponding well(s) in the OD plate. 5.Proceed to fragmentation with 45 µL in each well.
Fragmented PCR product is not the correct size		
PCR product is still visible in 200-1100 bp size region	Failed or incomplete fragmentation due to reduced DNase activity.	Check that you have selected the correct activity of DNase to add to fragmentation reaction. (See *Prepare the Fragmentation Master Mix* on page 67)
		Ensure fragmentation reagent is kept at –20°C. Do not reuse diluted working stock.
.CEL file can not be generated		
GCOS or AGCC is unable to align grid.	Unable to place a grid on the .dat file due to the absence of B2 signal.	Hybridization controls including oligo B2 must be added to hybridization cocktail for grid alignment.
.dat image is dim.	Insufficient signal intensity or staining failure.	Make fresh stain buffers.
	Incorrect wash buffers used on fluidics station.	Prime the fluidics station with the correct buffers prior to running the assay. Incorrect wash buffers will disrupt hybridization of the labeled, fragmented DNA.
High MAPD or Low Contrast QC Values		
Gel images and spectrophotometric quantitation indicate successful PCR reaction.	Over fragmentation of DNA sample due to incorrect dilution of Fragmentation Reagent stock.	Check that you have selected the correct activity of DNase to add to fragmentation reaction. (See *Prepare the Fragmentation Master Mix* on page 67.) Work quickly and on ice; mix thoroughly. Transfer reactions to pre-heated thermal cycler (37°C).
Extremely high MAPD or low Contrast QC values. Sample hybridization is absent on .cel and .dat images but B2 grid is bright.	Labeling reaction suboptimal.	Use a new vial of Terminal Dideoxynucleotidyl Transferase. Verify the labeling reagents and repeat labeling.
Positive control has good MAPD and Contrast QC values, but samples do not.	Genomic DNA not optimal.	Ensure DNA samples are of high quality (i.e., run in a 1 to 2% gel and compare to Reference 103 DNA control). Use positive control sample as a reference guide for assay procedures. Prepare master mixes for samples and controls.
Very high MAPD or low Contrast QC values.	Mixed up Nsp and Sty enzymes during the digestion or ligation stages.	Repeat the experiment, making sure the correct reagents are used for each digestion and ligation stage.

OD Troubleshooting Guidelines

Table 6.1 Sample OD > 1.4 (> 7 µg/µL)

If the sample OD is greater than 1.4 (calculated concentration greater than 7 µg/µL), a problem may exist with either PCR product elution or the OD reading. The limit on PCR yield is approximately 7 µg/µL, as observed in practice and as predicted by the mass of dNTPs in the reaction. Possible causes include: • Check the OD320 reading. If it is > 0.1, you may have bead slurry carryover. OK to proceed. • The purified PCR product was eluted in a volume less than 55 µL. • The purified PCR product was not mixed adequately before making the 1:100 dilution. • The diluted PCR product was not mixed adequately before taking the OD reading. • The plate spectrophotometer or NanoDrop may require calibration. • Pipettes may require calibration. • The settings on the plate spectrophotometer, NanoDrop, or the software may be incorrect. • OD calculations may be incorrect and should be checked.

Table 6.2 Sample OD < 0.9 (4.5 µg/µL)

If the sample OD is less than 0.8 (calculated concentration less than 4 µg/µL), a problem may exist with either the genomic DNA, the PCR reaction, the elution of purified PCR products, or the OD readings. Possible problems with input genomic DNA that would lead to reduced yield include: • The presence of inhibitors (heme, EDTA, etc.). • Severely degraded genomic DNA. • Inaccurate concentration of genomic DNA. Check the OD reading for the PCR products derived from Reference DNA 103 as a control for these issues.
Troubleshooting possible problems with the elution or OD readings. Possible causes include: • The purified PCR product was eluted in a volume greater than 55 µL. • The purified PCR product was not mixed adequately before making the 1:100 dilution. • The diluted PCR product was not mixed adequately before taking the OD reading.

Table 6.3 OD260/OD280 ratio is not between 1.8 and 2.0

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

Table 6.4 The OD320 measurement is > 0.2

Possible causes include:
• Magnetic beads may have been carried over into purified sample. *Action:* Spin down the sample for 5 min. Place on the MagnaRack and pipet out the eluate.
• Precipitate may be present in the eluted samples.
• There may be defects in the OD plate.
• Air bubbles in the OD plate or in solutions.

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

Chapter 7

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 hr 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 7.1 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 7.1 on page 102.
2. Prepare 500 mL of 0.525% sodium hypochlorite solution using deionized water.

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

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

IMPORTANT:

- **The shelf life of this solution is 24 hr. After this period, you must prepare fresh solution.**
- **Each fluidics station with 4 modules requires 500 mL of 0.525% sodium hypochlorite solution.**

