




GeneChip[®] CustomSeq[®] Resequencing Array Protocol

Version 2.1

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Contents

CHAPTER 1	GeneChip® CustomSeq® Resequencing Array Protocol	1
	Before You Start	3
	Assay Overview	3
	Reagents, Equipment and Other Materials Required	5
	Control Overview	10
	STAGE 1: PERFORMING LONG-RANGE PCR	12
	About This Stage	12
	Reagents Required	13
	Prepare the Reagents	13
	Add Genomic DNA and PCR Master Mix	17
	Perform PCR	19
	Verify the PCR Reaction	20
	STAGE 2: QUANTITATION AND POOLING OF PCR PRODUCTS	21
	About Quantitation and Pooling	21
	Reagents, Equipment and Other Materials Required for the PicoGreen® Method	23
	Equipment and Other Materials Required for Absorption Spectrophotometry	24
	PicoGreen® Method	25
	Absorption Spectrophotometry Method	31
	Extrapolating Values for PCR Product Pooling	34
	STAGE 3: PCR PURIFICATION AND ELUTION	37
	Reagents, Equipment and Other Materials Required	37
	Purification	38
	Elute the Samples	39
	What To Do Next	39
	STAGE 4: FRAGMENTATION	40
	Reagents Required	40
	Confirm Activity of the Fragmentation Reagent	41
	Quantitate and Dilute the Pooled PCR Products	41
	Prepare the Fragmentation Master Mix	44
	Fragment the Pooled PCR Products	45
	Ensure that Fragmentation is Complete	46

STAGE 5: LABELING	48
Reagents Required	48
Prepare the Labeling Master Mix	48
Add Labeling Master Mix to Each Reaction	49
STAGE 6: HYBRIDIZATION	51
Reagents Required	51
Prepare and Aliquot the Hybridization Master Mix	52
Prepare the Pre-Hybridization Buffer	53
Pre-Hybridize the Arrays	54
Denature the Samples	54
Load the Denatured Samples Onto Arrays	54
STAGE 7: WASHING, STAINING, AND SCANNING	56
Reagents and Other Materials Required	56
Reagent Preparation	57
Experiment and Fluidics Station Setup	60
Prepare the Arrays for Washing and Staining	63
Prepare the Buffers and Stain Solutions	63
Fluidics Station Protocols	67
Washing and Staining Arrays Using FS-450/250	69
Washing and Staining Arrays Using FS400	71
Scanning Arrays	74
Shutting Down the Fluidics Station	78
Customizing Fluidics Station Protocols	79

APPENDIX A PCR Protocols 67

LONG-RANGE PCR PROTOCOL	69
Long-Range PCR Controls (7.5 kb Amplicon)	69
STANDARD PCR PROTOCOL	71
Standard PCR Controls (1.0 kb Amplicon)	71

APPENDIX B Affymetrix and Supplier Contact List 73

AFFYMETRIX TECHNICAL SUPPORT	75
SUPPLIER CONTACT INFORMATION	76

Chapter 1



**GeneChip® CustomSeq® Resequencing
Array Protocol**

Chapter 1



Before You Start

Assay Overview

This manual provides an assay protocol for GeneChip® CustomSeq® Resequencing Arrays. This document assumes that you have:

- Completed the CustomSeq® array design process (refer to the *GeneChip® CustomSeq® Custom Resequencing Array Design Guide*, P/N 701263).
- Designed an appropriate amplification strategy.
- Tested your primers to verify robust performance.

A high-level overview of this protocol is shown in [Figure 1.1 on page 4](#).

Various strategies can be employed to amplify target sequences from genomic DNA. Affymetrix strongly recommends utilizing long-range PCR amplification when appropriate to reduce assay cost and complexity. The recommended long-range PCR protocol is described under [Stage 1: Performing Long-Range PCR on page 12](#).

If using a standard (short-range) PCR protocol that has been optimized in your lab:

- Refer to [page 10](#) and [Appendix A](#) for control recommendations.
- Begin with [Stage 2: Quantitation and Pooling of PCR Products on page 21](#).

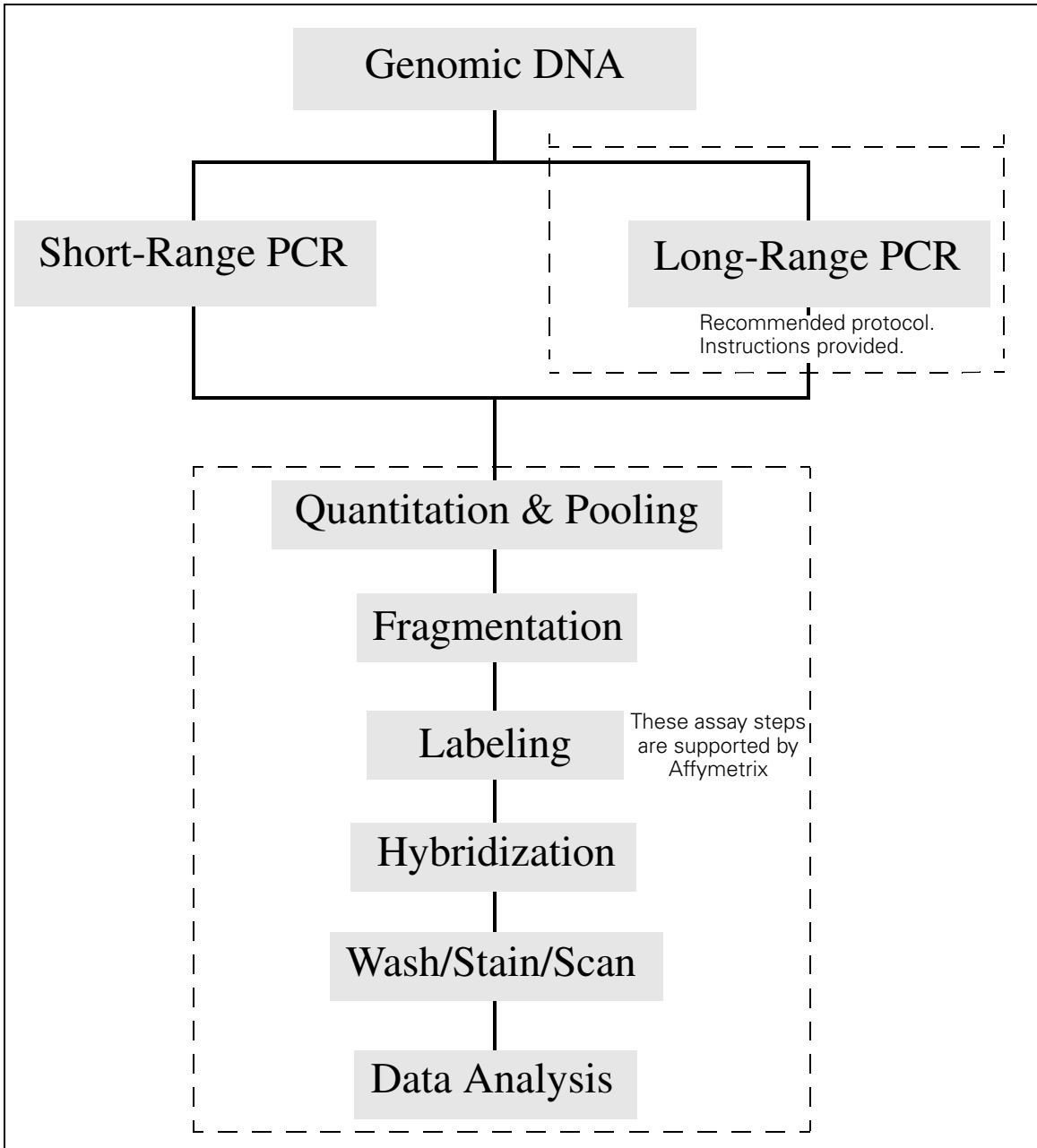


Figure 1.1
Overview of the GeneChip® CustomSeq® Resequencing Array Protocol

Reagents, Equipment and Other Materials Required

Resequencing Assay Workbook

The *Resequencing Assay Workbook* has been created to assist you with calculating the volumes required for various steps in the protocol including:

- Quantitation and pooling
- Fragmentation
- Labeling and hybridization

To use this workbook, navigate to the following address on the Affymetrix web site and download the workbook:

www.affymetrix.com/products/arrays/specific/custom_seq.affx

The workbook consists of the following Excel® worksheets, some of which require user input.

- UV Quantitation - PCR Product Pooling Worksheet
- PicoGreen® Quantitation - Protocol
- PicoGreen® Quantitation - Raw Data Input
- PicoGreen® Quantitation - Standard Curve
- PicoGreen® Quantitation - PCR Product Pooling Worksheet
- Fragmentation Worksheet: Pool Preparation
- Fragmentation Worksheet: Master Mix and Fragmentation
- Labeling and Hybridization Worksheet
- Staining Worksheet

Reagents, Equipment and Other Materials Required

The following reagents, equipment and other materials are required to process CustomSeq® arrays using this protocol.

Table 1.1
Reagents, Equipment and Other Materials Required

Assay Stage	Reagents	Equipment and Worksheets
Long-Range PCR	<ul style="list-style-type: none"> • LA PCR Kit Ver. 2.1: TaKaRa Bio Inc., P/N # RR013A; also available from Fisher, P/N TAKRR013A containing: <ul style="list-style-type: none"> • 10X LA PCR Buffer II (Mg²⁺): 1 mL/vial • dNTP Mixture: 800 µL/vial • TaKaRa LA Taq: 5 units/µL • Molecular Biology Grade Water: Cambrex, P/N 51200 • 1X TE, pH 8: Ambion, P/N 9849 • 99.9% DMSO: Sigma, P/N D-8418 • PCR primers • IQ-Ex Control Template (0.2 pg/µL): Affymetrix, available in the GeneChip® Resequencing Assay Kit, P/N 900447 • Control Primers (20 µM): Forward Primer 1.0 kb, Forward Primer 7.5 kb, Reverse Primer 	<ul style="list-style-type: none"> • Thermal Cycler

Table 1.1 (Continued)

Reagents, Equipment and Other Materials Required

Assay Stage	Reagents	Equipment and Worksheets
DNA Quantitation & Purification	<p>If using PicoGreen:</p> <ul style="list-style-type: none"> • Quant-iT™ PicoGreen® dsDNA Assay Kit, P/N P-7589 • 1X TE Buffer, pH 8: Ambion, P/N 9849 <p>If using Absorption Spectrophotometry:</p> <ul style="list-style-type: none"> • Spectrophotometer capable of reading 260 nm 	<ul style="list-style-type: none"> • Fluorescent plate reader, or UV/VIS Spec • Nunc 96 MicroWell flat-bottom plates: P/N 269620; also available from VWR, P/N 62409-069 • <i>Resequencing Assay Workbook</i> (PicoGreen only): <ul style="list-style-type: none"> • PicoGreen Quantitation - Protocol • PicoGreen Quantitation - Raw Data Input • PicoGreen Quantitation - Standard Curve • PicoGreen Quantitation - PCR Product Pooling Worksheet • <i>Resequencing Assay Workbook</i> (Absorption Spectrophotometry only): <ul style="list-style-type: none"> • UV Quantitation - PCR Product Pooling Worksheet
PCR Product Purification and Elution	<ul style="list-style-type: none"> • DNA Amplification Clean-Up Kit, to be used with Affymetrix DNA products: Clontech, P/N 636974 (1 plate) or P/N 636975 (4 plates) (each kit also contains RB Buffer) • Molecular Biology Grade Water: Cambrex, P/N 51200 	<ul style="list-style-type: none"> • Manifold - QIAvac multiwell unit: QIAGEN, P/N 9014579 • MicroAmp® Clear Adhesive Films, ABI P/N 4306311 • Jitterbug™ 115 VAC: Boekel Scientific, P/N 130000 • Vacuum Regulator for use during the PCR clean up step. QIAGEN Vacuum Regulator: QIAGEN, P/N 19530 • 96-well PCR Plate

Table 1.1 (Continued)

