

# **Array-Based CGH Procedures for Genomic DNA Analysis**

## **Protocol**

Version 1.0

January 2005

**Research Use Only**



**Agilent Technologies**

## Notices

© Agilent Technologies, Inc. 2005

No part of this manual may be reproduced in any form or by any means (including electronic storage and retrieval or translation into a foreign language) without prior agreement and written consent from Agilent Technologies, Inc. as governed by United States and international copyright laws.

### Manual Part Number

G4410-90010

### Edition

First edition, January 2005  
Version 1.0

Printed in USA

Agilent Technologies, Inc.  
3500 Deer Creek Road  
Palo Alto, CA 94304 USA

### Technical Support

Technical product support may be obtained by contacting your local Agilent Support Services representative. Access to Agilent's most up-to-date user guides, protocols as well as list of worldwide sales and support center telephone numbers can be obtained at the following website:  
[www.agilent.com/chem/dnasupport](http://www.agilent.com/chem/dnasupport)

### Notice to Purchaser

These products are intended for research use only. Agilent products may not be resold, modified for resale, or used to manufacture commercial products without written approval of Agilent Technologies, Inc.

### Label Licenses and User Restrictions

This license permits the person or legal entity to which this Nucleic Acid Microarray ("Microarray") has been provided (the "Buyer") to use the Microarray, and the data generated by the use of the Microarray, for research use only, including clinical trials and commercial purposes. In addition, Buyer agrees and understands that it is not licensed to use a Microarray or the data generated there from in a clinical diagnostic setting where data from an individual's sample is given to such individual or their caregiver for a fee or reimbursement. Neither Agilent nor its licensors grants any other licenses, expressed or implied, to permit the manufacture, use, sale or importation of the Microarray, any other arrays, or any nucleic acids on the Microarray. This license prohibits Buyer from selling data generated from the use of the Microarray to multiple other parties.

### Warranty

Each Product purchased will include a global warranty. A global warranty includes the warranty for the country of purchase. If the Product is moved to another country, Customer will receive the destination country's standard warranty, provided that: 1) the Product has a return to Agilent warranty, or 2) if the Product has an on-site warranty, Agilent has a Product-specific support presence in that country.

Product warranty information is available with Products, on quotations, or upon request. The warranty period begins on Delivery, or the date of installation if installed by Agilent. If Customer schedules or delays installation by Agilent more than 30 days after Delivery, the warranty period begins on the 31st day after Delivery. Customer may receive a different warranty when the Product is purchased as part of a system.

Agilent warrants that Software will not fail to execute its programming instructions due to defects in materials and workmanship when properly installed and used on the hardware designated by Agilent. Agilent further warrants that Agilent owned standard Software will substantially conform to Specifications. Agilent does not warrant that Software will operate in hardware and software combinations selected by Customer, or meet requirements specified by Customer. Agilent does not warrant that the operation of Products will be uninterrupted or error free.

If Agilent receives notice of defects or nonconformance as defined in Sections 7(c) and 7(d) during the warranty period, Agilent will, at its option, repair or replace the affected Product(s). Customer will pay expenses for return of such Product(s). Agilent will pay expenses for shipment of repaired or replacement Product(s). If Agilent is unable, within a reasonable time, to repair or replace the affected Product(s), Customer will be entitled to a refund of the purchase price upon prompt return of the Product(s) to Agilent.

Agilent warrants that Agilent Support will be provided in a professional and workmanlike manner. Agilent will replace, at no charge, parts which are defective and returned to Agilent within 90 days of delivery.

The above warranties do not apply to defects resulting from improper or inadequate maintenance, repair or calibration by Customer; Customer or third party supplied hardware or software, interfacing or supplies; unauthorized modification; improper use or operation outside of the Specifications for the Product; abuse, negligence, accident, loss or damage in transit; or improper site preparation.

THE ABOVE WARRANTIES ARE EXCLUSIVE AND NO OTHER WARRANTY, WHETHER WRITTEN OR ORAL, IS EXPRESSED OR IMPLIED. AGILENT SPECIFICALLY DISCLAIMS THE IMPLIED WARRANTIES OF MERCHANTABILITY AND FITNESS FOR A PARTICULAR PURPOSE.

# Contents

## 1 Introduction

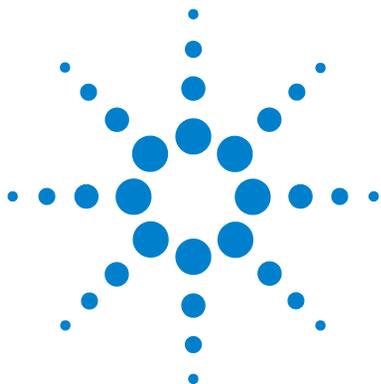
|                                       |    |
|---------------------------------------|----|
| Purpose                               | 5  |
| Agilent oligo microarray kit contents | 5  |
| General Procedural Notes              | 7  |
| Safety notes                          | 8  |
| Required equipment                    | 9  |
| Required reagents                     | 10 |
| Required software                     | 11 |

## 2 Procedures

|  |    |
|--|----|
| Genomic Amplification                                      | 15 |
| Restriction Digestion of Amplified DNA                     | 16 |
| Cleanup of Amplified/Digested DNA                          | 17 |
| Quantitation of DNA Product                                | 18 |
| Genomic DNA Labeling                                       | 19 |
| Cleanup of Labeled DNA                                     | 20 |
| Hybridization, Wash, and Dry                               | 21 |
| Preparation of reagents                                    | 21 |
| Preparation of hybridization samples                       | 23 |
| Hybridization  | 23 |
| Wash and dry   | 24 |
| Scanning Using Agilent Scanner                             | 27 |
| Image Processing of .tif Files Produced by Agilent Scanner | 28 |
| Feature Extraction   | 29 |

**A General Microarray Layout and Orientation**

**B Agilent Information Assets Access Agreement**



# 1 Introduction

Purpose 5

General Procedural Notes 7

## Purpose

This document describes Agilent's recommended procedures for the amplification, digestion, labeling of genomic DNA and hybridization, wash, scanning, and feature extraction of 60-mer oligo microarrays for array-based comparative genomic hybridization analysis (aCGH) using Agilent's Human Genome CGH Microarrays (G2519A, option 001).

### NOTE

Agilent cannot guarantee microarray performance and does not provide technical support to those who use nonAgilent protocols (for example, labeling/hybridization) in processing microarrays.

## Agilent oligo microarray kit contents

- Microarrays printed on 1" × 3" glass slides (44K)
- CD containing microarray design files in various file formats

### NOTE

Store entire kit at room temperature. After breaking foul on microarray pouch, store microarray slides at room temperature (in the dark) under a vacuum or under nitrogen.



**NOTE**

Please refer to application note “Quality control of DNA with the Agilent 2100 Bioanalyzer for Oligonucleotide Array CGH (aCGH)” for more information on using the Bioanalyzer to assess DNA quality. Application note is available at [www.agilent.com/chem/goCGH](http://www.agilent.com/chem/goCGH).