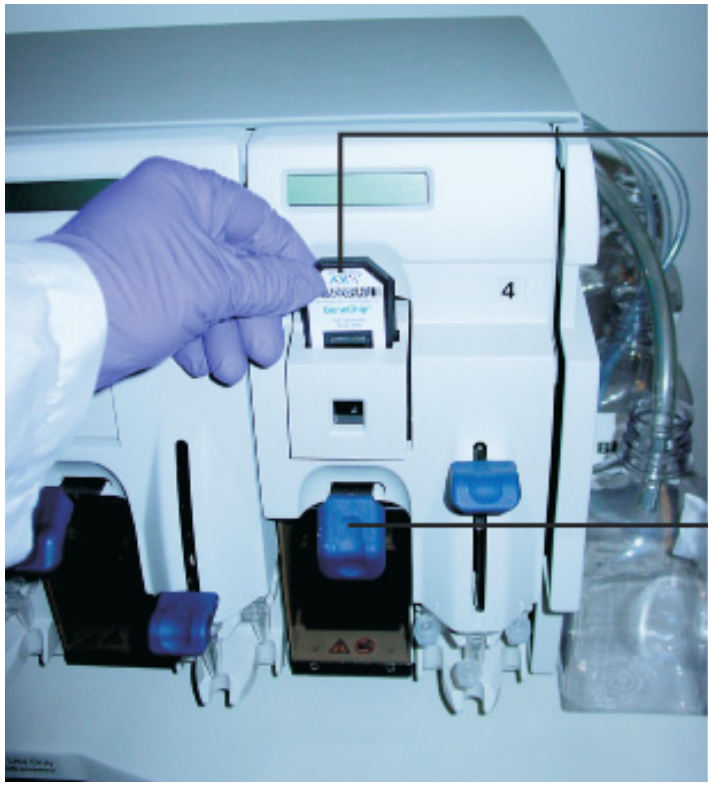


Figure 7.1 Disengaged washblocks showing cartridge levers in the down position. Remove any cartridges

3. As shown in Figure 7.2:
   - **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 (GCOS or AGCC).
5. Choose the current bleach protocol (as of the writing of this manual, it is BLEACHv2_450) for each module.

Figure 7.2 The bleach cycle. Immerse the tubes into the 0.525% sodium hypochlorite solution. The waste line remains in the waste bottle.

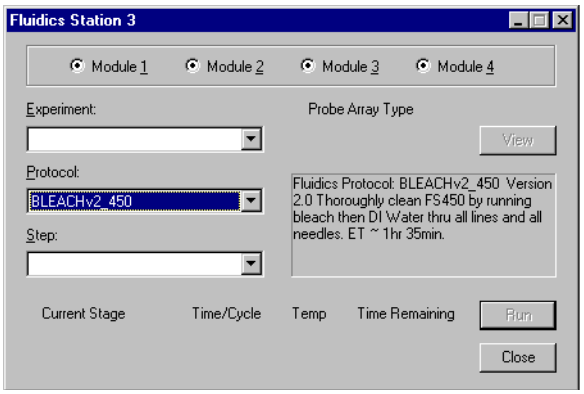


Figure 7.3 The Fluidics Station protocol window: select all modules.

6. In GCOS or AGCC, run the protocol for all modules.

NOTE: The fluidics station will not start until the needle lever is pressed down (Figure 7.4 on page 104). 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 7.4).

Figure 7.4 Press down on the needle levers to start the bleach protocol.

9. The fluidics station will begin the protocol, emptying the lines and performing the cleaning cycles using bleach solution.

10. After approximately 30 min, the LCD will prompt you when the bleach cycle is over and the rinse cycle is about to begin.

The Rinse Cycle

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

1. Follow the prompts on the LCD for each module. Lift up on the needle levers and remove the bleach vials. Load clean, empty vials onto each module.
2. Remove the three wash and water lines from the bleach bottle and transfer them to the DI water bottle (Figure 7.5).

   At this step, there is no need to be concerned about the bleach remaining in the lines.

Figure 7.5 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 7.2 below.