Reagents, Equipment and Other Materials Required

Assay Stage	Reagents	Equipment and Worksheets
Fragmentation and Labeling	<ul style="list-style-type: none"> • Invitrogen Pre-cast gels: 20% TBE Gel, P/N EC63155BOX • SYBR® Gold: Molecular Probes, P/N S11494 • DNA Marker 50-2500 bp: Cambrex, P/N 50631 • Molecular Biology Grade Water: Cambrex, P/N 51200 • From Box 3 of the GeneChip® Resequencing Assay Kit, Affymetrix, P/N 900447: <ul style="list-style-type: none"> • GeneChip® Fragmentation Reagent • 10X Fragmentation Buffer • GeneChip® DNA Labeling Reagent • Terminal Deoxynucleotidyl Transferase (30 U/μL) • 5X Terminal Deoxynucleotidyl Transferase Buffer 	<ul style="list-style-type: none"> • Thermal Cycler • XCell SureLock™ Mini-Cell 1 EI0001 (or comparable gel apparatus) • <i>Resequencing Assay Workbook</i>: <ul style="list-style-type: none"> • Fragmentation Worksheet: Pool Preparation • Fragmentation Worksheet: Master Mix and Fragmentation • Labeling and Hybridization Worksheet
Hybridization	<ul style="list-style-type: none"> • 5 M Tetramethylammonium chloride solution (TMAC): Sigma, P/N T3411 • Tween-20 (10% solution): Pierce, P/N 28320 • Acetylated Bovine Serum Albumin (BSA) solution conc. 50 μg/μL: Invitrogen, P/N 15561-020 • Herring sperm DNA: Promega Corp., P/N D1811 • Tris-HCl, 1 M, pH 7.8: Sigma, P/N T-2913 • Molecular Biology Grade Water: Cambrex, P/N 51200 • Oligonucleotide Control Reagent: Affymetrix, P/N 900440, available in Box 3 of the GeneChip® Resequencing Assay Kit, P/N 900447 	<ul style="list-style-type: none"> • GeneChip® Hybridization Oven • <i>Resequencing Assay Workbook</i>: <ul style="list-style-type: none"> • Labeling and Hybridization Worksheet

Table 1.1 (Continued)

Reagents, Equipment and Other Materials Required

Assay Stage	Reagents	Equipment and Worksheets
Washing and Staining	<ul style="list-style-type: none"> • SAPE: Molecular Probes, P/N S-866 • Anti-streptavidin antibody (goat), biotinylated: Vector Labs, P/N BA-0500 • 20X SSPE (3 M NaCl, 0.2M NaH₂PO₄, 0.02 M EDTA): Cambrex, P/N 51214; Invitrogen, P/N 15991 • Denhardt's Solution, 50X concentrate: Sigma, P/N D2532 • MES hydrate: Sigma-Aldrich, P/N M5287 • MES Sodium Salt: Sigma-Aldrich, P/N M5057 • 5 M NaCl, RNase-free, DNase-free: Ambion, P/N 9760G • Tween-20 (10% solution): Pierce, P/N 28320 • Molecular Biology Grade Water: Cambrex, P/N 51200 • Distilled Water: Invitrogen Life Technologies, P/N 15230147 	<ul style="list-style-type: none"> • GeneChip® Fluidics Station • <i>Resequencing Assay Workbook</i>: <ul style="list-style-type: none"> • Staining Worksheet
Scanning	—	• GeneChip® Scanner 3000

Control Overview

The GeneChip® Resequencing Assay Kit (P/N 900447) must be ordered with CustomSeq resequencing arrays. The purpose of this kit is to control four steps in the assay process: PCR amplification, fragmentation, labeling, and hybridization. The kit includes a plasmid vector containing synthetic (non-naturally occurring) DNA, PCR primers, fragmentation and labeling reagents, and an oligonucleotide hybridization control reagent.

PCR Controls

Affymetrix recommends that every PCR plate processed includes at least one positive control and one negative control.

Positive Controls

For positive control reactions, use the IQ-EX Control Template and a 7.5 or 1.0 kb primer pair (Forward Primer 7.5 kb or Forward Primer 1.0 kb and Reverse Primer). The appropriate primer pair to use is dependent upon the PCR strategy used.

- **For long-range PCR protocols:** use a 7.5 kb primer pair as described under [Stage 1: Performing Long-Range PCR on page 12](#) and in [Appendix A](#).
- **For short-range PCR protocols:** use a 1.0 kb primer pair. Guidelines are provided in [Appendix A](#).

Negative Controls

For negative control reactions, use the same primer pair as used for the positive control. Do **not** include the IQ-EX Control Template in negative controls.

After amplification, an aliquot from all PCR reactions should be run on an agarose gel to ensure amplification of target. The appropriately sized product should be observed for the positive control, and no product should be seen for the negative control. The control product facilitates troubleshooting PCR failure due to issues with PCR conditions versus possible genomic DNA quality issues. PCR controls should be carried throughout the entire assay to serve as controls for the fragmentation and hybridization procedures.

Fragmentation Control

The 7.5 kb or 1.0 kb PCR products should be fragmented, and the product run on a polyacrylamide gel. Appropriately fragmented samples consist largely of 20-200 bp fragments (see [Figure 1.15 on page 47](#)).

Hybridization Control

Hybridization controls should be added to every reaction prior to target hybridization to the CustomSeq array. The Oligonucleotide Control Reagent included in the GeneChip Resequencing Assay Kit contains a gridding control that serves as the hybridization control.

Remember to include this reagent in your hybridization cocktail.

Stage 1: Performing Long-Range PCR

About This Stage

Reaction Volumes

The instructions for the long-range PCR protocol are written for 100 μL reactions and have been optimized for 49-format arrays (30/300 kb).

Alternative volume reactions suitable for smaller format arrays have been validated and are listed below in [Table 1.2](#). If preferred, you can also use 50 μL reactions for 49-format arrays.

Table 1.2
Reaction Volumes

Reagent	Array Format		
	49 (100 μL rxn)	100 (50 μL rxn)	169 (25 μL rxn)
PCR Master Mix			
• Water	33.0 μL	16.7 μL	8.4 μL
• 2.5 mM dNTPs	16.0 μL	8.0 μL	4.0 μL
• 10X LA-Taq buffer w/ Mg	10.0 μL	5.0 μL	2.5 μL
• HS LA-Taq	1.0 μL	0.3 μL	0.1 μL
Primer Pair Solution, diluted to a final concentration of 3 μM /primer/PCR reaction	20.0 μL	10.0 μL	5.0 μL
Genomic DNA (5 ng/ μL)	20.0 μL	10.0 μL	5.0 μL
Total Volume for 1 Reaction	100.0 μL	50.0 μL	25.0 μL

Using 96-Well Plates

Long-range PCR amplification can be conducted in 96-well plates. One recommended plate layout is to keep the primer pairs together in rows so that the genomic DNA can be added using a multichannel pipette. Also consider downstream handling. For example, some robots process samples more efficiently in columns rather than rows.

Reagents Required

The reagents required for this long-range PCR protocol are listed below in [Table 1.3](#).

Table 1.3
Reagents Required

Reagent	Vendor	Part Number
LA PCR Kit Ver. 2.1 containing:	TaKaRa Bio Inc.	RR013A
<ul style="list-style-type: none"> • 10X LA PCR Buffer II (Mg²⁺; 1 mL/vial) • dNTP Mixture (800 µL/vial) • TaKaRa LA Taq (5 units/µL) 	Fisher	TAKRR013A
Molecular Biology Grade Water	Cambrex	51200
1X TE Buffer, pH 8 (dilute 10-fold in water to give 0.1X TE)	Ambion	9849 (or other TE)
99.9% DMSO (optional)	Sigma-Aldrich	D-8418 (optional)
GeneChip® DNA Resequencing Assay Kit	Affymetrix	900447

Prepare the Reagents

Location: Pre-PCR Clean Room

The Pre-PCR Clean Room/work space should be free from template DNA and PCR product. The use of gowns and gloves is recommended to prevent PCR carryover.

Prepare the PCR Primers

Affymetrix recommends ordering oligonucleotides from a qualified vendor. Standard salt-free purification is sufficient for this application. Primers should be tested prior to finalizing the array design to ensure robust amplification.

IMPORTANT !

Include at least one positive and one negative control with every set of PCR reactions. Use the IQ-EX Control Template and primer pairs included in the GeneChip DNA Resequencing Assay Kit. See [Appendix A](#) for more information on controls.

To prepare the PCR primers:

1. Resuspend the oligonucleotides in 0.1X TE Buffer to create a 100 μM stock solution.

Store the stock solution at -20°C .

2. Prepare a Primer Pair Working Solution by combining the reagents listed in [Table 1.4](#).

The Primer Pair Working Solution concentration is 10 μM per primer.

Table 1.4
Primer Pair Working Solution

Reagent	Volume
Forward primer (100 μM)	100 μL
Reverse primer (100 μM)	100 μL
0.1X TE Buffer	800 μL
TOTAL	1000 μL

3. For each reaction, aliquot the appropriate volume of Primer Pair Working Solution to one of the following:

- Each well to be used of a 96-well PCR plate
- A microcentrifuge tube appropriate for performing PCR (one tube per reaction)

100 μL rxns	50 μL rxns	25 μL rxns
6 μL	3 μL	1.5 μL

TIP 

Primer Pair Working Solution can be aliquoted into each well of a 96-well plate and stored at -20°C until ready to use.

Prepare the Genomic DNA

The genomic DNA used in this assay must be of high quality. In particular, ensure that the DNA is free from any PCR inhibitors or proteins. In some cases, you may want to include DMSO. See [Using DMSO](#) below for guidelines.

To prepare the genomic DNA:

1. Measure the concentration of genomic DNA by using a reagent such as PicoGreen® or by absorbance spectrophotometry.
2. Dilute the DNA to 5 ng/μL in molecular biology grade water.
3. Optional: Aliquot diluted DNA to 96-well PCR plates.

100 μL rxns	50 μL rxns	25 μL rxns
20 μL	10 μL	5 μL

Using this storage method, you can quickly add DNA to PCR mixes using a multichannel pipette.

4. Store the diluted DNA at –20°C.

IMPORTANT !

Do not subject frozen aliquots to more than 3 freeze/thaw cycles.

Using DMSO

DMSO is useful in some problematic PCRs. In others, it is unnecessary and even inhibitory. For templates with high GC content, we recommend using DMSO to a final concentration of up to 5% and reducing the volume of water in the reaction accordingly.

To prepare DMSO:

1. Dilute the DMSO to 50% with molecular biology grade water.
2. Store at 4°C.

Prepare the Pre-Aliquoted Primer Pair Solution

NOTE

To amplify the control plasmids (IQ-EX Control Template) for long-range PCR, use the Forward Primer 7.5 kb and Reverse Primer from the GeneChip DNA Resequencing Assay Kit.

To prepare the pre-aliquoted Primer Pair Solution:

1. If frozen, allow the aliquots of Primer Pair Working Solution to thaw.
2. Referring to [Table 1.5](#), dilute the Primer Pair Working Solution by adding the appropriate volume of molecular biology grade water to each tube/well.
3. Transfer the tubes/plate to the PCR Staging Room.

Table 1.5

Final Volume of Diluted Primer Pair Working Solution

Reagent	Array Format and Reaction Volume		
	49 (100 μ L)	100 (50 μ L)	169 (25 μ L)
Primer Pair Working Solution	6 μ L	3 μ L	1.5 μ L
Molecular Biology-Grade Water	14 μ L	7 μ L	3.5 μ L
Total Volume for 1 Reaction	20 μL	10 μL	5 μL

Add Genomic DNA and PCR Master Mix

Location: PCR Staging Room

The PCR Staging Room/work space should be free from any PCR product. The use of gowns and gloves is recommended to prevent PCR carryover.

Add Genomic DNA

IMPORTANT !

If processing more than one sample per plate, carefully track which wells are being used for each sample.

	1	2	3	4	5	6	7	8	9	10	11
A	JB100										
B	JB100										
C	JB100										
D	JB101										
E	JB101										
F	JB101										
G											
H											

Figure 1.2

Two Unique Samples Processed on the Same Plate

To add genomic DNA:

1. Add genomic DNA to each tube/well being used.

49 Format	100 Format	169 Format
20 μ L	10 μ L	5 μ L

The total volume of each tube/well should now be 40 μ L.