---

**NOTE**

First-time users of Agilent’s oligo microarray system, please refer to the following user manuals for detailed description and operation recommendations for each of the components used in the aCGH application workflow. The user manuals can be downloaded from the Agilent Web site at [www.agilent.com/chem/dna](http://www.agilent.com/chem/dna).

G2534-90001 Agilent Microarray Hybridization Chamber User Guide

G2545-80001 G2545A Hybridization Oven User Manual

G2566-90009 Agilent G2565AA and G2565BA Microarray Scanner System User Manual

G2566-90011 Agilent G2567AA Feature Extraction Software User Manual

---

## General Procedural Notes

- 1** Prevent contamination of reagents by nucleases by always wearing powder-free laboratory gloves, and using dedicated solutions and pipettors with nuclease-free aerosol-resistant tips.
- 2** Maintain a clean work area.
- 3** Minimize exposure to light; cyanine 3 and cyanine 5 are photolabile.
- 4** Mix genomic DNA samples by gently tapping the tube with your finger. Do not vortex stock solutions and reactions containing genomic DNA.
- 5** Prepare stock solutions that are stored frozen in 1.5-mL microcentrifuge tubes in the following way:
  - a** Thaw the aliquot as rapidly as possible without heating above room temperature.
  - b** Vortex briefly, then centrifuge for 5–10 s, to drive tube contents off of walls and lid.
  - c** Store on ice in a cold block until use.
- 6** In general, follow Biosafety Level 1 (BL1) safety rules.

## Safety notes

**CAUTION**

Wear appropriate personal protective equipment (PPE) when working in the laboratory.

---

**WARNING**

**Cyanine 3-dUTP and Cyanine 5-dUTP are potential carcinogens. Avoid inhalation, swallowing or contact with skin.**

---

**WARNING**

**LiCl is toxic and a potential teratogen. May cause harm to breastfed babies. Possible risk of impaired fertility. Target organ: central nervous system. Harmful by inhalation, by contact with skin and if swallowed. Wear suitable PPE. LiCl is a component of Agilent's 2× Hybridization Buffer.**

---

**WARNING**

**Lithium dodecyl sulfate (LLS) is harmful by inhalation and irritating to eyes, respiratory system and skin. Wear suitable PPE. LLS is a component of Agilent's 2× Hybridization Buffer.**

---

**WARNING**

**Triton X-100 is harmful if swallowed. Risk of serious damage to the eyes. Wear suitable PPE. Triton X-100 is a component of Agilent's 2× Hybridization Buffer.**

---

**WARNING**

**Triton X-102 is harmful if swallowed. Risk of serious damage to eyes. Wear suitable PPE.**

---

## Required equipment

| Description  | Company and part no.              |
|--|-----------------------------------|
| Agilent Scanner  | Agilent p/n G2565BA               |
| Human Genome CGH Microarray 44A  | Agilent p/n G2519A, option 001    |
| Hybridization Chamber, stainless   | Agilent p/n G2534A                |
| Hybridization Chamber gasket slides                                      | Agilent p/n G2534-60003           |
| Hybridization oven; temperature set at 65 °C                             | Agilent p/n G2545A                |
| Hybridization oven rotator for Agilent Microarray Hybridization Chambers | Agilent p/n G2530-60029           |
| Microcon YM-30 Filter Units  | Millipore p/n 42410               |
| Dry nitrogen source with filtered air gun                                | Millipore p/n WGGB01KAN           |
| QIAprep Spin Miniprep Kit  | Qiagen p/n 27106 or 27104         |
| 250-mL capacity Slide Staining Dish, with slide rack (x3)                | Wheaton p/n 900200, or equivalent |
| NanoDrop ND-1000 UV-VIS spectrophotometer                                |                                   |
| Powder-free gloves   |                                   |
| Pipetman micropipettors, (P-10, P-20, P-200, P-1000), or equivalent      |                                   |
| Sterile, nuclease-free aerosol barrier pipette tips                      |                                   |
| Sterile, nuclease-free 1.5-mL microfuge tubes                            |                                   |
| Sterile, nuclease-free 15-mL tubes                                       |                                   |
| Vortex mixer   |                                   |
| Ice bucket   |                                   |
| Eppendorf Microcentrifuge 5417R, or equivalent                           |                                   |
| Timer  |                                   |
| Clean forceps  |                                   |

| Description   | Company and part no. |
|---|----------------------|
| Circulating water bath set to 30 °C, 37 °C, 65 °C, and 95 °C    |                      |
| 1000 mL nuclease-free graduated cylinder                        |                      |
| 1000 mL capacity 0.2- $\mu$ m filtration units                  |                      |
| Magnetic stir plate (×2)  |                      |
| Magnetic stir bar (×2)  |                      |
| Vacuum dessicator or N <sub>2</sub> purge box for slide storage |                      |

## Required reagents

| Description                                | Company and part no.                           |
|--|--|
| 2x Hybridization Buffer, (25 mL)*          | Agilent p/n 5185-5973                          |
| 10% Triton X-102, (50 mL)*                 | Agilent p/n 5185-5975                          |
| 10× Control Targets, (100 hybridizations)* | Agilent p/n 5185-5976                          |
| 20× SSC                                    | Amresco p/n 0804-4L                            |
| 100% Ethanol                               | Amresco p/n E193                               |
| DNase/RNase-free distilled water           | Invitrogen p/n 10977015                        |
| BioPrime Array CGH Genomic Labeling Module | Invitrogen p/n 18095-012                       |
| Cot-1 DNA (1.0 mg/mL)                      | Invitrogen p/n 15279-011                       |
| Yeast tRNA (25 mg, lyophilized)            | Invitrogen p/n 15401-011                       |
| Cyanine-3-dUTP (1.0 mM)                    | Perkin Elmer p/n NEL578                        |
| Cyanine-5-dUTP (1.0 mM)                    | Perkin Elmer p/n NEL579                        |
| AluI (10 U/ $\mu$ L)                       | Promega p/n R6281                              |
| RsaI (10 U/ $\mu$ L)                       | Promega p/n R6371                              |
| Buffer C                                   | Promega p/n R003A<br>(supplied with p/n R6371) |

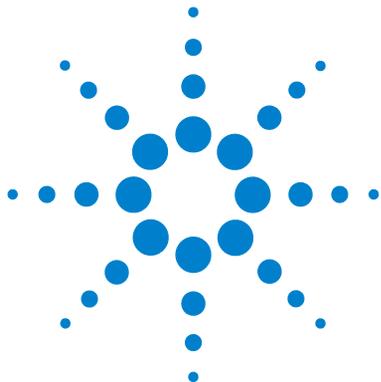
| Description  | Company and part no. |
|--|----------------------|
| Human Genomic DNA: Female (for possible use as a reference sample) | Promega p/n G1521    |
| 1× TE (pH 8.0), Molecular Grade                                    | Promega p/n V6231    |
| Repli-G Amplification Kit  | Qiagen 59043         |
| MilliQ water   |                      |

\* Same components, in smaller packagings, are available from Agilent's *In situ* Hybridization Kit PLUS (p/n 5184-3568), which includes one extra reagent that is not required for this procedure.