Table 7.2 Storage Suggestions for the Fluidics Station 450

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

Appendix A

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

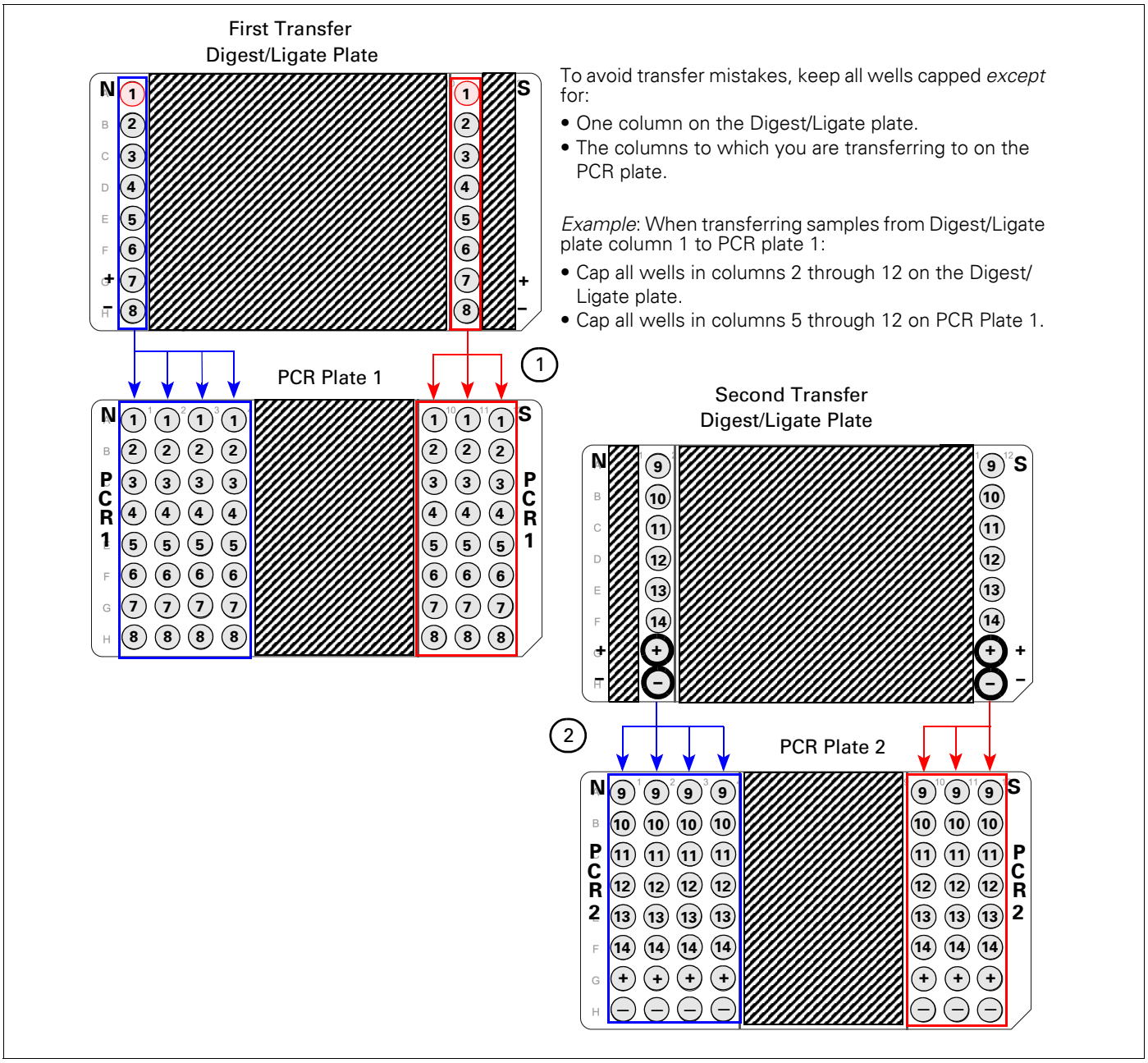


Figure A.1 16 reaction workflow — Digest/Ligate plate to 96-well plates

PCR to Purification