Carefully track which tubes/wells are to be pooled if processing more than one unique sample at a time (Figure 1.2).

2. For each set of PCR reactions, add one positive and one negative control.

Use the IQ-EX Control Template and primers included in the GeneChip DNA Resequencing Assay Kit. See [Appendix A](#) for more information.

Prepare and Add PCR Master Mix

Table 1.6
Final Volume in Each Tube/Well

Reagent	49 Format	100 Format	169 Format
Diluted Primer Pair Working Solution	20 μ L	10 μ L	5 μ L
Genomic DNA	20 μ L	20 μ L	5 μ L
Total Volume for 1 Reaction	40 μL	20 μL	10 μL

To minimize primer degradation by the polymerase, begin thermal cycling as soon as possible after adding the PCR Master Mix to the DNA/primers.

To prepare and add PCR Master Mix:

1. Prepare the PCR Master Mix as shown in [Table 1.7](#) and keep on ice.

Keeping the PCR Master Mix on ice prevents primer degradation from the proofreading activity of the polymerase.

Table 1.7
PCR Master Mix

Reagent	49 Format	100 Format	169 Format
Water	33 μ L	16.7 μ L	8.4 μ L
2.5 mM dNTPs (from TaKaRa Kit; final concentration 400 μ M)	16 μ L	8 μ L	4 μ L
10X LA PCR buffer(Mg ²⁺) (from TaKaRa Kit; final concentration 1X (2.5 mM Mg ²⁺))	10 μ L	5 μ L	2.5 μ L
LA Taq enzyme (from TaKaRa Kit; final concentration 5 U/100 μ L)	1 μ L	0.3 μ L	0.1 μ L
Total Volume for 1 Reaction	60 μL	30 μL	15 μL

2. Add the appropriate volume of PCR Master Mix to each tube/well.

49 Format	100 Format	169 Format
60 μ L	30 μ L	15 μ L

For each reaction:

Final Primer concentration = 600 nM (each primer)

Final DNA template = 100 ng/100 μ L Rxn

= 50 ng/50 μ L Rxn

= 25 ng/25 μ L Rxn

3. Seal the tubes/plate and keep on ice.
To avoid primer degradation by the polymerase, keep the PCR Master Mix and DNA/primer mix cold until the thermal cycling reaction starts.
4. Transfer the tubes/plate to the Main Lab and begin PCR as soon as possible.

Perform PCR

Location: Main Lab

The Main Lab has airborne contamination with PCR product and template. After entering the main lab it is not advisable to re-enter either the Pre-PCR Clean Room or the PCR Staging Area.

PCR Procedure

To minimize primer degradation by the polymerase, begin PCR as soon as possible after adding PCR Master Mix to the DNA/primers.

To perform PCR:

1. Preheat the thermal cycler block to 94°C.
2. When the block has reached 94°C, load the PCR reaction plates/tubes onto the thermal cycler and run the following program:

Table 1.8
Long-Range PCR Thermal Cycling Program

Temperature	Time	Cycles
94°C	2 minutes	1X
94°C 68°C	15 seconds 1 minute/kb	30X
68°C	5 minutes + 1 minute/kb	1X
4°C	HOLD	

NOTE 

Thermal cycling conditions are the same for all reaction volumes.

The following thermal cyclers¹ have been successfully used with this assay.

- DNA Engine Tetrad® PTC-225, MJ Research²
- GeneAmp® PCR System 9700

Thermal cyclers not listed here may still work; however, only the ones listed above have been tested and verified for use with this assay.

Verify the PCR Reaction

To verify the PCR reaction:

1. Run 2 to 4 µL of each reaction on a 1% TBE agarose gel.
2. Confirm that bands of an appropriate size are present.
3. If not proceeding directly to the next step, store samples at –20°C.

¹ The PCR process is covered by patents owned by Roche Molecular Systems, Inc. and F. Hoffmann-LaRoche Ltd (“Roche”). A license to use the PCR process for certain research and development activities accompanies the purchase of certain reagents from licensed suppliers when used in conjunction with an authorized thermal cycler. If you are using an MJ Research thermal cycler, your thermal cycler may not be an authorized thermal cycler. You should obtain authorization from Roche or ABI (see PCR licensing information in the MJ Research User Manual, if you are not already licensed). For information about obtaining a license contact The Director of Licensing at Applied Biosystems, 850 Lincoln Center Drive, Foster City, CA 94404 or the Licensing Department, Roche Molecular Systems, Inc., 1145 Atlantic Avenue, Alameda, CA 94501.

² In 2004 MJ GeneWorks, Inc. and its subsidiary MJ Research, Inc. were purchased by Bio-Rad Laboratories, Inc. This model is no longer available. Bio-Rad has indicated that the DNA Engine Tetrad 2 gives similar performance with the same programs. Affymetrix has not tested the newer version.

Stage 2: Quantitation and Pooling of PCR Products

The efficiency of a PCR reaction can vary between samples. **Assay performance may be compromised if amplicon concentration during hybridization varies by more than tenfold.** Therefore to achieve the maximum amount of sequence information from a single hybridization, similar quantities of each PCR reaction should be applied to the CustomSeq array.

Both of the quantitation methods described here are based on applying each PCR amplicon to the array at a concentration of 250 picomolar.

About Quantitation and Pooling

Quantitation and pooling are critical steps in the CustomSeq assay. Two methods are available for quantifying PCR products:

- PicoGreen®
- Absorption spectrophotometry

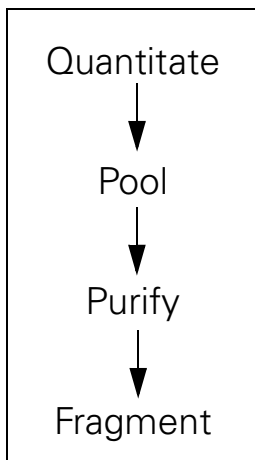
Generally, we recommend using the PicoGreen method over absorption spectrophotometry, particularly when working with a large number of PCR amplicons.

The basic mechanics of quantitation and pooling are described in this section. The instructions include using the *Resequencing Assay Workbook* which must be downloaded from the Affymetrix website.

The workbook contains Excel worksheets with embedded formulas. Several of the worksheets require user input. Once you have input the required data, the information (e.g., volumes) required to continue with the protocol are calculated automatically. Worksheets for both the PicoGreen and absorption spectrophotometry methods are included.

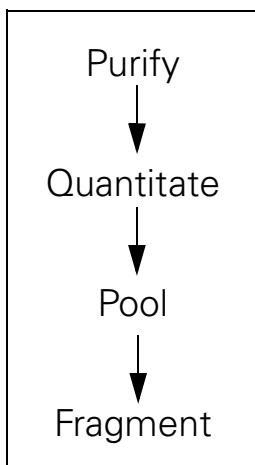
About the PicoGreen® Method

PicoGreen is a double-stranded DNA-specific dye. Using PicoGreen allows for quantitation and pooling of amplicons before proceeding to the purification step for the removal of primers and dNTPs. The advantage of this method is that it reduces the total number of columns used for purification.



About the Absorption Spectrophotometry Method

If using absorption spectrophotometry, the primers and dNTPs must be removed prior to quantitation and pooling to avoid interference in the quantitation step. Therefore, each amplicon be purified separately.



PCR Product Pooling Table

The PCR product size ranges from 1,000 to 30,000 basepairs (bp). Use the information in [Table 1.11 on page 36](#) to extrapolate the amount (ng) of PCR product to pool for each quantitation method.

The values in the PicoGreen column represent twice the amount of PCR product needed to compensate for any loss of product during the cleanup step.

Reagents, Equipment and Other Materials Required for the PicoGreen® Method

Reagents and Equipment Required

The reagents and equipment required for the PicoGreen method are listed in [Table 1.9](#).

Table 1.9

Reagents and Equipment required for the PicoGreen Method

Reagent	Vendor	Part Number
From the Quant-iT™ PicoGreen® dsDNA Assay Kit: <ul style="list-style-type: none"> • PicoGreen reagent • Lambda DNA standard 	Invitrogen	P-7589
1X TE Buffer	Ambion	9849
Fluorescence spectrophotometer or plate reader	—	—
96 MicroWell™ plates, flat-bottom	Nunc	269620
	VWR	62409-068

Worksheets Required

Use the following worksheets from the *Resequencing Assay Workbook*

- PicoGreen® Quantitation - Protocol
- PicoGreen® Quantitation - Raw Data Input
- PicoGreen® Quantitation - Standard Curve
- PicoGreen® Quantitation - PCR Product Pooling Worksheet

Equipment and Other Materials Required for Absorption Spectrophotometry

Equipment Required

The equipment required for the absorption spectrophotometry method are listed in [Table 1.10](#).

Table 1.10

Equipment and Other Materials Required for the Absorption Spectrophotometry Method

Reagent	Vendor	Part Number
Spectrophotometer capable of reading 260 nm	—	—
96 MicroWell™ plates, flat-bottom	Nunc	269620
	VWR	62409-068

Worksheets Required

Use the following worksheet from the *Resequencing Assay Workbook*

- UV Quantitation - PCR Product Pooling Worksheet

PicoGreen® Method

Quantitate the PCR Products

Determine the concentration of each PCR product in nanograms per microliter (ng/μL) by following the steps below.

To quantitate PCR products using PicoGreen:

1. Download the *Resequencing Assay Workbook* from:
www.affymetrix.com/products/arrays/specific/custom_seq.affx
See [Resequencing Assay Workbook on page 5](#) for more information.
2. Open the workbook and select the tab *PG Protocol*.
The *PicoGreen Quantitation - Protocol* worksheet summarizes this procedure.
3. Label one flat-bottom plate *Master*, and another flat-bottom plate *PicoGreen*.
4. To the plate labeled Master:
 - A. Pipet 300 μL of Lambda DNA standard at a concentration of 5.120 ng/μL into well A12.
 - B. Add 150 μL of 1X TE Buffer to column 12, wells B12 through H12 (see [Figure 1.3](#)).
 - C. Make 2-fold serial dilutions by transferring 150 μL of DNA standard into each subsequent well until you reach a concentration of 0.04 ng/μL.
 - D. Add 298 μL of 1X TE Buffer to each empty well.
 - E. Transfer 2 μL of each PCR product to an empty well on the Master plate (one amplicon per well) and mix well.

IMPORTANT !

Remember to include the IQ-EX Control Template.

Master Plate		1	2	3	4	5	6	7	8	9	10	11	12		
A	JB100	Aliquot 2 μ L/well of each PCR product including controls (one amplicon per well)											5.12	std, ng/uL	
B	JB100												2.56		
C	JB100												1.28		
D	JB101												0.64		
E	JB101												0.32		
F	JB101												0.16		
G													0.08		
H													0.04		

DNA Standard
in 1X TE Buffer
↓

Figure 1.3
Master Plate Layout for PicoGreen Quantitation Protocol

5. Prepare 20 mL of 2X PicoGreen by adding 100 μ L of concentrate to 19.9 mL of 1X TE Buffer.
6. To the plate labeled PicoGreen:
 - A. Add 100 μ L of 2X PicoGreen to each well.
 - B. Select the *PG Raw Data Input* tab in the workbook.
The *PicoGreen Quantitation – Raw Data Input* worksheet is displayed.
 - C. Take a blank reading of the plate.
 - D. Enter the readings in the first table of the worksheet ([Figure 1.4](#)).
 - E. Using a multichannel pipette, transfer 100 μ L from each well of the Master plate to the corresponding well of the PicoGreen plate.

1. Enter Blank PicoGreen Readings Here							
Blank	1	2	3	9	10	11	12
A	10715						9032
B	9518						10112
C	10460						8999
D	10861						10900
E	8987						9350
F	10163						10488
G							9000
H							9800

Columns 4 through 8 intentionally removed for display purposes only.

Figure 1.4
Example of Blank PicoGreen Readings

7. Take a reading of the PicoGreen plate.
8. Enter the readings into the second table of the worksheet (Figure 1.5).

2. Enter Amplicon PicoGreen Readings Here							
Amplicon	1	2	3	9	10	11	12
A	16422						41821
B	18701						32482
C	16873						21134
D	17854						17055
E	18633						13489
F	16056						12540
G							10378
H							10836

Columns 4 through 8 intentionally removed for display purposes only.