## Required software

| Description  |
|--|
| Windows NT 4.0 SP6, Windows 2000 SP2 or XP Operating System      |
| Agilent's Feature Extraction software, version A.7.5.1 or higher |





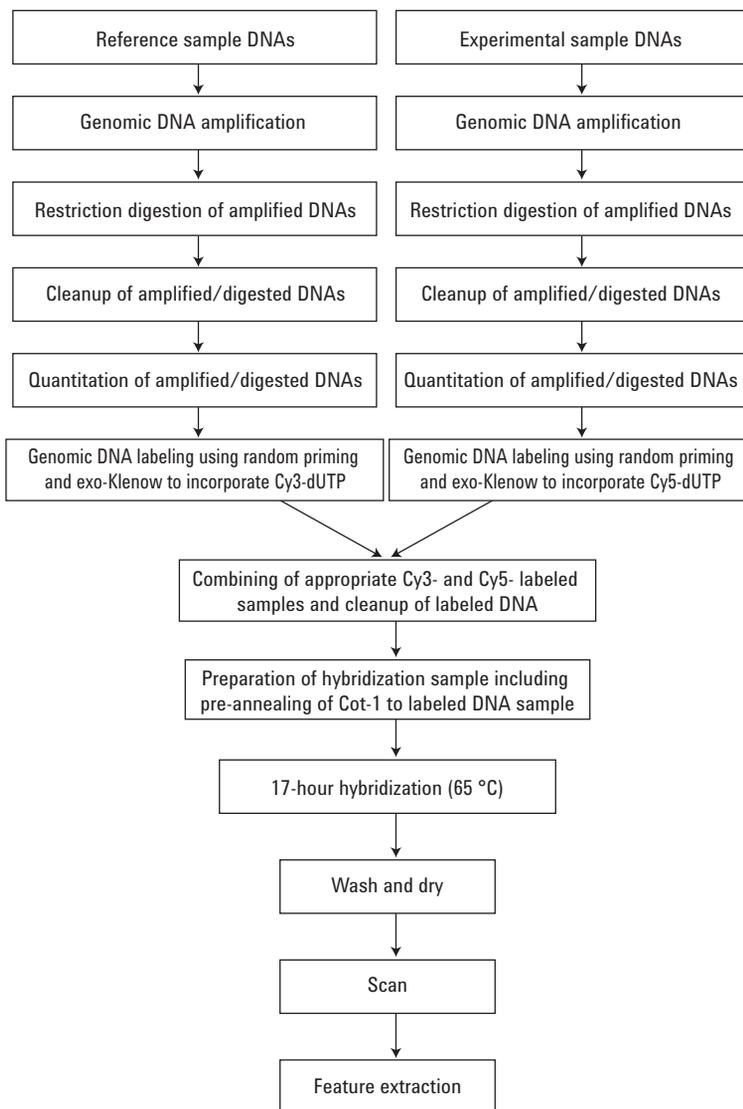
## 2 Procedures

|  |    |
|--|----|
| Genomic Amplification                                      | 15 |
| Restriction Digestion of Amplified DNA                     | 16 |
| Cleanup of Amplified/Digested DNA                          | 17 |
| Quantitation of DNA Product                                | 18 |
| Genomic DNA Labeling                                       | 19 |
| Cleanup of Labeled DNA                                     | 20 |
| Hybridization, Wash, and Dry                               | 21 |
| Scanning Using Agilent Scanner                             | 27 |
| Image Processing of .tif Files Produced by Agilent Scanner | 28 |
| Feature Extraction   | 29 |

Agilent's array-based Comparative Genomic Hybridization (aCGH) application uses a “2-color” process to measure genomic alterations in an experimental sample relative to a reference sample. The type of sample used as a reference is a matter of experimental choice, however, many experimenters use normal human (female) genomic DNA as a commercially available reference sample. [Figure 1](#) is a standard workflow for sample preparation and array hybridization designs.



## 2 Procedures



**Figure 1** Workflow for sample preparation and array processing.

## Genomic Amplification

The Repli-G Amplification Kit provides a highly uniform amplification across the entire genome with minimal sequence bias. The method uses phi29 DNA polymerase and exonuclease-resistant primers in an isothermal amplification reaction. A typical Repli-G amplification reaction yields approximately 25–30 µg of DNA, after digestion and cleanup, from 25 ng of input genomic DNA.

- 1 Add 25-ng genomic DNA to 1.5-mL nuclease-free microfuge tube. Add nuclease-free water to bring to a final volume of 20 µL.
- 2 Thaw all Repli-G kit components immediately before use and maintain on ice.

**CAUTION**

Combine Repli-G kit components on ice immediately prior to addition to sample. The 4× Mix may form a precipitate when thawed. The precipitate will fully dissolve upon vortexing for 10 s.

---

- 3 Mix the components in [Table 1](#) on ice in the order indicated. Quick freeze remainder of Repli-G kit components on dry ice, then return to –80 °C.

**Table 1** Preparation of Repli-G Master Mix

| Component                          | Volume ( $\mu\text{L}$ )/<br>reaction | Volume ( $\mu\text{L}$ )/<br>12 reactions |
|------------------------------------|---------------------------------------|---|
| Nuclease-free water                | 17.0                                  | 212.5                                     |
| 4 $\times$ Mix                     | 12.5                                  | 156.25                                    |
| DNA Polymerase                     | 0.5                                   | 6.25                                      |
| Final volume of Repli-G Master Mix | 30.0                                  | 375                                       |

\* Volume is calculated based on 12.5 reactions.

- 4 Dispense 30- $\mu\text{L}$  aliquots of Repli-G Master Mix into each reaction tube containing genomic DNA.
- 5 Transfer sample tubes to circulating water bath at 30 °C. Incubate at 30 °C for 16 h.
- 6 Transfer sample tubes to circulating water bath at 65 °C. Incubate at 65 °C for 10 min, then move to ice.

**NOTE**

We recommend using the Agilent 2100 Bioanalyzer in combination with the RNA 6000 Nano LabChip Kit (product number 5065-4476) for amplified genomic DNA analysis. Save an aliquot from [step 6](#) above for QC analysis.

## Restriction Digestion of Amplified DNA

- 1 Mix the components in [Table 2](#) on ice in the order indicated.

**Table 2** Preparation of Alul/RsaI Mix

| Component                     | Volume ( $\mu\text{L}$ )/<br>reaction | Volume ( $\mu\text{L}$ )/<br>12 reactions |
|-------------------------------|---------------------------------------|---|
| Nuclease-free water           | 30.0                                  | 375                                       |
| 10 $\times$ Reaction Buffer C | 10.0                                  | 125                                       |
| Alul (10 U/ $\mu\text{L}$ )   | 5.0                                   | 62.5                                      |
| RsaI (10 U/ $\mu\text{L}$ )   | 5.0                                   | 62.5                                      |
| Final volume of Alul/RsaI Mix | 50.0                                  | 625                                       |

\* Volume is calculated based on 12.5 reactions.