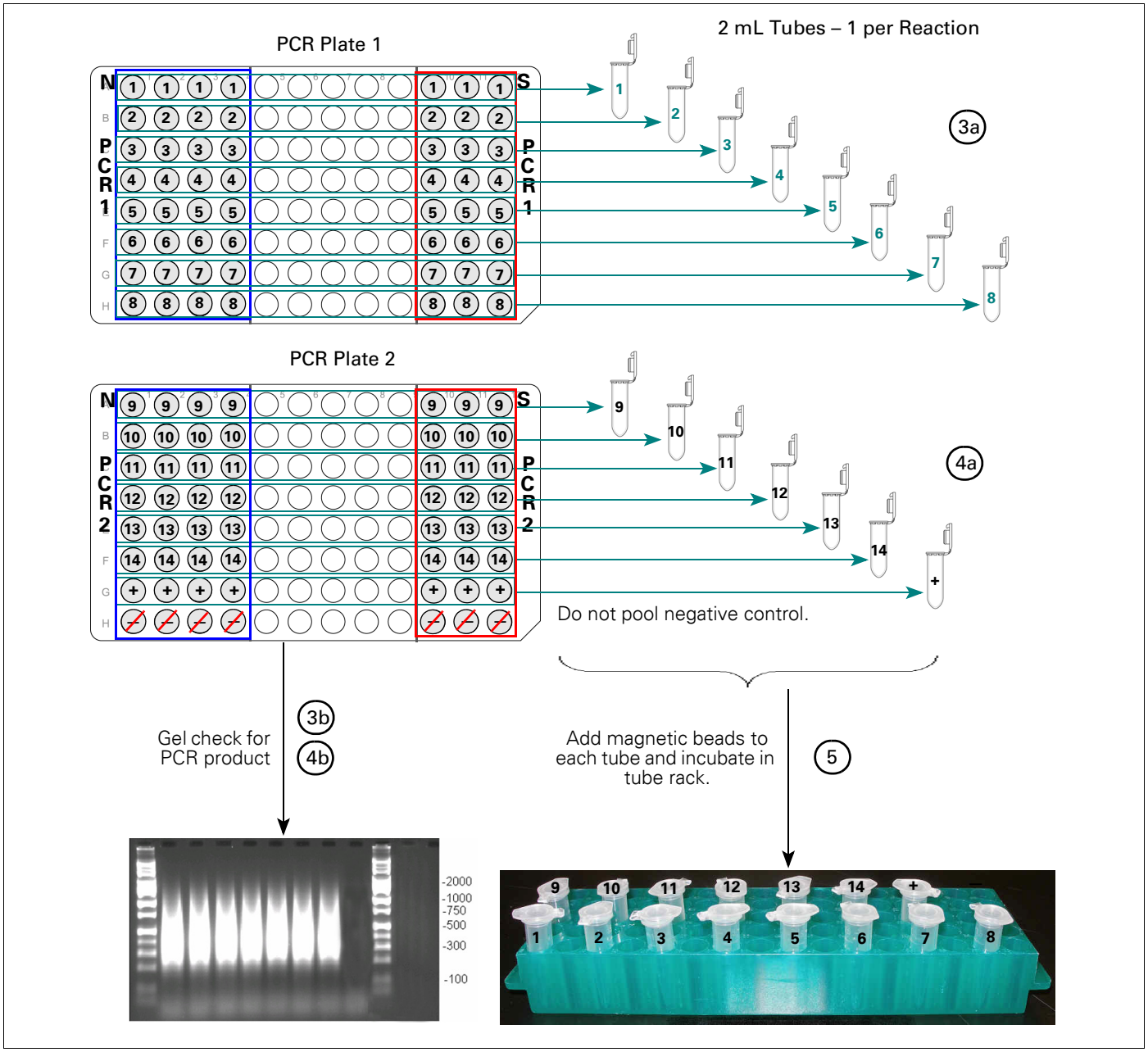


Figure A.2 16 reaction workflow — PCR to purification

Purification continued to Fragmentation and Labeling

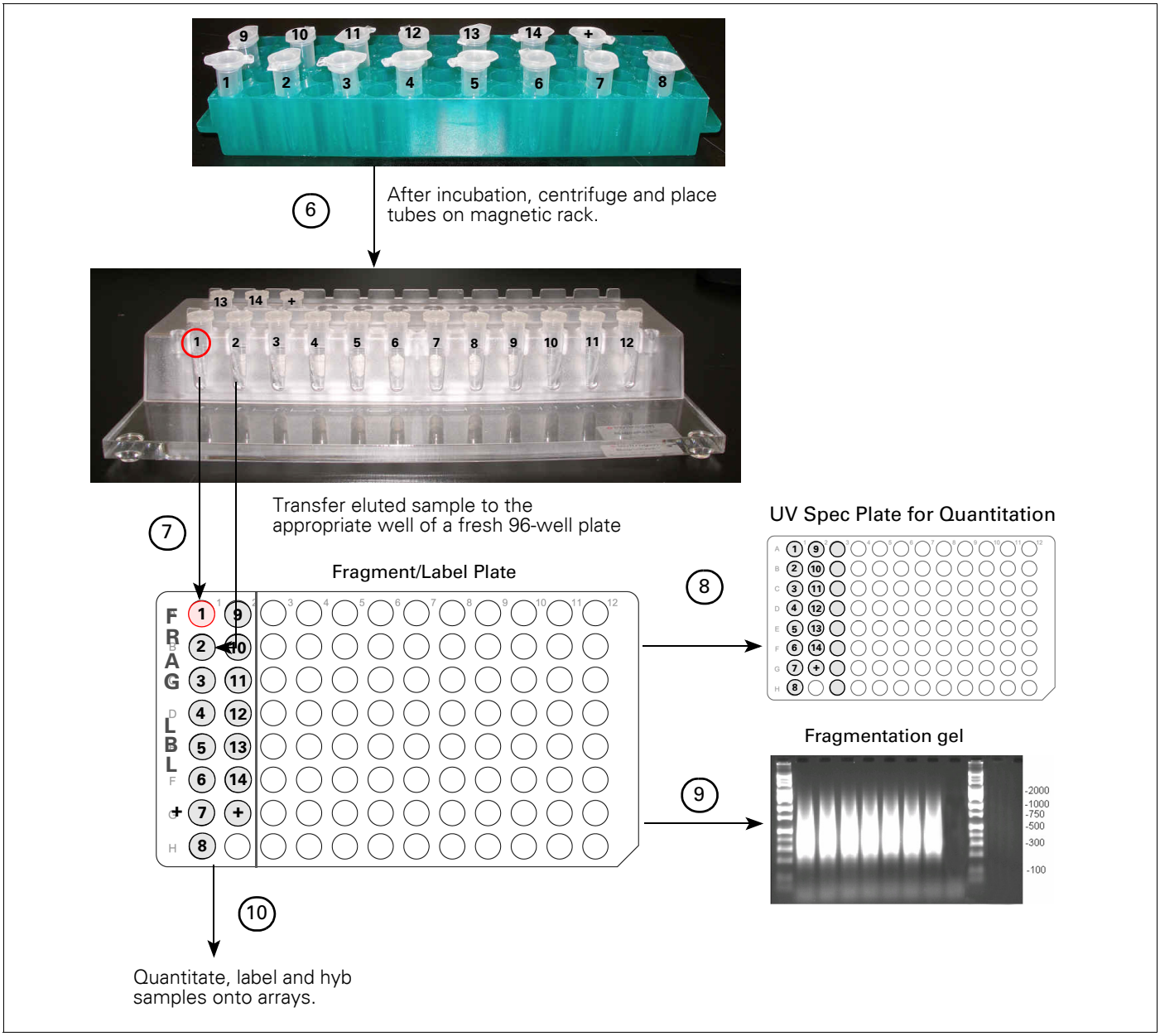


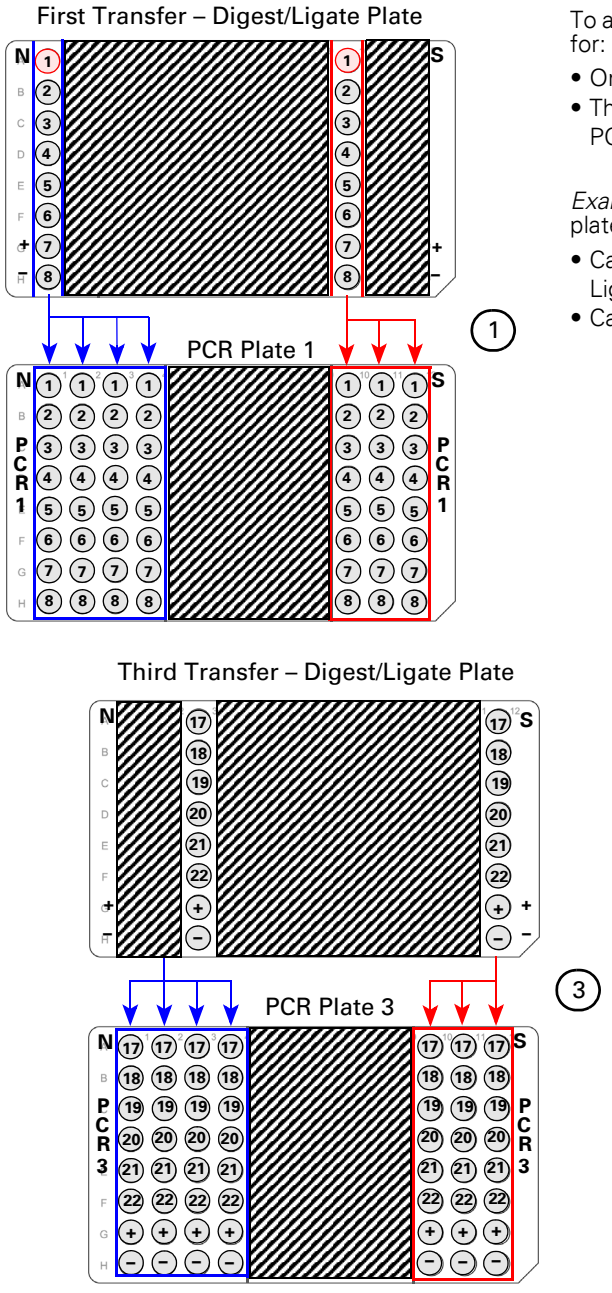
Figure A.3 16 reaction workflow — Purification to Labeling

Appendix B

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



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

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

Example: When transferring samples from Digest/Ligate plate column 1 to PCR plate 1:

- Cap all wells in columns 2 through 12 on the Digest/Ligate plate.
- Cap all wells in columns 5 through 12 on PCR Plate 1.