Figure 1.5
Example of Amplicon/PicoGreen Mixture Readings

Pool the PCR Products

To pool the PCR products:

1. Select the *PG Standard Curve* tab in the workbook to view the *PicoGreen Quantitation - Standard Curve* worksheet.

Notice that the standard curve has been calculated automatically based on the information entered on the *PicoGreen Quantitation - Raw Data Input* worksheet.

2. Ensure that:
 - The curve is linear
 - The R value is > 0.9

If the R value is less than 0.9, read the plate again. If still less than 0.9, reject the plate and repeat the experiment.

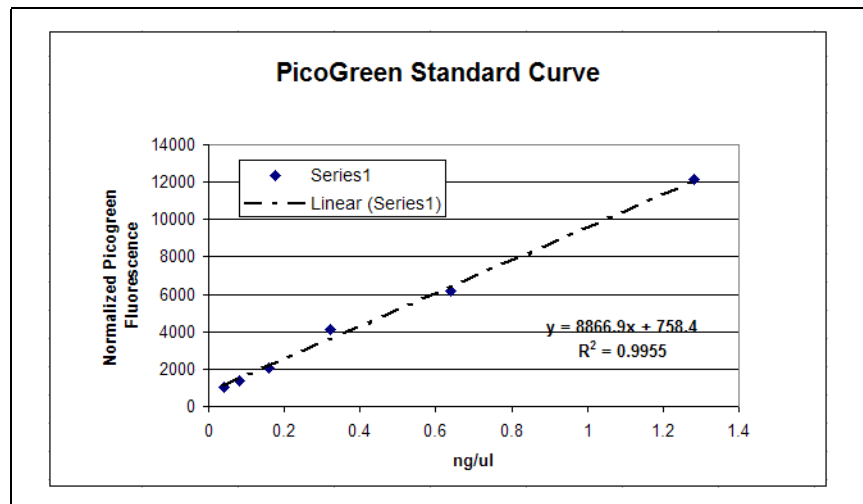


Figure 1.6
Example of Standard Curve

3. Select the *PG Pooling* tab in the workbook and enter the following on the *PicoGreen® Quantitation - PCR Product Pooling Worksheet* (Figure 1.7 and Figure 1.8):
 - Net PCR volume (after gel analysis and quantitation)
 - DNA Amount/Amplicon pM/Array (250, 150 or 111.7)
 - Pico Dilution Factor
 - Number of Replicates (number of arrays per unique sample)

- Sample Name and Lot Number (optional)
- In the Amplicon Name and Size columns, enter a meaningful identifier for each amplicon and its size in bp.
IMPORTANT If more than one sample per plate, carefully track which PCR reactions go with which sample.
Remember to include the IQ-EX Control Template.

14	Net PCR Volume:	100	ul		
15					
16		49 Format	100 Format	169 Format	
17	DNA Amount / Amplicon pM / Array:	250	150	111.7	
18					
19	DNA Amount / Amplicon pM / Array:	250.0	pM		Enter a value from row 17.
20					
21	Pico Dilution Factor:	150			
22					
23	Number of Replicates:	3	0		
24					
25	Sample Name (optional):				
26	Lot Number (optional):				

Figure 1.7

Example of User Entered Information on PicoGreen Quantitation – PCR Product Pooling Worksheet

You must enter information in these columns			Data in these columns calculated automatically based on user input					
Wells	Amplicon Name	Size (bp)	Concentration		mol/L [M]	fmol/ul	Number of Replicates Possible	ul/Amplicon to Pool for # of Replicates (row 23)
			[ng/ul]	[ug/ul]				
A01	JB100-1	5500	83.71	0.083715	2.31E-08	23.1	9.2	14.3
B01	JB100-2	5500	142.52	0.142518	3.93E-08	39.3	15.7	8.4
C01	JB100-3	5500	95.66	0.095658	2.64E-08	26.4	10.5	12.5
D01	JB101-1	7800	105.47	0.10547	2.05E-08	20.5	8.2	16.1
E01	JB101-2	7800	150.35	0.15035	2.92E-08	29.2	11.7	11.3
F01	JP101-3	7800	86.86	0.086861	1.69E-08	16.9	6.7	19.6

Figure 1.8

Example of User Entered Information and Automatic Calculations on the PicoGreen Quantitation – PCR Product Pooling Worksheet

4. Pool the PCR products for each sample in a new 1.5 mL tube/well by adding the volumes displayed on the worksheet (column labeled *μL/Amplicon to Pool for Number of Replicates*).
5. Repeat this process for each sample.
6. Optional: Store the pooled PCR products and any remaining unpooled products at -20°C .

What to do Next

1. Clean up the pooled PCR products as described in [Stage 3: PCR Purification and Elution on page 37](#).
2. Quantify and fragment the purified, pooled PCR products as described in [Stage 4: Fragmentation on page 40](#).

Absorption Spectrophotometry Method

NOTE 

We recommend repeating the steps listed for absorption spectrophotometry for each unique DNA sample.

A dilution of your product may be needed if the volumes to pool are below the stated accuracy of the pipettes. Such dilutions can be made using Clontech RB buffer.

Clean Up the PCR Products

PCR products must be cleaned up before absorption spectrophotometry and pooling.

To cleanup the PCR products:

1. Clean up each PCR product by following the instructions in *Stage 3: PCR Purification and Elution* on page 37.
2. Once the products have been cleaned up, proceed to *Quantitate the Purified PCR Products* below.

Quantitate the Purified PCR Products

NOTE 

Use RB Buffer as a blank when determining concentration.

To quantitate the purified PCR products:

1. Select the *UV Pooling* tab in the *Resequencing Assay Workbook*. The *UV Quantitation - PCR Product Pooling Worksheet* is displayed.
2. Follow the absorption spectrophotometry manual instructions.
3. Enter the UV absorbance reading for each amplicon in the UV Absorbance column (*Figure 1.9* on page 32).

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

Pool the PCR Products

To pool the PCR products:

- Enter the following on the *UV Quantitation - PCR Product Pooling Worksheet*:
 - Net PCR Volume (after running the gel)
 - DNA Amount/Amplicon pM/Array
 - UV Dilution Factor
 - Number of Replicates (arrays per sample)
 - Sample Name and Lot Number (optional; assumes one unique sample per plate)
 - In the Amplicon Name and Size columns, enter a meaningful identifier for each amplicon and its size in bp (Figure 1.9).

IMPORTANT If more than one unique sample per plate, carefully track which PCR reactions go with which sample.

IMPORTANT !

Remember to include the IQ-EX Control Template.

You must enter information in these columns				Data in these columns calculated automatically based on user input		
Wells	Amplicon Name	Size (BP)	UV Absorbance	Concentration (ng/ul)	Number of Replicates Possible	ul/Amplicon to Pool for # of Replicates (row 26)
A01	XM100-1	4000	0.3	1,500.00	227.3	18.6
B01	XM100-2	5500	0.2	1,000.00	110.2	38.3
C01	XM100-3	7800	0.4	2,000.00	155.4	27.2

Figure 1.9

Example of table from the UV Quantitation - PCR Product Pooling Worksheet

- Pool the PCR products for each unique sample in a new 1.5 mL tube/well by adding the volumes displayed on the worksheet (column labeled *ul/Amplicon to Pool for Number of Replicates*).
- If necessary, repeat this process for each unique sample.

-
4. Bring the volume of each pool to 38.3 μ L by adding Clontech RB buffer.

IMPORTANT 

Pooled samples must be suspended in Clontech RB buffer, as the fragmentation reaction has been optimized for this buffer.

5. Do one of the following:
 - Quantify and fragment the purified, pooled PCR samples as described in [Stage 4: Fragmentation on page 40](#).
 - Optional: Store the pooled PCR products and any remaining unpooled products at -20°C .

Extrapolating Values for PCR Product Pooling

The nanogram amount for any size amplicon can be determined by plotting the values from either of the PCR product pooling worksheets (*UV Quantitation – PCR Product Pooling Worksheet* or *PicoGreen Quantitation – PCR Product Pooling Worksheet*).

In [Figure 1.11](#), the nanogram values for the absorption spectrophotometry method have been plotted against their size in base pairs. Using the equation for that line, the values for additional PCR amplicons can be determined (example shown in [Table 1.11 on page 36](#)).

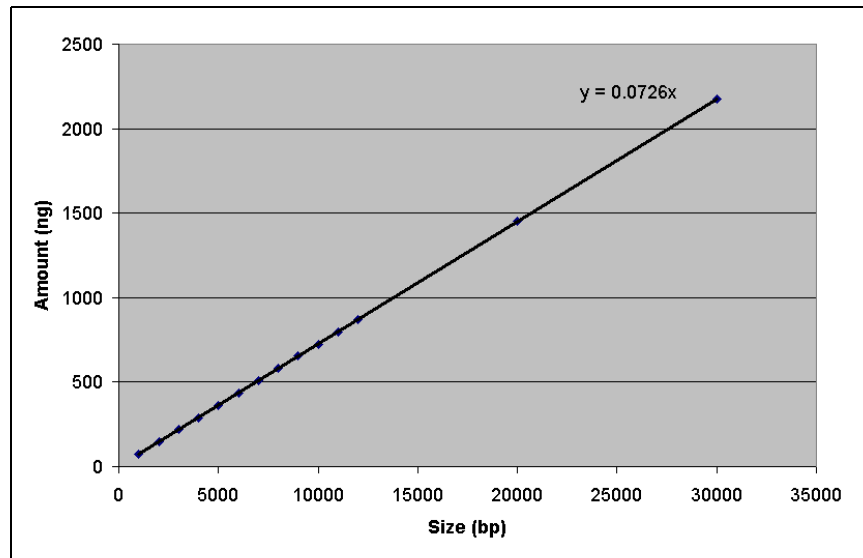


Figure 1.10
PicoGreen® Method: Plotting the Amount of PCR Product to Pool

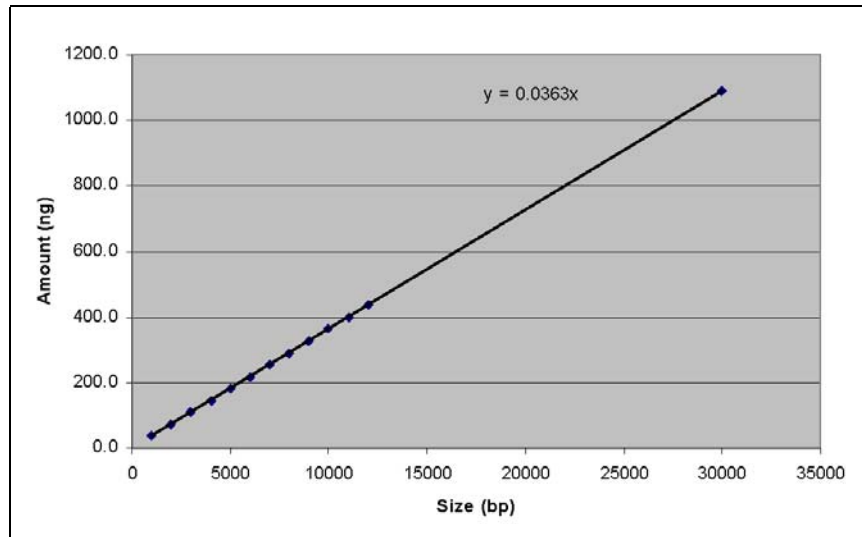


Figure 1.11
Absorption Spectrophotometry: Plotting the Amount of PCR Product to Pool

Table 1.11
 Extrapolating the Nanogram Amount of Additional PCR Products

	Size of Amplicon (bp)	PicoGreen® Method from Figure 1.8 on page 29	Absorption Spectrophotometry Method from Figure 1.9 on page 32
Values calculated automatically using the worksheets	1,000 bp	72.6 ng	36.3 ng
	2,000 bp	145.2 ng	72.6 ng
	3,000 bp	217.8 ng	108.9 ng
	4,000 bp	290.4 ng	145.2 ng
	5,000 bp	363.0 ng	181.5 ng
	6,000 bp	435.6 ng	217.8 ng
	7,000 bp	508.2 ng	254.1 ng
	8,000 bp	580.8 ng	290.4 ng
	9,000 bp	653.4 ng	326.7 ng
	10,000 bp	726.0 ng	363.0 ng
	11,000 bp	798.6 ng	399.3 ng
	12,000 bp	871.2 ng	435.6 ng
	20,000 bp	1,452.0 ng	726.0 ng
	30,000 bp	2,178.0 ng	1,089.0 ng
Formula	Size of Amplicon	y = 0.0726x	y=0.0363x
Values calculated using formula	4,000 bp	290.4 ng	145.2 ng
	5,500 bp	399.3 ng	199.7 ng
	7,800 bp	566.3 ng	283.1 ng

Stage 3: PCR Purification and Elution

Reagents, Equipment and Other Materials Required

Reagents Required

The reagents required for PCR purification and elution are listed in [Table 1.12](#).