- 2 Dispense 50- $\mu\text{L}$  aliquots of Alul/RsaI Mix into each reaction tube from [step 6](#) of “[Genomic Amplification](#)”.
- 3 Transfer sample tubes to circulating water bath at 37 °C. Incubate at 37 °C for 2 h, then move to ice.

## Cleanup of Amplified/Digested DNA

- 1 **To prepare Buffer PE:** Add ethanol (100%) to buffer PE (supplied with QIAprep Miniprep Kit) (see bottle label for volume). Mark appropriate checkbox to indicate that ethanol was added to bottle.
- 2 Add 500  $\mu\text{L}$  of **Buffer PB** (supplied with QIAprep Miniprep Kit) to each sample.
- 3 Apply sample to QIAprep Miniprep column. Centrifuge 60 s at  $17,900 \times g$  (13,000 rpm in Eppendorf 5417R) in a microcentrifuge. Discard flow-through.
- 4 Add 750- $\mu\text{L}$  **Buffer PE** to each spin column. Centrifuge 60 s at  $17,900 \times g$  in a microcentrifuge. Discard flow-through.
- 5 Centrifuge 60 s at  $17,900 \times g$  in a microcentrifuge again.

### CAUTION

Residual wash buffer will not be completely removed unless the flow-through is discarded before this additional centrifugation. Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions.

---

- 6 Place the QIAprep spin column in a clean 1.5-mL microfuge tube. To elute DNA, add 50  $\mu\text{L}$  of **Buffer EB** (10-mM Tris-Cl, pH 8.5) to the center of each spin column. Let stand at room temperature (RT) for 60 s. Centrifuge 60 s at  $17,900 \times g$  in a microcentrifuge to collect purified DNA.
- 7 Samples may be stored at  $-20\text{ }^{\circ}\text{C}$  prior to labeling.

### NOTE

We recommend using the Agilent 2100 Bioanalyzer in combination with the DNA 12000 LabChip Kit (product number 5064-8231) for QC analysis. Save an aliquot from [step 6](#) above for QC analysis.

---

## Quantitation of DNA Product

- 1 For DNA concentration measurements using the NanoDrop ND-1000 UV-VIS spectrophotometer, select **Nucleic Acid Measurement**, then select **Sample Type** to be **DNA-50**.
- 2 Use 1.5  $\mu\text{L}$  of Buffer EB to blank instrument.
- 3 Use 1.5  $\mu\text{L}$  of each DNA sample from [step 6](#) of “[Cleanup of Amplified/Digested DNA](#)” to measure DNA concentration. Record the DNA concentration ( $\text{ng}/\mu\text{L}$ ) for each sample.
- 4 Calculate amplification yield ( $\mu\text{g}$ ) by multiplying DNA concentration ( $\text{ng}/\mu\text{L}$ ) by the sample volume (50  $\mu\text{L}$ ) and dividing by 1000.

### NOTE

A typical amplification reaction yields approximately 25–30  $\mu\text{g}$  of DNA, after digestion and cleanup, from 25 ng of input genomic DNA.

---

## Genomic DNA Labeling

The BioPrime Array CGH Genomic Labeling Module uses random primers and a mutant form of the Klenow fragment of DNA polymerase I (exo-Klenow) to differentially label genomic DNA samples with fluorescently labeled nucleotides. For Agilent's aCGH application, the experimental sample is labeled using one dye, and the reference sample is labeled using the other dye. The "polarity" of the sample labeling is a matter of experimental choice.

- 1 Calculate volume ( $\mu\text{L}$ ) of amplified DNA for a final 10.0  $\mu\text{g}$  (by dividing 10,000 by the DNA concentration [ $\text{ng}/\mu\text{L}$ ]).
- 2 Transfer volume of amplified DNA calculated in step 1 to a nuclease-free 1.5-mL microfuge tube. Add nuclease-free water to bring sample volume to a final volume of 21  $\mu\text{L}$ .
- 3 Dispense 20- $\mu\text{L}$  aliquots of 2.5 $\times$  Random Primers Solution (supplied with BioPrime Kit) into each reaction tube.
- 4 Transfer sample tubes to circulating water bath at 95  $^{\circ}\text{C}$ . Incubate at 95  $^{\circ}\text{C}$  for 5.0 min, then move to ice and incubate on ice 5.0 min.
- 5 Mix the components in [Table 3](#) on ice.

**Table 3 Preparation of exo-Klenow Mix**

| Component                          | Volume ( $\mu\text{L}$ )/<br>reaction | Volume ( $\mu\text{L}$ )/<br>12 reactions* |
|------------------------------------|---------------------------------------|--|
| 10 $\times$ dUTP Mix               | 5.0                                   | 62.5                                       |
| Cy3-dUTP 1.0 mM or Cy5-dUTP 1.0 mM | 3.0                                   | 37.5                                       |
| exo-Klenow                         | 1.0                                   | 12.5                                       |
| Final volume of exo-Klenow Mix     | 9.0                                   | 112.5                                      |

\* Volume is calculated based on 12.5 reactions.

- 6 Dispense 9.0- $\mu\text{L}$  aliquots of exo-Klenow Mix into each reaction tube.

**NOTE**

Each 50- $\mu$ L labeling DNA reaction contains 50-mM Tris-HCl (pH 6.8), 5-mM  $MgCl_2$ , 10-mM 2-mercaptoethanol, 300- $\mu$ g/mL random octamers, 120- $\mu$ M dATP, 120- $\mu$ M dGTP, 120- $\mu$ M dCTP, 60- $\mu$ M dTTP, 60- $\mu$ M Cy3-dUTP, 40-U *exo*-Klenow.

- 7 Transfer sample tubes to circulating water bath at 37 °C. Incubate at 37 °C for 2 h.

**NOTE**

Cy3-dUTP and cy5-dUTP are light sensitive. Minimize light exposure by covering the water bath.

- 8 Remove sample tubes from water bath. Add 5.0  $\mu$ L of Stop Buffer (supplied with BioPrime Kit) to each reaction tube.
- 9 Reactions can be stored at -20 °C overnight (O/N) in dark.

## Cleanup of Labeled DNA

- 1 Combine appropriate Cy5-labeled sample and Cy3-labeled sample for a mixture volume of approximately 100  $\mu$ L.
- 2 Add 400  $\mu$ L of 1 $\times$  TE (pH 8.0) to each reaction tube.
- 3 Place Microcon YM-30 filters in a 1.5-mL microfuge tube, and load each sample into filter. Centrifuge 10 min at 8,000  $\times$  g (8,700 rpm in Eppendorf 5417R) in a microcentrifuge at room temperature. Discard flow-through.
- 4 Add 500- $\mu$ L 1 $\times$  TE (pH 8.0) to each filter. Centrifuge 10 min at 8,000  $\times$  g in a microcentrifuge at room temperature. Discard flow-through.
- 5 Invert the filter into a fresh 1.5-mL microfuge tube. Centrifuge 1.0 min at 8,000  $\times$  g in microcentrifuge at room temperature to collect purified sample.
- 6 Measure volume ( $\mu$ L) of each eluate until each sample volume is <130  $\mu$ L. Bring total sample volume to 130  $\mu$ L with nuclease-free water.