Figure B.1 24 reaction workflow — Digest/Ligate plate to PCR plates

PCR to Purification

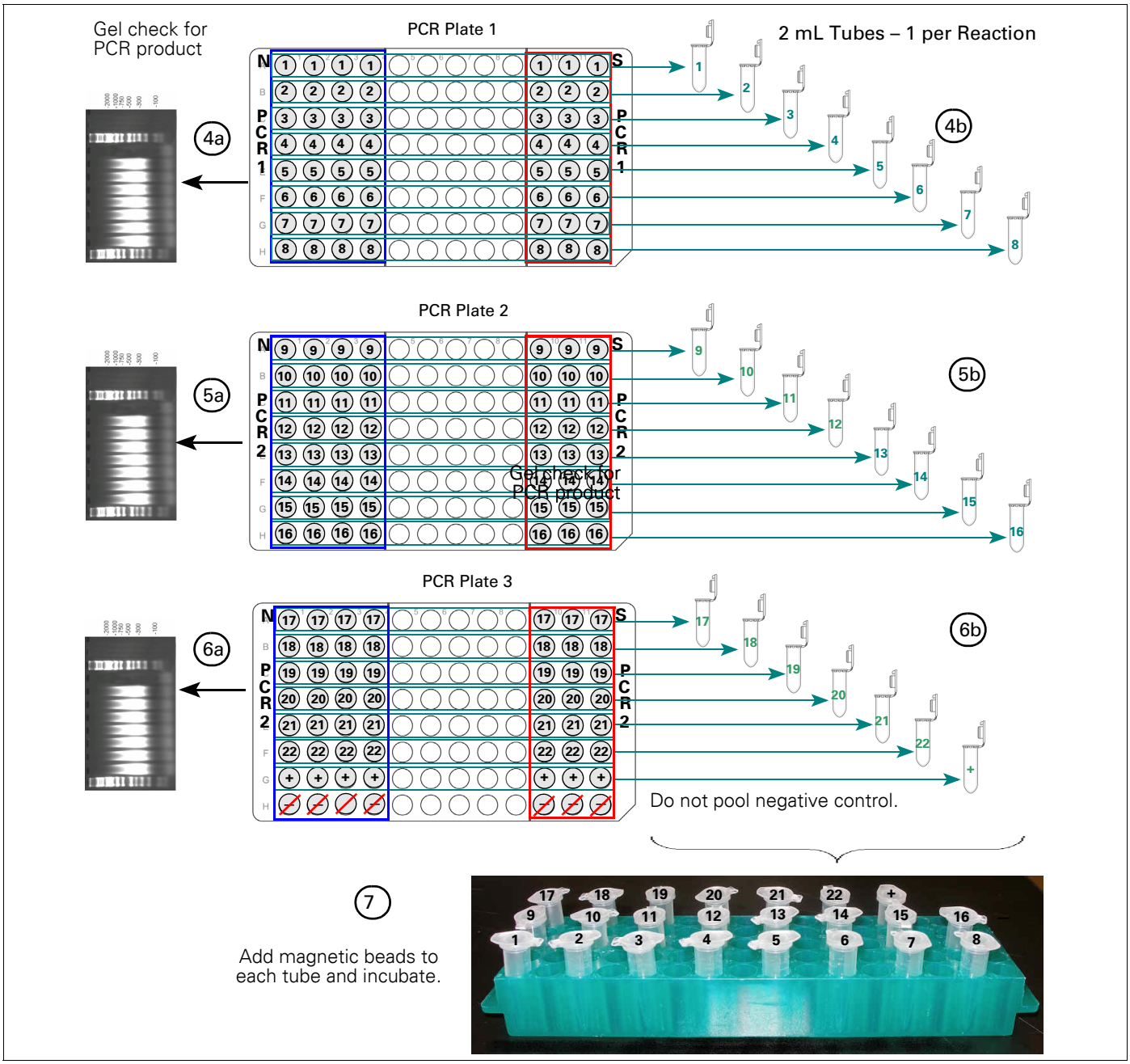


Figure B.2 24 reaction workflow — PCR to purification

Purification continued to Fragmentation and Labeling

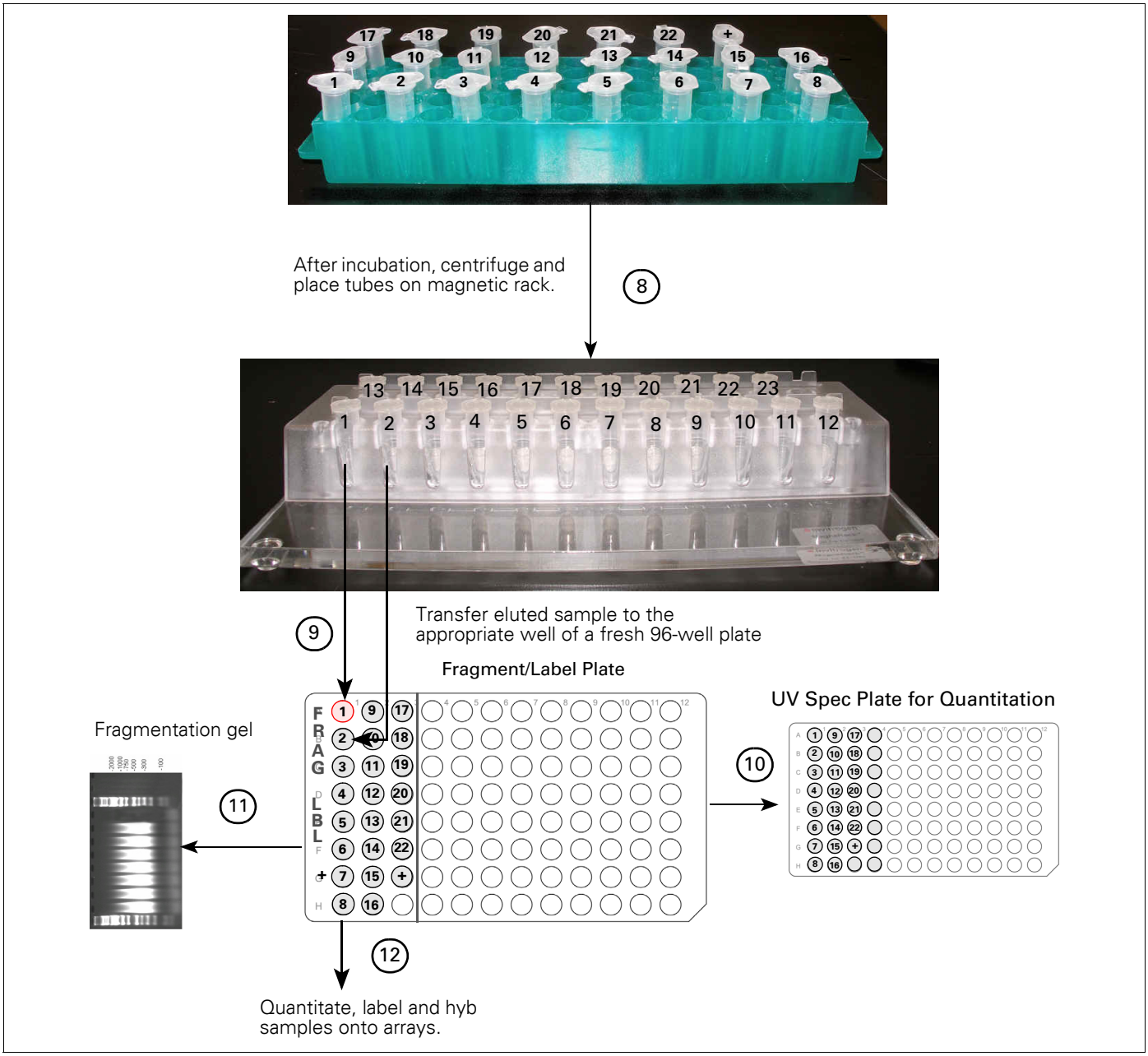


Figure B.3 24 reaction workflow — PCR to purification

Appendix C

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® SNP 6.0 Cytogenetics Copy Number 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.**

From Affymetrix

Affymetrix Equipment Required

Table C.1 Affymetrix Equipment Required

✓	Item	Part Number
❒	GeneChip® Fluidics Station 450	00-0079
❒	GeneChip® Hybridization Oven 640 or 645	800139 (640) 00-0331 (645)
❒	GeneChip® 3000 Scanner with 7G upgrade	901153

Affymetrix Software Required

Table C.2 Affymetrix Software Required

✓	Item	Part Number
❒	One of the following instrument control applications: • GeneChip® Operating Software • GeneChip® Command Console	Latest version
❒	Genotyping Console	Latest version

Affymetrix Arrays Required

Table C.3 Affymetrix® Genome-Wide Human SNP Array 6.0

✓	Item	Part Number
❒	100 arrays	901150
	50 arrays	901153
	5 arrays	901182

Affymetrix Reagents Required

Table C.4 Affymetrix® Genome-Wide Human SNP Nsp/Sty Assay Kit 5.0/6.0 – 30 reaction kit