Table 1.12
Reagents Required for Purification and Elution

Reagent	Vendor	Part Number
DNA Amplification Clean-Up Kit (to be used with Affymetrix DNA products; each kit contains RB Buffer)	Clontech	636974 (1 plate) or 636975 (4 plates)
Molecular Biology Grade Water	Cambrex	51200

Equipment Required

The equipment and other materials required for PCR purification and elution are listed in [Table 1.13](#).

Table 1.13
Equipment and Other Materials Required for PCR Purification and Elution

Reagent	Vendor	Part Number
Manifold - QIAvac multiwell unit	QIAGEN	9014579*
96-well Plate	—	—
MicroAmp® Clear Adhesive Films	Applied Biosystems	4306311
Jitterbug™ 115 VAC	Boekel Scientific	130000
QIAGEN Vacuum Regulator (For use during the PCR clean up step)	QIAGEN	19530†

* Clontech also supports the Millipore MultiScreen®, Promega Vac-Man® 96, and Bio-Rad Aurum vacuum manifolds for use with the Clean-Up Plates.

† The Clontech protocol requires ~600 mbar vacuum. If your lab does not have an internally regulated vacuum source, this vacuum regulator is strongly suggested.

Purification

Follow the steps as outlined below. Consult the Clontech Clean-Up Plate Handbook for the general procedure and ordering information.

NOTE

If the PicoGreen method was used for quantitation, the volume of pooled sample may exceed the capacity of one well. If this is the case, split the pooled sample between two to four wells and elute each well with 50 μ L of RB buffer ([Step 1 on page 39](#)).

CAUTION

When pipetting into the wells of the Clean-Up Plate, be careful not to pierce the membrane.

Make sure the orientation of PCR plates is consistent. Use a method of distinguishing between used and unused wells on the plate.

To purify the PCR products:

1. Place a Clean-Up Plate on top of the vacuum manifold.
2. Seal any wells that are not to be used.
A PCR plate seal/film or an aluminum foil lid can be used. Place the seal/lid on top of the Clean-Up Plate. Apply pressure to make the cover stick to the plate. Then, using a razor, cut between the used and unused wells. Remove the portion that covers the wells you want to use.
3. Transfer each amplicon or pooled sample into one well of the Clean-Up Plate.
4. Apply a vacuum and maintain at ~600 mbar until the wells are completely dry.
5. Wash the PCR products by adding 50 μ L molecular biology grade water and dry the wells completely (approximately 20 minutes).
6. Repeat the wash 1 additional time for a total of 2 water washes.
7. Switch off vacuum source and release the vacuum.
8. Carefully remove the Clean-Up Plate from the vacuum manifold.
9. Gently tap the Clean-Up Plate on a stack of clean absorbent paper to remove any liquid that might remain on the bottom of the plate.

Elute the Samples

To elute the samples:

1. Add 50 μ L RB Buffer to each well.
2. Tightly seal the plate a PCR plate film/seal.
3. Moderately shake the Clean-Up Plate on a plate shaker at 1000 rpm for 10 minutes at room temperature.
Use the Jitterbug (Boekel Scientific, model 130000).
4. Working one well at a time, pipette the eluate from each well and transfer it to the corresponding well of a fresh 96-well plate.
For easier recovery, hold the plate at a slight angle.

What To Do Next

Do one of the following:

- If you used the PicoGreen method for sample quantitation, proceed to *Stage 4: Fragmentation* on page 40.
- If you are using absorption spectrophotometry for sample quantitation, proceed to *Absorption Spectrophotometry Method* on page 31.

Stage 4: Fragmentation

The fragmentation reaction is an enzymatic reaction that is sensitive to time and temperature. It is important to strictly observe the conditions of this reaction, as any changes in conditions can lead to sub-optimal product.

Reagents Required

Table 1.14
Reagents Required

Reagent	Vendor	Part Number
From Box 3 of the GeneChip® Resequencing Assay Kit: <ul style="list-style-type: none"> • GeneChip® Fragmentation Reagent • GeneChip® 10X Fragmentation Buffer 	Affymetrix	900447
RB Buffer	Clontech	DNA Amplification Clean-Up Kit P/N 636974 or P/N 636975
20% TBE Gel: Invitrogen	Invitrogen	EC63155BOX
DNA Marker 50-2500 bp	Cambrex	50631
SYBR® Gold	Molecular Probes	S11494

The formulations below are optimized for 49-format arrays (30/300 Kb). Formulations for alternative reaction volumes for use with small array formats can be found [Table 1.15 on page 45](#). If preferred, 50 µL reactions could also be used for 49-format arrays.

IMPORTANT !

The GeneChip® Fragmentation Reagent contains an active enzyme. The following precautions are recommended when handling this reagent:

- Store the Fragmentation Reagent at -20°C until ready to use.
- Minimize repeated exposure to temperatures $>0^{\circ}\text{C}$.
- Keep in a bench top cooler when handling the reagent.
- Prepare all dilutions, additions, and mixing steps ON ICE.

Confirm Activity of the Fragmentation Reagent

The activity of the Fragmentation Reagent is listed on the reagent tube label.

To confirm activity of the Fragmentation Reagent:

Test the fragmentation reaction with a control sample prior to fragmenting all of the PCR products in the experiment.

A small titration range of the Fragmentation Reagent (for example, 0.010, 0.015, 0.020, 0.025, 0.030 U/ μg) can be employed to optimize fragmentation conditions for the particular PCR and purification conditions that exist in your laboratory.

[Figure 1.15 on page 47](#) includes an example of a properly fragmented amplicon (expect a smear in the range of approximately 20-200 bp).

Quantitate and Dilute the Pooled PCR Products

You will now quantitate and dilute the pooled PCR products.

Complete the worksheet, *Fragmentation Worksheet: Pool Preparation*, for pools of the same size. For example, if the amplicon sizes for 30 pools total 16500 bp each, and the amplicon sizes for 20 pools total 14000 bp each, complete the worksheet twice: once for the pools that total 16500 bp each; and once for the pools that total 14000 bp each.

To quantitate and dilute the pooled PCR products:

1. Select the *Fragmentation Pool Prep* tab in the workbook.

Fragmentation Worksheet: Pool Preparation is displayed.

2. Enter the following values under *Pool Preparation* (Figure 1.12 on page 43):

- Sample (PCR Pool) Designation: A name for the PCR pool
- Sum of Amplicon Sizes (bp) per Pool

This value should be the same for each pool. Do not combine pools of varying sizes on the same worksheet.

Add the size of each amplicon in one pool for a unique sample (e.g. $5500 + 5500 + 5500 = 16500$ bp)

- UV₂₆₀ Absorbance: the UV absorbance reading for this pool
- Dilution Factor: the dilution factor

The volume of pooled PCR product required for fragmentation is calculated automatically (μL of Pool Required).

3. Based on the array format you are using, dilute the pooled PCR product as follows:

- A. Transfer the volume of the pool indicated on the worksheet (μL of Pool Required) to a new PCR plate/tube, one aliquot per pool per well/tube.
- B. Add the volume of RB Buffer and 10X Fragmentation Buffer indicated on the worksheet to each aliquot from each pool.

4. Place the tubes/plate on ice.

Fragmentation Worksheet: Pool Preparation

** Enter information in all GREEN HIGHLIGHTED cells. Calculations are made automatically based on data input.

IMPORTANT: The size of each pool (sum of amplicon sizes in bp) must be the same for each pool entered. If pools vary in size, you will need to complete this worksheet multiple times.

Experiment Name (optional):

Sample sequence (optional):

Pool Preparation

Sample (PCR Pool) Designation	Sum of Amplicon Sizes (bp) per Pool	UV ₂₆₀ Absorbance	Dilution Factor	ug/ul	49 Format		
					pmol/Array		
						250	
					uL of Pool Required	uL RB	uL 10x Frag Buffer
JB100	16500	0.3749	10	0.2	3.2	35.1	4.3
JB101	16500	0.4450	10	0.2	2.7	35.6	4.3
JB102	16500	0.6650	10	0.3	1.8	36.5	4.3

This value must be the same for each pool entered. If pools vary in size, you will need to complete the worksheet multiple times.

Figure 1.12

Example of Pool Preparation Worksheet (Columns for 100 and 169 Format Arrays Not Shown)

Prepare the Fragmentation Master Mix

To prepare the Fragmentation Master Mix:

1. Select the *Fragmentation* tab in the workbook.

Fragmentation Worksheet: Master Mix and Fragmentation is displayed.

2. In Table 1. *Calculate the Number of Samples Required for 1 μ L Fragmentation Reagent*, enter the activity of the Fragmentation Reagent (as confirmed under *Confirm Activity of the Fragmentation Reagent* on page 41).

Once this value is entered, the volume of each reagent required for the Fragmentation Master Mix is calculated automatically in Table 2 on the worksheet.

1. Calculate the Number of Samples Required for 1 μ L Fragmentation Reagent*		49 Format	100 Format	169 Format
Total ug product to be applied to the array	ug	0.60	0.4	0.27
Units Fragmentation Reagent/ug Product **	U/ug	0.150	0.150	0.150
Units of Fragmentation reagent U required	Total U	0.09	0.054	0.04
Activity of Fragmentation reagent (on the label of the reagent tube) **	U/ul	3.00	3.0	3.00
Volume of Fragmentation reagent required (ul)	ul	0.030	0.0180	0.0134
Volume of water	ul	3.570	2.1	1.61
Minimum Number of Reactions to Prepare		33	56	75

Figure 1.13

Enter Activity of Fragmentation Reagent

3. Prepare the Fragmentation Master Mix ON ICE by following the recipe in Table 2. *Prepare the Fragmentation Master Mix* on the worksheet.

IMPORTANT !

Prepare the Fragmentation Master Mix on ice. The master mix must be chilled before adding to DNA.

Due to the small amount of Fragmentation Reagent required, the master mix is scaled accordingly. There will be excess master mix. Discard all excess master mix. Prepare fresh master mix for each set of pooled PCR products.

Fragment the Pooled PCR Products

To fragment the pooled PCR products:

1. Keep the Fragmentation Master Mix and pooled PCR products on ice.
2. Referring to Table 3. *Fragmentation Reaction Assembly* on the worksheet or Table 1.15 (both below), add the appropriate volume of Fragmentation Master Mix to each pool based on the array format you are using.

3. Fragmentation Reaction Assembly		49 Format
Prepared Pool (from 'Fragmentation Pool Prep')	ul	42.6
Fragmentation Master Mix	ul	4.0
Total Reaction Volume	ul	46.6

Figure 1.14

Adding Fragmentation Master Mix to the Prepared Pools

Table 1.15

Fragmentation Reaction

	49 Format	100 Format	169 Format
Volume of Prepared PCR Pool (quantitated and diluted)	42.6	25.6 µL	19.1 µL
Fragmentation Master Mix	4.0 µL	2.4 µL	1.8 µL
Total Volume in each tube/well	46.6 µL	28.0 µL	20.9 µL

3. Preheat the thermal cycler to 37°C.

- Once preheated, load the tubes/plate onto the thermal cycler and run the following program:

Table 1.16
Thermal Cycling Conditions

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

- When the program is finished, spin down the tubes/plate.

Ensure that Fragmentation is Complete

To ensure that fragmentation is complete, you will run an aliquot of each reaction on a gel.