**NOTE**

If sample volume exceeds 130 mL, return sample to its filter and centrifuge 5.0 min at  $8,000 \times g$  in microcentrifuge at room temperature. Discard flow-through. Repeat steps 5 and 6 until each sample volume <130 mL. Bring total sample volume to 130 mL with nuclease-free water.

7 Reactions can be stored at  $-20\text{ }^{\circ}\text{C}$  O/N in dark.

**NOTE**

We recommend using the Agilent 2100 Bioanalyzer in combination with the DNA 7500 LabChip Kit (product number 5064-8230) for labeled DNA analysis. Save an aliquot from [step 6](#) above for QC analysis.

## Hybridization, Wash, and Dry

### Preparation of reagents

#### Yeast tRNA 5.0 mg/mL

- 1 Add 1.0-mL nuclease-free water to a vial containing 25 mg of lyophilized yeast tRNA. Store at room temperature for 60 min to reconstitute sample.
- 2 Transfer sample to sterile 15-mL tube. Add 4.0-mL nuclease-free water to dilute sample to 5.0 mg/mL final concentration. Mix well. Dispense into 1.0-mL aliquots in 1.5-mL nuclease-free microfuge tubes. Store at  $-20\text{ }^{\circ}\text{C}$ .

#### Wash 1: 0.5× SSC, 0.005% Triton X-102

- 1 Add the components in [Table 4](#) in the order indicated to a nuclease-free graduated cylinder:

**Table 4 Wash 1: 0.5x SSC, 0.005% Triton X-102**

| Component        | Volume (mL) |
|------------------|-------------|
| 20× SSC          | 25          |
| 10% Triton X-102 | 0.5         |
| MilliQ water     | 975         |
| Final volume     | 1000        |

- 2 Pass solution through 0.2- $\mu$ m sterile filtration unit. Store at room temperature.

### Wash 2: 0.1× SSC, 0.005% Triton X-102

- 1 Add the components in [Table 5](#) in the order indicated to a nuclease-free graduated cylinder:

**Table 5 Wash 2: 0.1x SSC, 0.005% Triton X-102**

| Component        | Volume (mL) |
|------------------|-------------|
| 20× SSC          | 5           |
| 10% Triton X-102 | 0.5         |
| MilliQ water     | 994         |
| Final volume     | 1000        |

- 2 Pass solution through 0.2- $\mu$ m sterile filtration unit. Store at 37 °C to equilibrate temperature of wash solution to 37 °C.

### CAUTION

The temperature of Wash 2 solution must be at 37 °C for optimal performance. Prepare Wash 2 solution a day in advance of performing the wash procedure and store the solution in an incubator set to 37 °C. Before its use, verify that the temperature of Wash 2 solution is 37 °C.

## Preparation of hybridization samples

- 1 Add the components in [Table 6](#) in the order indicated to reaction tubes containing 130  $\mu\text{L}$  of purified Cy5- and Cy3-labeled sample mixture from [step 6](#) of “[Cleanup of Labeled DNA](#)”.

**Table 6 Assembly of Hybridization Samples**

| Component                         | Volume ( $\mu\text{L}$ ) |
|-----------------------------------|--------------------------|
| Cot-1 DNA (1.0 mg/mL)             | 50                       |
| Yeast tRNA (5 mg/mL)              | 20                       |
| Agilent 10x Control Targets       | 50                       |
| Agilent 2x Hybridization Buffer   | 250                      |
| Final Hybridization Sample volume | 500                      |

- 2 Transfer sample tubes to circulating water bath at 95 °C. Incubate at 95 °C for 3 min, then move to ice.
- 3 Transfer sample tubes to circulating water bath at 37 °C. Incubate at 37 °C for 30 min.
- 4 Remove samples from water bath. Centrifuge 5 min at  $17,900 \times g$  (13,000 rpm in Eppendorf 5417R) in a microcentrifuge to pellet any precipitates. Carefully remove supernatant materials.

## Hybridization

- 1 Load a clean SureHyb gasket slide into the chamber base with the label facing up and aligned with the rectangular section of the chamber base. Ensure that the gasket slide is flush with the chamber base and is not ajar.
- 2 Slowly dispense 490  $\mu\text{L}$  of hybridization mixture onto the gasket well in a "drag and dispense" manner.
- 3 Place an array active side down onto the Surehyb gasket, so the numeric barcode side is facing up. Assess that the sandwich-pair is properly aligned.

- 4 Place the chamber cover onto the sandwiched slides and slide the clamp assembly onto both pieces.
- 5 Hand-tighten the clamp onto the chamber.
- 6 Vertically rotate the assembled chamber to wet the gasket and assess the mobility of the bubbles. Tap the assembly on a hard surface if necessary to move stationary bubbles.
- 7 Place assembled slide chamber in rotisserie in hybridization oven set to 65 °C. Turn on rotisserie to rotate at setting 5.
- 8 Hybridize at 65 °C for 17 h.

### Wash and dry

#### CAUTION

For optimal performance, the temperature of Wash 2 solution must be 37 °C . Prepare Wash 2 solution a day in advance of performing the wash procedure and store the solution in an incubator or circulating water bath set to 37 °C. Before its use, verify that the temperature of the Wash 2 solution is 37 °C.

---

#### CAUTION

A maximum of four disassembly procedures yielding four slides is advised if you are using a 10-slide rack. This ensures that there is an empty slot between each slide and between the slide and the side of the slide rack. This facilitates uniform washing, which is important for optimum performance.

---

**Table 7 Wash Conditions**

|             | <b>Buffer</b> | <b>Temperature</b> | <b>Time (min)</b> |
|-------------|---------------|--------------------|-------------------|
| Disassembly | Wash 1        | Room temperature   |                   |
| 1st wash    | Wash 1        | Room temperature   | 5                 |
| 2nd wash    | Wash 2        | 37 °C              | 5                 |

- 1** Completely fill slide-staining dish #1 with Wash 1 at room temperature.
- 2** Place slide rack into slide-staining dish #2. Add magnetic stir bar. Fill slide-staining dish #2 with enough Wash 1 at room temperature to cover the slide rack.
- 3** Place slide rack into slide-staining dish #3. Add magnetic stir bar. Fill the slide-staining dish with enough Wash 2 at 37 °C to cover the slide rack. Place slide-staining dish #3 on a magnetic stirrer with a heating element to maintain temperature of Wash 2 solution at 37 °C during wash.
- 4** Remove one hybridization chamber from incubator and record time. Record whether a bubble formed during hybridization, and if all bubbles are rotating freely.
- 5** Prepare the hybridization chamber assembly.
  - a** Place the hybridization chamber assembly on a flat surface and loosen the thumbscrew, turning counter-clockwise.
  - b** Slide off the clamp assembly and remove the chamber cover.
  - c** With gloved fingers, remove the “sandwiched slides” from the chamber base by grabbing the slides from their ends. Keep the oligo microarray slide (numeric barcode facing