✓	Item	Part Number
❒	30 reactions Components contained in this kit: • Adaptor, Nsp (50 µM) • Adaptor, Sty (50 µM) • PCR Primer, 002 (100 µM) • GeneChip® Fragmentation Reagent • 10X Fragmentation Buffer • GeneChip® DNA Labeling Reagent (30 mM) • Terminal Deoxynucleotidyl Transferase (30 U/µL) • 5X Terminal Deoxynucleotidyl Transferase Buffer • Oligonucleotide Control Reagent 0100 • Reference Genomic DNA, 103 (50 ng/µL)	901013

Optional Affymetrix Equipment

Table C.5 Optional Affymetrix® Equipment

✓	Item	Part Number
❒	GeneChip® System 3000Dx v.2 with Data Transfer Server	00-0349
❒	GeneChip® Hybridization Oven 645	00-0331

Equipment Required from Other Suppliers

Pre-PCR Clean Area Equipment Required

When performing the pre-PCR stages of the SNP 6.0 Cytogenetics Copy Number 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 C.6 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	Stratagene	400012
❒	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	—
❒	Ice Bucket, 4 to 9 liters (Magic Touch Icewares)	Fisher Scientific	—
❒	Microfuge (for tubes and strip tubes)	Any vendor	—
❒	Pipet, single-channel, 2–20 µL	Rainin	L-20
❒	Pipet, single-channel, 20–200 µL	Rainin	L-200
❒	Pipet, single-channel, 100–1000 µL	Rainin	L-1000
❒	Pipet, 8-channel, 2–20 µL	Rainin	L8-20
❒	Pipet, 8-channel, 20–200 µL	Rainin	L8-200
❒	Plate centrifuge, multipurpose	Eppendorf	5804 or 5810
❒	Vortexer (with plate pad)	VWR	58816-1212
❒	Select one of these thermal cyclers: • GeneAmp PCR System 9700 (gold/silver block)	Applied Biosystems	4314878
	• 2720 Thermal Cycler	Applied Biosystems	4359659
	• MJ Tetrad PTC-255	Bio-Rad	—
	• DNA Engine Tetrad 2	Bio-Rad	PTC-0240G

Post-PCR Area Equipment Required

Table C.7 Post-PCR Area Equipment Required

✓	Item	Vendor	Part Number
❒	Benchtop Cooler, –20°C	Stratagene	400012
❒	Cooling Chamber, single	Diversified Biotech	CHAM-1020
❒	Freezer, –20°C; deep freeze; manual defrost; 17 cu ft	Any vendor	—
❒	Gel box for electrophoresis	Any vendor	—
❒	Gel imager	Any vendor	—
❒	Ice Bucket, 4 to 9 liters (Magic Touch Icewares)	Fisher Scientific	—
❒	MagnaRack, magnetic stand	Invitrogen	CS15000
❒	Microcentrifuge 5414D or R	Eppendorf	022621408
❒	Microcentrifuge Standard Rotor F-45-24-11, 24 bores	Eppendorf	022621502
❒	Microfuge (for tubes and strip tubes)	Any vendor	—
❒	Microtube Foam Insert with stem (Multiple Sample Starter Set, Model H301) (you will attach to one of the vortexers)	Scientific Industries	504-0233-00
❒	Pipet, single-channel, 2–20 µL	Rainin	L-20
❒	Pipet, single-channel, 20–200 µL	Rainin	L-200
❒	Pipet, single-channel, 100–1000 µL	Rainin	L-1000
❒	Pipet, 8-channel, 2–20 µL	Rainin	L8-20
❒	Pipet, 8-channel, 20–200 µL	Rainin	L8-200
❒	Pipet, 8-channel, 100–1000 µL	Rainin	L8-1000
❒	Plate centrifuge, multipurpose	Eppendorf	5804 or 5810
❒	Refrigerator, 4°C, 6 cu ft	Any vendor	—
❒	Spectrophotometer – select one of the following: • SpectraMax spectrophotometer	Molecular Devices	Spectramax Plus384
	• NanoDrop	NanoDrop	ND-1000
❒	Select one of these thermal cyclers: (if routinely processing > 8 samples, you may to use additional thermal cyclers for PCR) • GeneAmp PCR System 9700 (gold/silver block)	Applied Biosystems	4314878
	• MJ Tetrad PTC-255	Bio-Rad	—
	• DNA Engine Tetrad 2	Bio-Rad	PTC-0240G
❒	Vortexer (2 required) One vortexer must have a plate pad. The Microtube Foam Insert listed above will be attached to the other vortexer.	VWR	58816-1212