To ensure that fragmentation is complete:

- For each fragmentation reaction, remove:
 - 4 μL for 49- and 100-format arrays
 - 2 μL for 169-format arrays
- Add 4 μL of 2X gel loading dye.
- Run along with Cambrex DNA Marker 50-2500 on a 20% TBE PAGE gel to ensure that the fragmentation is complete.
- Stain the gel with SYBR Gold diluted 1:10,000 in TE for 15 minutes.
- View the gel under the same conditions used for ethidium bromide visualization.

Properly fragmented DNA runs between 20-200 bp. See [Figure 1.15](#).

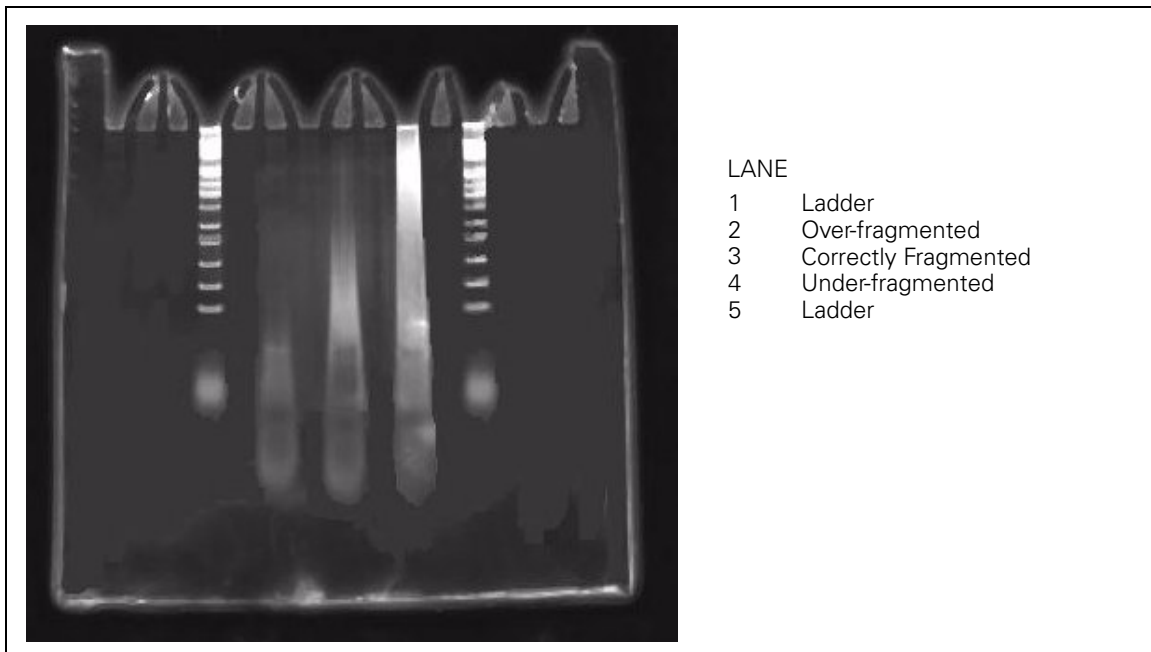


Figure 1.15
Fragmentation of CustomSeq® Control PCRs

Stage 5: Labeling

Reagents Required

Table 1.17
Reagents Required

Reagent	Vendor	Part Number
From Box 3 of the GeneChip® Resequencing Assay Kit:	Affymetrix	900447
<ul style="list-style-type: none"> • GeneChip® DNA Labeling Reagent • Terminal Deoxynucleotidyl Transferase (30 U/μL) • 5X Terminal Deoxynucleotidyl Transferase Buffer 		

Prepare the Labeling Master Mix

To prepare the Labeling Master Mix:

1. Select the *Labeling and Hyb* tab in the workbook.
The *Labeling and Hybridization Worksheet* is displayed.
2. In Table 1. *Prepare Labeling Master Mix*, enter the number of arrays to be processed in the appropriate cell ([Figure 1.16](#)).
Notice that the volumes for each reagent are calculated automatically, including 5% extra.
3. Prepare the Labeling Master Mix.

Add Labeling Master Mix to Each Reaction

1. Referring to the worksheet or [Table 1.18](#), add the appropriate volume of Labeling Master Mix to each reaction.

Enter number of arrays to be processed. The volume for each reagent in the Labeling Master Mix (including 5% extra) is calculated automatically.

1. Prepare Labeling Master Mix		49-format	100-format	169-format
n= number of arrays		1	6	1
5X Tdt Buffer	ul	12.6	45.4	5.7
DNA Labeling Reagent	ul	2.1	7.6	0.9
Tdt	ul	3.6	12.9	1.6
Total Volume (includes 5% extra)		18.3	65.8	8.2
Volume to Add to Each Reaction		17.4	10.4	7.8
Total Volume / Reaction		60.0	34.4	26.9

Figure 1.16

Using the Labeling and Hybridization Worksheet to Prepare the Labeling Master Mix

Table 1.18

Adding Labeling Master Mix to Each Reaction

Array Format	Labeling Master Mix	Total Volume Each Tube/Well
49	17.4 μ L	60 μ L
100	10.4 μ L	34.4 μ L
169	7.8 μ L	26.9 μ L

2. Place the tubes/plate on a thermal cycler and run the following program:

Table 1.19
Labeling Thermal Cycler Program

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

3. When the program is finished, spin down the tubes/plate.
4. Do one of the following:
 - Proceed to *Stage 6: Hybridization* on [page 51](#)
 - Store the samples at -20°C

Stage 6: Hybridization

Allow the arrays to equilibrate to room temperature prior to hybridization. While the arrays equilibrate, prepare the Pre-Hybridization Buffer and the Hybridization Master Mix.

Reagents Required

WARNING



Tetramethylammonium chloride solution (TMAC) is toxic. Consult the MSDS associated with this product for safe handling and disposal procedures.

Table 1.20
Reagents Required

Reagent	Vendor	Part Number
From Box 3 of the GeneChip® Resequencing Assay Kit: • Oligonucleotide Control Reagent	Affymetrix	900447
Tetramethylammonium chloride solution (TMAC)	Sigma	T3411
Acetylated Bovine Serum Albumin (BSA) solution	Invitrogen	15561-020
Herring sperm DNA	Promega Corp.	D1811
Tris-HCl, 1 M, pH 7.8	Sigma	T2913
Tween-20 (10% solution), diluted to 1% in molecular biology grade water	Pierce	28320
Molecular Biology Grade Water	Cambrex	51200

Prepare and Aliquot the Hybridization Master Mix

IMPORTANT !

Be sure the Hybridization Controls are included in the Hybridization Master Mix.

To prepare and aliquot the Hybridization Master Mix:

1. Select the *Labeling and Hyb* tab in the workbook.
The *Labeling and Hybridization Worksheet* is displayed.
2. In Table 3. *Prepare Hyb Master Mix*, enter the number of arrays to be processed in the appropriate cell (Figure 1.17).
3. Notice that the volumes for each reagent are calculated automatically, including 5% extra.
4. Prepare the Hybridization Master Mix.
The master mix can be stored at -20°C for up to four weeks.
5. Referring to the worksheet, add the appropriate amount of Hybridization Master Mix to each reaction (Figure 1.17 or Table 1.21).

Enter number of arrays to be processed based on format. The volume for each reagent in the Labeling Master Mix (including 5% extra) is calculated automatically.

3. Prepare Hyb Master Mix		49-format	100-format	169-format
n= number of arrays		1	6	1
Volumes include 5% extra.				
5 M TMAC	ul	138.6	499.0	62.4
1 M tris, 7.8	ul	2.3	8.3	1.0
1% Tween 20	ul	2.3	8.3	1.0
50 mg/mL BSA	ul	2.3	8.3	1.0
10 mg/mL HS DNA	ul	2.3	8.3	1.0
130X oligo control reagent	ul	1.8	6.4	0.8
Water	ul	18.4	66.2	8.3
Total Volume	ul	168.0	604.8	75.6
Volume to Apply to Labeled Sample	ul	160.0	96.0	72.0
Final Volume (Labeled Sample + Hyb Mix)	ul	220.0	130.4	98.9

Figure 1.17

Using the Labeling and Hybridization Worksheet to Prepare the Hyb Master Mix

Table 1.21
Adding Hybridization Master Mix

Array Format	Hybridization Master Mix	Total Volume in Tube/Well
49	160 μ L	220 μ L
100	96 μ L	130.4 μ L
169	72 μ L	98.9 μ L

Prepare the Pre-Hybridization Buffer

To prepare the Pre-Hybridization Buffer:

1. Based on [Table 1.22](#) and the number of arrays you will process, calculate the volume of each reagent required.

Table 1.22
Pre-Hybridization Buffer – Volumes Shown are for 1 Array

Reagent	Stock	Final	1 Array
Water	—	—	196 μ L
Tris, pH 7.8	1M	10mM	2 μ L
Tween-20	1%	0.01%	2 μ L
Total			200 μ L

2. Prepare the buffer.
The Pre-Hybridization Buffer can be stored at room temperature.

Pre-Hybridize the Arrays

To pre-hybridize the arrays:

1. Load each array with the appropriate volume of Pre-Hybridization Buffer (Table 1.23).

Table 1.23
Adding Pre-Hybridization Buffer

Array Format	Pre-Hybridization Buffer
49	200 μ L
100	120 μ L
169	80 μ L

2. Load the arrays in the hybridization oven at 49°C rotating at 60 RPM for 15 minutes.

Denature the Samples

To denature the samples:

1. Load the tubes/plate onto a thermal cycler at 95°C for 5 minutes.
2. Equilibrate the tubes/plate by placing them at 49°C for 5 minutes.
3. Vortex the tubes/plate and briefly spin to collect any condensation from the side of the tubes/wells.

Load the Denatured Samples Onto Arrays

To load the denatured samples onto arrays:

1. Remove the arrays from the hybridization oven.
2. Remove and discard the Pre-Hybridization Buffer from the arrays.
3. Load each array with the appropriate volume of denatured sample (Table 1.24).

Table 1.24

Loading Denatured Samples Onto Arrays

Array Format	Denatured Sample
49	200 μ L
100	120 μ L
169	80 μ L

4. Return the arrays to the hybridization oven for 16 hours at 49°C rotating at 60 RPM.

Stage 7: Washing, Staining, and Scanning

The arrays should be washed using the GeneChip® Fluidics Station 400 or 450/250, and scanned using the GeneChip® Scanner 3000. Refer to the GeneChip® Operating Software for installation and setup information.

After completing the procedures described in this chapter, the scanned array image (.dat file) is ready for analysis. Refer to the *GeneChip® Sequence Analysis Software User's Guide* (P/N 702084) for analysis information.

Reagents and Other Materials Required

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

Table 1.25
Reagents and Materials Required

Reagent	Vendor	Part Number
Water, Molecular Biology Grade	Cambrex	51200
Distilled water	Invitrogen	15230147
20X SSPE (3 M NaCl, 0.2 M NaH ₂ PO ₄ , 0.02 M EDTA)	Cambrex	51214
Anti-streptavidin antibody (goat), biotinylated (reconstitute according to product instructions)	Vector Laboratories	BA-0500
R-Phycoerythrin Streptavidin	Invitrogen	S-866
10% surfact-Amps20 (Tween-20)	Pierce Chemical	28320
Bleach (5.25% Sodium Hypochlorite)	VWR	21899-504 (or equivalent)
Denhardt's Solution, 50X concentrate	Sigma	D2532
MES hydrate	Sigma-Aldrich	M5287
MES Sodium Salt	Sigma-Aldrich	M5057
5 M NaCl, RNase-free, DNase-free	Ambion	9760G

Table 1.25
Reagents and Materials Required

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

Reagent Preparation

Wash A: Non-Stringent Wash Buffer

(6X SSPE, 0.01% Tween 20)

For 1000 mL:

300 mL of 20X SSPE

1.0 mL of 10% Tween-20

699 mL of water

Filter through a 0.2 µm filter.

Store at room temperature.

Wash B: Stringent Wash Buffer

(0.6X SSPE, 0.01% Tween 20)

For 1000 mL:

30 mL of 20X SSPE

1.0 mL of 10% Tween-20

969 mL of water

Filter through a 0.2 µm filter.