- up) as you quickly transfer the sandwiched slides to slide-staining dish #1.
- d** Without letting go of the “sandwich slides,” submerge the slides into slide-staining dish #1 containing **Wash 1**.
  - e** With the “sandwiched slides” **completely submerged** in the wash solution, pry the two slides apart from the **barcode end only**. Do this by slipping one of the blunt ends of the tweezers between the slides and then gently turn the tweezers upwards or downwards to separate the slides. Let the gasket slide drop to the bottom of the staining dish.
  - f** Remove the oligo microarray slide quickly and place into the slide rack contained in slide-staining dish #2 containing **Wash 1** at RT. Minimize exposure of the slide to air. Touch only the barcoded portion of the slide or its edges!
- 6** Repeat steps [step 4](#) and [step 5](#) for all remaining hybridization chambers in the group. A maximum of four disassembly procedures yielding four slides is advised at one time in order to facilitate uniform washing.
  - 7** Transfer slide-staining dish #2 containing slides to a magnetic stirrer, and stir using setting 4 for 5 minutes.
  - 8** Transfer slide rack containing all slides to slide-staining dish #3 filled with Wash 2 at 37 °C, and stir using setting 4 for 5 minutes.
  - 9** The slide rack containing slides must stay immersed in the **Wash 2** during the individual slide drying process. Dry each slide, one slide at a time. Slowly remove one slide from the slide rack, trying to minimize wash buffer droplets on the slide surface. Using a nitrogen-filled air gun, quickly blow drops of solution from the slide surface. Repeat procedure for each individual slide in the slide rack.

### NOTE

Take care to avoid allowing drops of solution to travel back over the slide once the microarray is dried.

- 10 Scan slides immediately to minimize impact of environmental oxidants on signal intensities. If necessary, store slides in orange slide boxes in a N<sub>2</sub> purge box, in the dark, or in a vacuum desiccator prior to scanning.

## Scanning Using Agilent Scanner

- 1 Assemble slides into appropriate slide holder. For version B slide holders, slides should be placed into slide holder with Agilent barcode facing up.
- 2 Place assembled slide holders into scanner carousel.
- 3 Verify Default Scan Settings.
  - a Choose **Settings** and then **Modify Default Settings**.
  - b Verify that **Scan region** is set to **Scan Area (61 × 21.6 mm)**, **Dye channel** is set to **Red & Green**, the **Scan resolution (μm)** is set to **10**, and the **Red PMT** and **Green PMT** are each set to **100%**.
  - c Verify that defaults settings are selected for the automatic file naming: **Instrument Serial Number** for **Prefix 1** and **Array Barcode** for **Prefix 2**.
- 4 Verify that the Scanner status in the main window says Scanner Ready.
- 5 Click **Scan Slot m-n** on the Scan Control main window where the letter **m** represents the Start slot where the first slide is located and the letter **n** represents the End slot where the last slide is located.

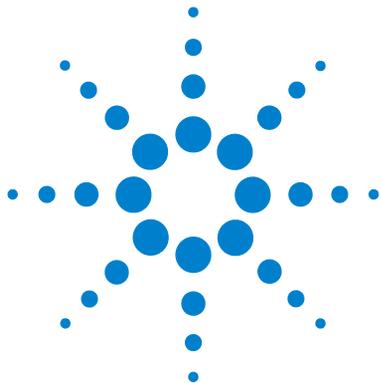
### Image Processing of .tif Files Produced by Agilent Scanner

- 1 Open Raw Image file from Agilent's Feature Extraction Software.
- 2 Verify PMT voltage settings for scanned images.
  - a Choose **View** and then **FileInfo**, and select the **Image** tab.
  - b The radio buttons **Image 1** and **Image 2** display the PMT voltage for the red and the green scan, respectively. The default voltage setting will depend on the scanner used.
  - c Verify that PMT voltage settings are the "default" settings specified for the scanner used. If not, rescan the slides using the proper PMT voltage settings.
- 3 Crop the Raw Images.
  - a To crop an array image, first turn cropping mode **On** by making sure that the crop-mode is depressed on the toolbar, then press down the left mouse button in one corner and move the mouse to the opposite corner while holding down the left mouse button. Select the entire array, while leaving a border on all sides.
  - b Choose **File** and then **SaveModifiedImage** to save the cropped image file as a .tif file.
  - c The radio buttons **Left or single array** and **Right array** indicate which image was cropped when multiple arrays are present on one slide. For "football field" type arrays, verify that **Left or single array** is selected.
  - d Click **Browse** to select a destination for the cropped files.
  - e Click **SaveModifiedImage**. Cropped Images will be saved with the suffix **\_A01**.
  - f Repeat [step a](#) through [step e](#) until all array images are processed.

## Feature Extraction

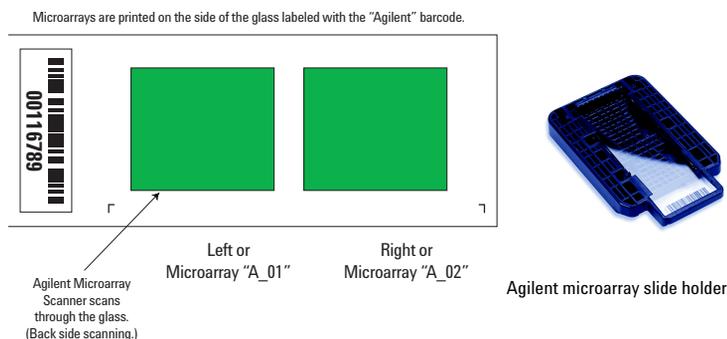
- 1 Open one Cropped Image file from Image Analysis application.
- 2 Specify design file path and file output preferences.
  - a Choose **Tools**, then **Preferences**, then **Feature Extraction**.
  - b Click **Design File first**, then **Design File Search Path**. Click **Browse** to select the appropriate folder containing the design files.
  - c Click **Add** to add the directory path to the design files.
  - d Verify the .XML file specifying the corresponding array design is in this directory. If not, copy the design file to this location from the CD supplied with the array kit.
  - e Verify that the **Same as the image file** box is checked to specify the default directory for the output files. Verify that the **Save log files** box is checked. The output files should be placed in the same directory as the Cropped Images files to enable the visualization of Extraction results.
  - f Click **OK** to exit Preferences.
- 3 Choose **Feature Extraction**, then **Feature Extractor** to open application.
- 4 When the **Feature Extraction Configuration** window appears, select the desired file types for the result files by checking the appropriate boxes.
- 5 Set settings for FE parameters.
  - a Click **More**.
  - b Select the **FindSpots** tab. Click **Load Defaults**. Verify **Autofind Corners on** and **Dev Limit = 30** microns.
  - c Select the **SpotAnalyzer** tab. Click **Load Defaults**. Verify that **CookieCutter** is selected for the **Spot analysis method**, the **Local Background** Radius is set to **127** microns, and the **Reject based on IQR** radio is selected for Pixel outlier rejection (Feature = **1.42**, Background = **1.42**).
  - d Select the **PolyOutlierFlagger** tab. Click **Load Defaults**. Verify that **Non-Uniformity Outlier Flagging** is **On** and that values for