Reagents Required from Other Suppliers

Table C.8 Reagents Required from Other Suppliers

✓	Item	Vendor	Part Number
❒	Nsp I Enzyme • NE Buffer 2 (10X) • BSA (100X)	New England Biolabs	R0602L
❒	Sty I Enzyme • NE Buffer 3 (10X) • BSA (100X)	New England Biolabs	R0500S
❒	T4 DNA Ligase	New England Biolabs	M0202L
❒	Magnetic Beads	Beckman Coulter	A63881 (60 mL) A63882 (450 mL)
❒	TITANIUM DNA Amplification Kit (300 rxn)	Clontech	639240
❒	Reduced EDTA TE Buffer, pH 8.0	TekNova	T0223
❒	AccuGENE Water	Lonza Group LTD	51200
❒	SSPE	Lonza Group LTD	51214
❒	EB Buffer	Qiagen	19086
❒	Human Cot-1 DNA (1 mg/mL; 500 µL per tube)	Invitrogen	15279-011
❒	10% Tween-20	Pierce	28320
❒	Herring Sperm DNA (HSDNA) (10 mg/mL; 100 mg)	Promega	D1815
❒	Absolute Ethanol (for dilution to 75%)	Sigma-Aldrich	459844
❒	Denhardt's Solution	Sigma-Aldrich	D2532
❒	DMSO	Sigma-Aldrich	D5879
❒	MES Hydrate SigmaUltra (50 g or 250 g)	Sigma-Aldrich	M5287
❒	MES Sodium Salt (10 g, 25 g, or 100 g)	Sigma-Aldrich	M5057
❒	Tetramethyl Ammonium Chloride (TMAC)	Sigma-Aldrich	T3411
❒	5 M NaCl, RNase-free, DNase-free	Ambion	9760G
❒	EDTA	Ambion	9260G
❒	R Phycoerythrin Streptavidin	Molecular Probes	S-866
❒	Antistreptavidin-antibody	Vector Labs	BA-0500
❒	Bleach (5.25% Sodium Hypochlorite)	VWR	21899-504 (or equivalent)
❒	Distilled water	Invitrogen	15230147

Consumables Required from Other Suppliers

Table C.9 Consumables Required from Other Suppliers

✓	Item	Vendor	Part Number
❒	Adhesive film for 96-well plates – use on of these:		
	• MicroAmp Clear Adhesive Film	Applied Biosystems	4306311
	• Microseal 'B' Film	Bio-Rad	MSB1001
❒	DNA Marker, All Purpose Hi-Lo	Bionexus	BN2050
❒	Gel Loading Solution	Sigma	G2526
❒	Pipet tips, 20 µL filter tips	Rainin	GP-L10F
❒	Pipet tips, 200 µL filter tips	Rainin	GP-L200F
❒	Pipet 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
❒	Solution Basin, 55 mL sterile	Labcor	730-004
❒	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	
❒	Tough Spots, 1/2”	Diversified Biotech	T-SPOTS-50
❒	Tube, Eppendorf Safe-Lock Microcentrifuge 1.5 mL	VWR	21008-959
❒	Tube, Eppendorf Safe-Lock Microcentrifuge 2.0 mL (must be round bottom tubes)	VWR	20901-540
❒	Tube, centrifuge 50 mL	VWR	21008-178
❒	Tube strips, 8-well, 0.2 mL	VWR	20170-004

Supplier Contact List

Table C.10 Supplier Contact List

Supplier	Web Site Address
Affymetrix	www.affymetrix.com
Agencourt Bioscience Corp.	agencourt.com
Ambion	ambion.com
Applied Biosystems	www.appliedbiosystems.com
Bionexus Inc.	www.bionexus.net
Bio-Rad	bio-rad.com
Bio-Smith	biosmith.com
Clontech	www.clontech.com
Diversified Biotech	divbio.com
E&K Scientific	eandkscientific.com
Eppendorf	eppendorf.com
ESCO	www.escoglobal.com
Fisher Scientific	www.fishersci.com
Invitrogen Life Technologies	invitrogen.com
Labcor	labcorproducts.com
Lonza	www.lonza.com
Molecular Devices	moleculardevices.com
Molecular Probes	molecularprobes.com
NanoDrop	nanodrop.com
New England Biolabs	www.neb.com
Pierce Biotechnology (part of Thermo Fisher Scientific)	piercenet.com
Promega	www.promega.com
QIAGEN	www1.qiagen.com
Rainin	www.rainin.com
Scientific Industries	www.scientificindustries.com
Sigma-Aldrich	www.sigma-aldrich.com
Stratagene	stratagene.com
Teknova	teknova.com
VWR	vwr.com
Vector Labs	vectorlabs.com

Appendix D

THERMAL CYCLER PROGRAMS

This appendix includes the thermal cycler programs required for the Affymetrix® SNP 6.0 Cytogenetics Copy Number Assay.

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

Cyto Digest

Cyto Digest Program	
Temperature	Time
37ºC	120 min
65ºC	20 min
4ºC	Hold

Cyto Ligate

Cyto Ligate Program	
Temperature	Time
16ºC	180 min
70ºC	20 min
4ºC	Hold

Cyto 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

Cyto PCR Program for GeneAmp® PCR System 9700		
Temperature	Time	Cycles
94°C	3 min	1X
94°C 60°C 68°C	30 sec 45 sec 15 sec	} 30X
68°C	7 min	1X
4°C	HOLD (Can be held overnight)	

For the MJ Tetrad PTC-225 and Tetrad 2

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

Cyto PCR Program for MJ Tetrad PTC-225 and Tetrad 2		
Temperature	Time	Cycles
94°C	3 min	1X
94°C 60°C 68°C	30 sec 30 sec 15 sec	} 30X
68°C	7 min	1X
4°C	HOLD (Can be held overnight)	

Cyto Fragment

Cyto Fragment Program	
Temperature	**Time**
37°C	35 min
95°C	15 min
4°C	Hold

Cyto Label

Cyto Label Program	
Temperature	**Time**
37°C	4 hr
95°C	15 min
4°C	Hold
Samples can remain at 4°C overnight.	

Cyto Hyb

Cyto Hyb Program	
Temperature	**Time**
95°C	10 min
49°C	Hold