Store at room temperature.

0.5 mg/mL Anti-Streptavidin Antibody

Resuspend 0.5 mg in 1 mL of water.

Store at 4°C.

12X MES Stock Buffer

(1.22M MES, 0.89M [Na⁺])

For 1,000 mL:

70.4g of MES hydrate

193.3g of MES Sodium Salt

800 mL of Molecular Biology Grade water

Mix and adjust volume to 1,000 mL.

The pH should be between 6.5 and 6.7.

Filter through a 0.2 µm filter.

IMPORTANT !

Do not autoclave 12X MES. 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 5M NaCl

0.1 mL of 10% Tween-20

73.1 mL of water

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

Experiment and Fluidics Station Setup

The following instructions are for GeneChip Operating Software (GCOS) 1.4 or GeneChip Operating Software Server (GCOS Server).

Register a New Experiment in GCOS

To register a new experiment in GCOS:

1. From the **File** menu click **New Experiment**.
 - The New Experiment window appears in the display pane ([Figure 1.18](#)).
 - The top half of the display pane refers to the sample and the bottom half refers to the experiment.

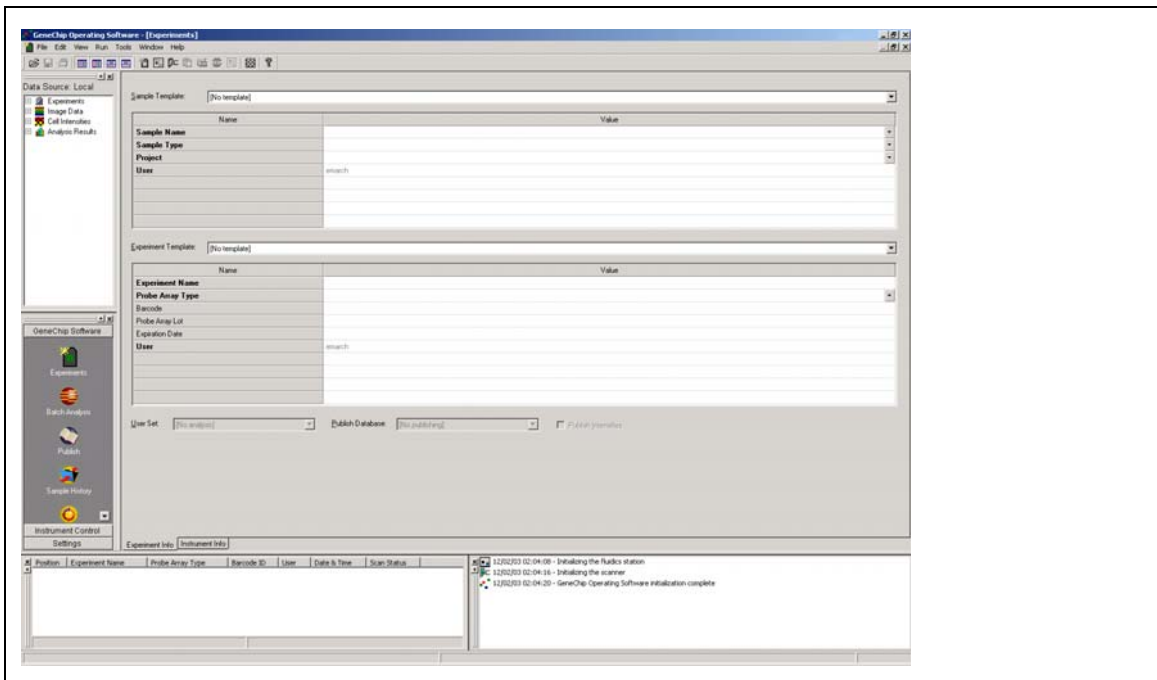


Figure 1.18
GCOS Sample Entry Pane

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

TIP

The **Sample Information** fields can be customized. See the *GeneChip® Operating Software User's Guide (P/N 701439)* for further information.

Set Up and Prime the Fluidics Station

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

To set up the fluidics station:

1. Turn on the Fluidics Station using the toggle switch on the lower left side of the machine.
2. Select **Run** → **Fluidics** from the menu bar in GCOS.
 - The Fluidics Station dialog box appears with a drop-down list for selecting the experiment name for each of the fluidics station modules. A second drop-down list is accessed for choosing the Protocol for each of the fluidics station modules. Use the radio buttons to access each module.

NOTE

Refer to the *GeneChip® Fluidics Station 450/250 User's Guide (P/N 08-0092)* for instructions on connecting and addressing multiple fluidics stations.

To prime the fluidics station:

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

Priming should be done:

- when the fluidics station is first started.
 - when wash solutions are changed.
 - before washing, if a shutdown has been performed.
 - if the LCD window instructs the user to prime.
1. To prime the fluidics station, select **Protocol** in the Fluidics Station dialog box.
 2. Choose **Prime** for the respective modules in the **Protocol** drop-down list.
 3. Change the intake buffer reservoir A to **Non-Stringent Wash Buffer**, and intake buffer reservoir B to **Stringent Wash Buffer**.
 4. Click **Run** for each module to begin priming.
 5. Follow LCD instructions.

NOTE 

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

Prepare the Arrays for Washing and Staining

The Affymetrix staining protocol for CustomSeq arrays is a three stage process consisting of:

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

Once stained, the array is filled with Array Holding Buffer prior to scanning as outlined in [Table 1.31](#) and [Table 1.33](#).

Prepare the Arrays

To prepare arrays:

1. After 16 hours of hybridization, remove the hybridization cocktail from the array and set it aside in a microcentrifuge vial.
2. Store the hybridization cocktail on ice during the procedure or at -80°C for long-term storage.
3. Fill the array completely with 250 μL of Array Holding Buffer.

NOTE

If necessary, arrays can be stored in Array Holding Buffer at 4°C for up to 3 hours before washing and staining. Equilibrate arrays to room temperature before washing and staining.

Prepare the Buffers and Stain Solutions

Prepare the following buffers and stain solutions:

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

Stain Buffer

The volume given is sufficient for one array. Mix well.

Table 1.26
Stain Buffer

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

SAPE Stain Solution

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

The volume given is sufficient for one array. Mix well.

Table 1.27
SAPE Stain Solution

Components	Volume	Final Concentration
Stain Buffer	495 µL	1X
1 mg/mL Streptavidin Phycoerythrin (SAPE)	5.0 µL	10 µg/mL
Total	500 µL	

NOTE 

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

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

Antibody Stain Solution

The volume given is sufficient for one array. Mix well.

Table 1.28
Antibody Solution Mix

Components	Volume	Final Concentration
Stain Buffer	495 μ L	1X
0.5 mg/mL biotinylated antibody	5 μ L	5 μ g/mL
Total	500 μ L	

NOTE 

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

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

Array Holding Buffer

Table 1.29
Array Holding Buffer

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

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

NOTE 

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

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

Fluidics Station Protocols

Fluidics Station 450/250

Table 1.30
Fluidics Station 450/250 Protocols

Array Format	Protocols Available
49 (standard)	DNAArray_WS5_450 FLEXDNAArray_WS5_450
100	Midi_DNAArray_WS5_450 FLEXMidi_DNAArray_WS5_450
169	Mini_DNAArray_WS5_450 FLEXMini_DNAArray_WS5_450

Table 1.31
FS-450 Fluidics Protocol - Antibody Amplification for Targets

49 Format (standard) Protocol: DNAArray_WS5_450	
Post Hyb Wash #1	6 cycles of 5 mixes/cycle with Wash Buffer A at 25°C
Post Hyb Wash #2	6 cycles of 5 mixes/cycle with Wash Buffer B at 45°C
Stain	Stain the array for 10 minutes in SAPE solution at 25°C
Post Stain Wash	6 cycles of 5 mixes/cycle with Wash Buffer A at 25°C
2nd Stain	Stain the array for 10 minutes in antibody solution at 25°C
3rd Stain	Stain the array for 10 minutes in SAPE solution at 25°C
Final Wash	10 cycles of 6 mixes/cycle with Wash Buffer A at 30°C. The final holding temperature is 25°C
Filling Array	Fill the array with Array Holding Buffer.
Wash Buffer A = non-stringent wash buffer Wash Buffer B = stringent wash buffer	

Fluidics Station 400

Table 1.32
Fluidics Station 400 Protocols

Array Format	Protocols Available
49 (standard)	DNAArray_WS5 FLEXDNAArray_WS5
100	Midi_DNAArray_WS5 FLEXMidi_DNAArray_WS5
169	Mini_DNAArray_WS5 FLEXMini_DNAArray_WS5

Table 1.33
FS-400 Fluidics Protocol - Antibody Amplification for Targets

49 Format (standard) Protocol: DNAArray_WS5	
Post Hyb Wash #1	6 cycles of 5 mixes/cycle with Wash Buffer A at 25°C
Post Hyb Wash #2	6 cycles of 5 mixes/cycle with Wash Buffer B at 45°C
Stain	Stain the array for 10 minutes in SAPE solution at 25°C
Post Stain Wash	6 cycles of 5 mixes/cycle with Wash Buffer A at 25°C
2nd Stain	Stain the array for 10 minutes in antibody solution at 25°C
3rd Stain	Stain the array for 10 minutes in SAPE solution at 25°C
Final Wash	10 cycles of 6 mixes/cycle with Wash Buffer A at 30°C. The holding temperature is 25°C
Filling Array	Fill the array with Array Holding Buffer.
Wash Buffer A = non-stringent wash buffer Wash Buffer B = stringent wash buffer	

Washing and Staining Arrays Using FS-450/250

IMPORTANT !

The wash and stain buffers are different from the GeneChip expression buffers.

To wash and stain arrays using the FS-450/250:

1. In the Fluidics Station dialog box on the workstation, select the correct experiment name from the drop-down **Experiment** list. The Probe Array Type appears automatically.
2. In the **Protocol** drop-down list, select the appropriate fluidics script ([Table 1.30 on page 67](#)).
3. Choose **Run** in the Fluidics Station dialog box to begin the washing and staining.
4. Follow the instructions in the LCD window 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 FS-450/250 fluidics stations).
5. Insert the appropriate array into the designated module of the fluidics station while the cartridge lever is in the down or eject position.
6. When finished, verify that the cartridge lever is returned to the up or engaged position.
7. Remove any microcentrifuge vials remaining in the sample holders of the fluidics station module(s) being used.
8. When prompted to “Load Vials 1-2-3,” place the three vials into the sample holders 1, 2 and 3 on the fluidics station as follows:
 - A. Place one vial containing 500 μ L Streptavidin Phycoerythrin (SAPE) stain solution mix in sample holder 1.
 - B. Place one vial containing 500 μ L anti-streptavidin biotinylated antibody stain solution in sample holder 2.
 - C. Place one vial containing 800 μ L Array Holding Buffer in sample holder 3.
 - D. Press down on the needle lever to snap needles into position and

to start the run.

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

9. When staining is finished, remove the microcentrifuge vials containing stain and replace with three empty microcentrifuge vials as prompted.
10. Remove the arrays from the fluidics station modules by first pressing down the cartridge lever to the eject position.
11. Check the array window for large bubbles or air pockets.
 - If bubbles are present, the array should be filled with Array Holding Buffer manually, using a pipette. Take out one-half of the solution and then manually fill the array with Array Holding Buffer.
 - If the array has no large bubbles, it is ready to scan on the GeneChip Scanner 3000. Pull up on the cartridge lever to engage wash block and proceed to [Scanning Arrays on page 74](#).

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.

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

If no more samples require washing and staining, shut down the fluidics station following the procedure outlined in the section, [Shutting Down the Fluidics Station beginning on page 78](#).

Washing and Staining Arrays Using FS400

IMPORTANT !