- Term (A) = 0.0081** (Feature), **Term (A) = 0.0225** (Background), **Term (B) = 320** (Feature and Background), **Term (C) = 600** (Feature and Background). Verify **Population Outlier Flagging** is **On** (Reject based on IQR = **1.42** and Min population = **10**).
- e Select the **BGSub** tab. Select **Average of negative control features** for the **Background Subtraction Method**. Verify that for **Feat Significance** the value for **2-sided t-test of feature vs. background max p-value = 0.01** and **WellAbove SD Multi = 2.6**. Uncheck **Spatial detrend** to turn **Off**. Verify that **Adjust background globally** is **Off**.
  - f Choose the **Dye Norm** tab. Verify **Rank Consistency Filter** radio is checked as the **Normalization Feature Method**. Verify that **Omit feature with background PopnOL** is **Off**. Select **Linear** for the **Normalization Method**.
  - g Choose **Ratio**. Click **Load Defaults**. Verify **Use most conservative estimate of error between Universal Error Model and propagated error** radio is selected (Multiplicative error: Red = **0.08**, Green = **0.08**, Additive error: Red = **25**, Green = **25**). Verify **Use Surrogates** is checked **On** and that **Additive error Auto. estimate** for Red and Green is **Off**.
- 6 Press **Run** and Feature Extraction will start. When Feature Extraction is complete, the results are displayed in a separate **Results** window. To close this window, click **OK**. Visual results are then displayed on the array image.
  - 7 Inspect the Visuals results to verify that the landing lights were appropriately identified and that the grid was properly placed. If a warning message appears starting with "Description: (Fit2BrightSpots)", Feature Extraction failed to locate the grid and corner locations must be input manually. To manually locate corners, choose **FindSpots** and uncheck **Autofind corners** to select **Manual corner location**. Manually locate the **X** and **Y** positions of the **Upper Left**, **Upper Right** and **Lower Left** fiducial features.
  - 8 Close Cropped Image file by clicking **X** at the upper-right corner of window.



## A General Microarray Layout and Orientation

### Agilent oligo microarray (2 microarrays/slide format) as imaged on the Agilent microarray scanner (G2565BA)



**Figure 2** Microarray slide and slide holder

Agilent oligo microarray formats and the resulting “microarray design files” are based on how the Agilent microarray scanner images 1-in × 3-in glass slides. Agilent designed its microarray scanner to scan through the glass slide (back side scanning). The glass slide is securely placed in an Agilent slide holder with the “Agilent-labeled” barcode facing upside down. In this orientation, the “active side” containing the microarrays is protected from potential damage by fingerprints and other elements. Once securely placed, the “numeric barcoded” nonactive side of the slide is visible.

Figure 2 depicts how the Agilent microarray scanner reads the microarrays and how this relates to the “microarray design files” that Agilent generates during the manufacturing process



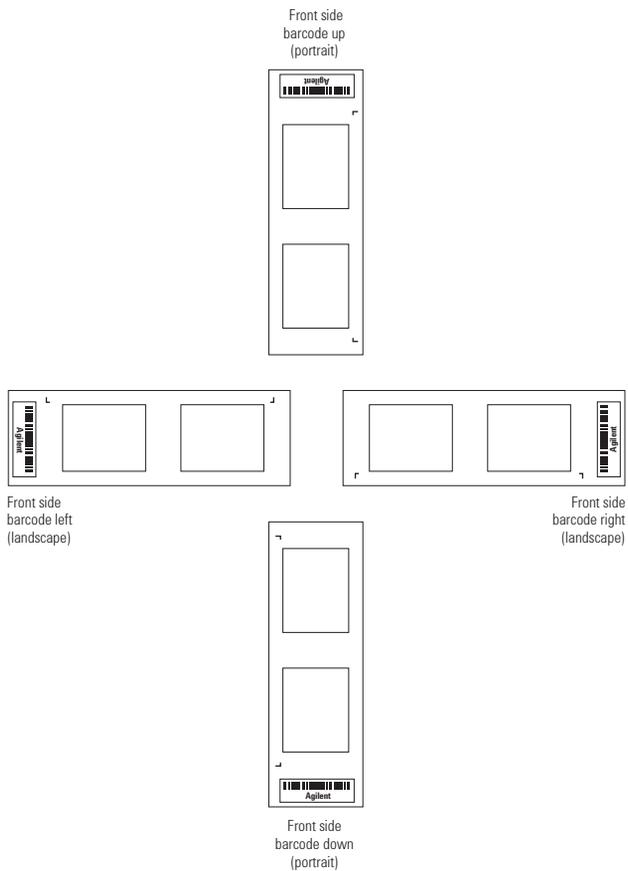
of its *in situ*-synthesized oligonucleotide microarrays. Thus, if you have a scanner that reads microarrays from the “front side” of the glass slide, the collection of microarray data points will be different in relation to the “microarray design files” supplied with the Agilent oligo microarray kit you purchased. Therefore, please take a moment to become familiar with the microarray layouts for each of the Agilent oligo microarrays and the layout information as it pertains to scanning using a “front side” scanner.

### **NonAgilent front side microarray scanners**

When imaging Agilent oligo microarray slides, the user must determine:

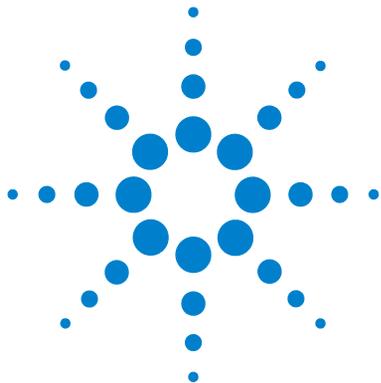
- If their scanner images microarrays by reading them on the “front side” of the glass slide (“Agilent-labeled” barcode side of the slide) and
- If the image produced by the nonAgilent scanner is oriented in a “portrait” or “landscape” mode, “Agilent-labeled” barcode left-side, right-side, up or down, as viewed as an image in the imaging software (see [Figure 3](#) below).

This changes the feature numbering and location as it relates to the “microarray design files” found on the CD in each Agilent oligo microarray kit. Microarray layout maps are available from Agilent. For more information, visit [www.agilent.com/chem/dnasupport](http://www.agilent.com/chem/dnasupport) for access to scanner compatibility Agilent Microarray Maps User Guide (G4140-90020) for visual references and guides that will help you determine the feature numbering as it pertains to your particular scanner configuration.