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

To wash and stain arrays using the FS-400:

1. In the Fluidics Station dialog box on the workstation, select the correct experiment name in the drop-down **Experiment** list.
The array type appears automatically.
2. In the **Protocol** drop-down list, select the appropriate fluidics script ([Table 1.32 on page 68](#)).
3. Choose **Run** in the Fluidics Station dialog box to begin the washing and staining.
4. Follow the instructions on the LCD window on the fluidics station.
If you are unfamiliar with inserting and removing arrays from the fluidics station modules, refer to the *Fluidics Station 400 User's Guide* (P/N 700103), or Quick Reference Card (P/N 08-0072).
5. Insert the appropriate array into the designated module of the fluidics station while the cartridge lever is in the **EJECT** position.
6. When finished, verify the cartridge lever is returned to the **ENGAGE** position.
7. Remove any microcentrifuge vials remaining in the sample holders of the fluidics station module(s) being used.
8. When the LCD window indicates, place the microcentrifuge vial containing 500 µL Streptavidin Phycoerythrin (SAPE) solution into the sample holder.
Verify that the metal sampling needle is in the vial with its tip near the bottom.
9. When the LCD window indicates, replace the microcentrifuge vial containing the SAPE stain solution with a microcentrifuge vial containing 500 µL antibody stain solution into the sample holder, making sure that the metal sampling needle is in the vial with its tip near the bottom.
Do not discard. You will need this vial again.

10. When the LCD window indicates, replace the microcentrifuge vial containing the antibody stain solution with the first microcentrifuge vial containing 500 μ L Streptavidin Phycoerythrin (SAPE) stain solution into the sample holder.

Verify the metal sampling needle is in the vial with its tip near the bottom.

11. When the LCD window indicates, replace the microcentrifuge vial containing the SAPE solution with a microcentrifuge vial containing 800 μ L Array Holding Buffer.

The Fluidics Station dialog box and the LCD window display the status of the washing and staining as they progress. When the wash is complete, the LCD window displays the message EJECT CARTRIDGE.

12. Remove microcentrifuge vial containing Array Holding Buffer and replace with an empty microcentrifuge vial.

13. Remove the arrays from the fluidics station modules by first moving the array holder lever to the EJECT position.

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

- If bubbles are present, the array should be filled with Array Holding Buffer manually, using a pipette. Take out one-half of the solution and then manually fill the array with Array Holding Buffer.
- If the array has no large bubbles, it is ready to scan on the GeneChip Scanner 3000. Pull up on the cartridge lever to engage wash block and proceed to [Scanning Arrays on page 74](#).

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.

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

If there are no more samples to wash and stain, shut down the fluidics station following the procedure outlined in the section, *Shutting Down the Fluidics Station* beginning on page 78.

NOTE 

For proper cleaning and maintenance of the fluidics station including the bleach protocol, refer to *GeneChip® Fluidics Station 400 User's Guide* (P/N 700103).

Scanning Arrays

The scanner is controlled by GeneChip Operating Software 1.4 or higher. The array is scanned after the wash protocols are complete. Make sure the laser is warmed up prior to scanning by turning the GeneChip Scanner 3000 laser on at least 10 minutes before use. If the array was stored at 4°C, allow to warm to room temperature before scanning.

NOTE 

Refer to the GCOS online help, tutorials, and User's Guide for more information on scanning.

WARNING 

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

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

Prepare Arrays for Scanning

Before you scan the array, follow the directions in this section on handling the array. 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.

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

IMPORTANT 

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

To prepare arrays for scanning:

1. On the back of the array cartridge, clean excess fluid from around the septa.
2. Carefully apply one Tough-Spot to each of the two septa. Press to ensure the spots remain flat. If the Tough-Spots do not apply smoothly; that is, if you observe bumps, bubbles, tears or curled edges, do not attempt to smooth out the spot. Remove the spot and apply a new spot. See [Figure 1.19](#).

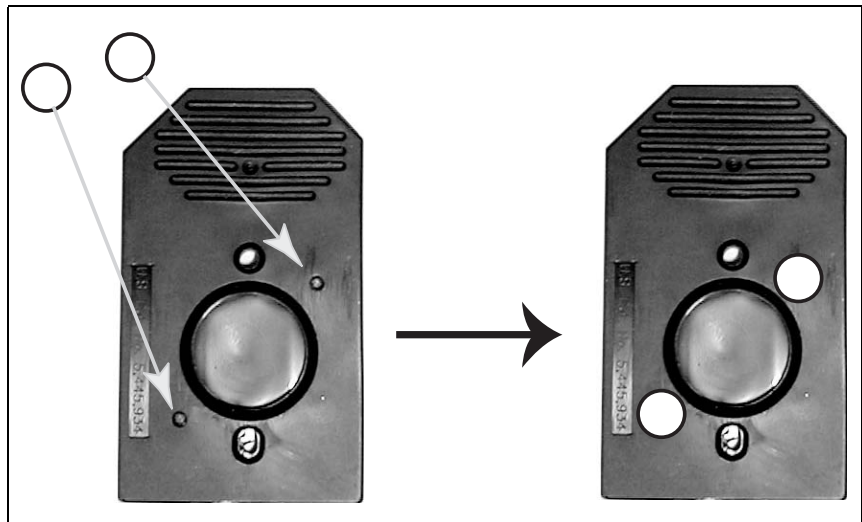


Figure 1.19
Applying Tough-Spots™ to the array cartridge

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

Scan the Arrays

NOTE 

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

To scan the arrays:

1. Select **Run** → **Scanner** from the menu bar. Alternatively, click the **Start Scan** icon in the tool bar.
 - The Scanner dialog box appears with a drop-down list of all unscanned experiments.
2. Select the experiment name that corresponds to the array you wish to scan.

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

NOTE 

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

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

WARNING 

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

4. Once the experiment has been selected, click the **Start** button.
 - A dialog box prompts you to load the array into the scanner.
5. Pixel resolution and wavelength for the GeneChip Scanner 3000 are preset and cannot be changed.

6. Open the sample door of the scanner and insert the array into the holder. Do not force the array into the holder. The door of the GeneChip Scanner 3000 closes automatically.

IMPORTANT !

Do not force the array into the holder.

7. Click **OK** in the Start Scanner dialog box.
 - The scanner begins scanning the array. When **Scan in Progress** is selected from the **View** menu, the array image appears on the screen as the scan progresses.

Shutting Down the Fluidics Station

To shut down the fluidics station:

1. After removing an array from the array holder, the LCD window displays the message **ENGAGE WASHBLOCK**.
2. If you are using the FS-400, engage the wash block by pushing up on the cartridge lever to the **ENGAGE** position.
If you are using the FS-450/250, gently lift up the cartridge lever to engage, or close, the washblock.
The fluidics station automatically performs a Cleanout procedure. The LCD window indicates the progress of the Cleanout procedure.
3. When the fluidics station LCD window indicates **REMOVE VIALS**, the Cleanout procedure is complete.
4. Remove the sample microcentrifuge vial(s) from the sample holder(s).
5. If no other processing is to be performed, place wash lines into a bottle filled with deionized water.
6. Choose **Shutdown** for all modules from the drop-down **Protocol** list in the Fluidics Station dialog box. Click the **Run** button for all modules.
The Shutdown protocol is critical to instrument reliability. Refer to the appropriate Fluidics Station User's Guide for more information.
7. After Shutdown protocol is complete, flip the ON/OFF switch of the fluidics station to the OFF position.

IMPORTANT !

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

Customizing Fluidics Station Protocols

There may be times when the fluidics protocols need to be modified. Modification of protocols must be done before downloading the protocol to the fluidics station. Protocol changes will not affect runs in progress. For more specific instructions, refer to the GeneChip Operating Software online help.

To customize a fluidics station protocol:

1. Select **Tools** → **Edit Protocol** from the menu bar.
The Edit Protocol dialog box appears.
2. Select the protocol to be changed from the **Protocol Name** drop-down list.
The name of the protocol is displayed in the **Protocol Name** box. The conditions for that protocol are displayed on the right side of dialog box.
3. Select the item to be changed and input the new parameters as needed.

Table 1.34

Valid Ranges for Wash/Stain Parameters

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

4. To return to the default values for the protocol selected, click the **Defaults** button.
5. After all the protocol conditions are modified as desired, change the name of the edited protocol in the **Protocol Name** box.

CAUTION 

If the protocol is saved without entering a new Protocol Name, the original protocol parameters will be overwritten.

6. Click **Save**, then close the dialog box.

Enter 0 (zero) for hybridization time if hybridization step is not required. Likewise, enter 0 (zero) for the stain time if staining is not required. Enter 0 (zero) for the number of wash cycles if a wash solution is not required.

Appendix **A**

PCR Protocols

Appendix *A*



Long-Range PCR Protocol

Long-Range PCR Controls (7.5 kb Amplicon)

Use the following control template and primers when the average expected product is greater than 5 kb. These components are in the GeneChip® Resequencing Assay Kit, P/N 900447.

1. Prepare the following control mix:

Table A.1
Long-Range PCR Control Mix

Component	Volume for 1 Control Reaction
Forward primer 7.5 kb (20 μ M)	3 μ L
Reverse primer (20 μ M)	3 μ L
IQ-EX Control Template (0.2 pg/ μ L)	5 μ L
Water, molecular biology grade	29 μ L
AmpliTaq Gold DNA polymerase (Applied Biosystems)	5 units
MgCl ₂	2.5 mM
dNTP	200 μ M (each)
1X PCR buffer II (Applied Biosystems)	Total volume = 100 μ L

2. Amplify using the following PCR program:
 - 95°C 10 minutes
 - 30 cycles (94°C 30 sec. - 68°C 30 sec. - 72°C 60 sec.)
 - 72°C 5 minutes

3. Quantify as described in *Stage 2: Quantitation and Pooling of PCR Products* on page 21, and include as one of your amplicons to pool.

NOTE 

To produce the 1.0 kb PCR product from this kit using the long-range PCR protocol, follow the instructions for the 7.5 kb amplicon with the 1.0 kb Forward Primer, and substitute a 1 minute extension time per cycle.

Standard PCR Protocol

Standard PCR Controls (1.0 kb Amplicon)

Use the following control template and primers when the average expected product is less than 5 kb. These components are in the GeneChip® Resequencing Assay Kit, P/N 900447.

1. Prepare the following control mix:

Table A.2
Standard PCR Control Mix

Component	Volume for 1 Control Reaction
Forward primer 1.0 kb (20 μ M)	3 μ L
Reverse primer (20 μ M)	3 μ L
IQ-EX Control Template (0.2 pg/ μ L)	5 μ L
AmpliTaq Gold DNA polymerase (Applied Biosystems)	5 units
MgCl ₂	2.5 mM
dNTP	200 μ M (each)
1X PCR buffer II (Applied Biosystems)	Total volume = 100 μ L

2. Amplify using the following PCR program:
 - 95°C 10 minutes
 - 30 cycles (94°C 30 sec. - 68°C 30 sec. - 72°C 60 sec.)
 - 72°C 5 minutes

3. Quantify as described in *Stage 2: Quantitation and Pooling of PCR Products* on page 21, and include as one of your amplicons to pool.

NOTE 

To produce the 1.0 kb PCR product from this kit using the long-range PCR protocol, follow the instructions for the 7.5 kb amplicon with the 1.0 kb Forward Primer, and substitute a 1 minute extension time per cycle.

Appendix **B**

Affymetrix and Supplier Contact List

Appendix **B**



Affymetrix Technical Support

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

Affymetrix Inc.
3420 Central Expressway
Santa Clara, CA 95051
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Tel: 1-888-362-2447 (1-888-DNA-CHIP)
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Supplier Contact Information

Supplier	Web Site
Ambion	www.ambion.com
GE Healthcare Life Sciences (formerly Amersham Biosciences)	www.amershambiosciences.com
Brinkmann Instruments	www.brinkmann.com
Cambrex	www.cambrex.com
Clontech	www.clontech.com
Fisher Life Sciences	US: www.fishersci.com Europe: www.fisher.co.uk
Invitrogen Life Technologies	www.invitrogen.com
Molecular Probes	www.probes.com
New England Biolabs	www.neb.com
PerkinElmer Life Sciences	lifesciences.perkinelmer.com/
Pierce Chemical	www.piercenet.com
Promega Corporation	www.promega.com
QIAGEN	www.qiagen.com
Sigma-Aldrich	www.sigma-aldrich.com
TaKaRa Bio Inc.	www.takara-bio.com
Vector Laboratories	www.vectorlabs.com
VWR Scientific Products	www.vwrsp.com