**Figure 3** Microarray slide orientation

## General Microarray Layout and Orientation



## **B** **Agilent Information Assets Access Agreement**

### **I. PURPOSE**

- 1** Agilent Technologies (“Agilent”) and User have entered into a business relationship that would be fostered by granting User access to certain of Agilent's Information Assets (“Assets”). Accordingly, User and Agilent have entered into this Information Assets Access Agreement (IAAA) in order to protect the confidentiality, integrity, availability, and physical security of Agilent 's various information assets and to prevent unauthorized individuals from gaining access to Agilent's various networks, computers and information assets. Access is granted only for the purposes of fulfilling the specific business relationship between User and Agilent which gives rise to this agreement. The use of Agilent information for any other purpose is expressly prohibited.
- 2** User and Agilent acknowledge that they have entered into other agreements or contractual arrangements defining their business relationship. These agreements are independent of this IAAA and in the event of any conflict between them, the terms of this IAAA shall prevail only as applies to this IAAA.

### **II. PHYSICAL SECURITY OF AGILENT INFORMATION ASSETS**

- 1** User agrees to exercise no less than a reasonable degree of care to ensure that access to Agilent's information assets is limited solely to its authorized personnel who have been granted access by Agilent to specific information assets. Under no circumstance may User allow an individual to access an Agilent information asset without a properly authorized grant of access from Agilent for that individual and for that information asset. Only those Users provided



access to the information assets via an Agilent authorized authentication procedure may access the information assets.

USE OF AGILENT INFORMATION ASSETS FOR OTHER THAN INTERNAL USE AND/OR FOR COMMERCIAL PURPOSES WITHOUT AGILENT'S PRIOR WRITTEN CONSENT IS EXPRESSLY PROHIBITED.

Use of any other computer systems or Agilent information assets is expressly prohibited in the absence of written authorization from Agilent. This prohibition applies even when an information resource which User is authorized to access serves as a gateway to other information resources outside of User's scope of authorization. These prohibitions apply even if Agilent fails to implement physical or logical security measures which prevent the access.

USE OF AGILENT INFORMATION ASSETS BY OTHER INDIVIDUALS NOT AUTHORIZED IN WRITING OR VIA ELECTRONIC MAIL BY AGILENT IS EXPRESSLY PROHIBITED.

- 2** Without limiting the foregoing, User specifically agrees to:
  - a** Control physical access to passwords, tokens, log-on scripts and other such hardware/data as may be used to access Assets. No passwords, security tokens or other authentication information may be shared between individuals without written approval from Agilent. User agrees to access Agilent information resources only from the specific locations that have been approved for access by Agilent management. For access outside of Agilent premises, Agilent will specify in writing or via electronic mail the specific network connections that will be used to access Agilent information assets.
  - b** Periodically review personnel granted access under this IAAA to insure that access remains necessary to the underlying business purpose.
  - c** Coordinate within User's organization the proper implementation of this agreement and ensure that all the rules and obligations contained in this agreement are strictly followed and complied with at all times in User's organization.

- 3 In its sole discretion and without incurring liability, Agilent reserves the right to deny, limit, or terminate access of any person or firm or access to any system(s) without notice or cause.

### **III. PROTECTION OF ELECTRONIC CONFIDENTIAL INFORMATION**

- 1 User and its employees or agents (collectively referred to as User, unless the context requires otherwise) shall not disclose any Electronic Confidential Information (ECI) received from Agilent to any other person without the express prior written consent of Agilent. User shall use such information only for purposes necessary for its relationship with Agilent or consistent with this Agreement, and shall use reasonable care to avoid unauthorized disclosure or use of such ECI.
- 2 Electronic Confidential Information obtained from Assets shall be defined as any information marked as confidential or proprietary, and any information, while not marked as confidential or proprietary, which is of such a nature that a reasonable person would believe it to be confidential or proprietary. Agilent shall take reasonable precautions to prevent the User from receiving unmarked confidential or proprietary information. However, User acknowledges that due to the nature of electronic systems, such information may be delivered to User inadvertently or with an inadvertent omission of the markings. If User receives information that a reasonable person would believe to be confidential or proprietary, User shall have an affirmative duty to inquire of Agilent as to the confidentiality of the information before treating it as non-confidential information.
- 3 This Agreement imposes no obligation upon User with respect to any confidential or proprietary information disclosed to User pursuant to the terms of a separately executed Confidential Disclosure Agreement or other contractual arrangement specifically addressing such information. The obligations of the parties with respect to such information shall be governed solely by the terms of

such Confidential Disclosure Agreement or contractual arrangement.

#### **IV. LIMITED RIGHTS TO PUBLISH IN PEER-REVIEWED JOURNALS ONLY**

- 1** User may publish the following only in peer-reviewed scientific journals [“Publication.”]
- 2** User may publish only fifty (50) probe-sequences in any single Publication.
- 3** User or its permitted publisher, including without limitation the investigator, scientists, and/or researchers, hereby agrees to add a statement in a prominent location within such publication; provided User publishes the specific design file number that Agilent shall provide, stating the following: ANY PUBLICATION OR PUBLIC DISCLOSURE OF OLIGONUCLEOTIDE SEQUENCES FOR USE WITH MICROARRAYS MAY ONLY BE REPRODUCED OR MANUFACTURED FOR YOU BY AGILENT TECHNOLOGIES, INC. BY REFERENCING THE CATALOG MICROARRAY PRODUCT NUMBER OR THE CUSTOM MICROARRAY DESIGN IDENTIFICATION NUMBER SUPPLIED BY YOU BY AGILENT.
- 4** User hereby grants Agilent all rights to reproduce products based upon User's Confidential Information for Third Parties requesting such Products.

#### **V. ADMINISTRATION**

- 1** Audit: Upon reasonable notice, User shall allow Agilent access to such information as may reasonably be necessary to ascertain User's compliance with the obligations contained herein.
- 2** Loss/Damage: User shall indemnify and hold Agilent harmless from all loss, damage, cost and expense arising from User's breach of this Agreement.
- 3** User warrants and represents that each employee or agent of User who is granted access under this agreement has been informed of the obligations contained herein and has agreed in writing to be bound by them. Without limiting the foregoing, each individual must be informed of the specific information assets he is authorized to access and the duty to

refrain from accessing other information assets outside his scope of authorization even if logical or physical security measures are not in place to prevent the access.

**VI. MISCELLANEOUS**

- 1** This Agreement is made under and shall be construed according to the laws of the State or Country in which the signing Agilent entity is located.
- 2** This Agreement constitutes the entire and exclusive agreement of the parties regarding the subject matter of this Agreement. This Agreement may not be varied.







**Agilent Technologies**

Information, descriptions, and specifications are subject to change without notice. Please register online with Agilent to receive new product updates at [www.agilent.com/chem/dnasupport](http://www.agilent.com/chem/dnasupport).

Agilent Genomics Solution websites:  
[www.agilent.com/chem/genomics](http://www.agilent.com/chem/genomics)  
[www.agilent.com/chem/dna](http://www.agilent.com/chem/dna)

© Agilent Technologies, Inc.  
Printed in USA, January 2005



G4410-90010