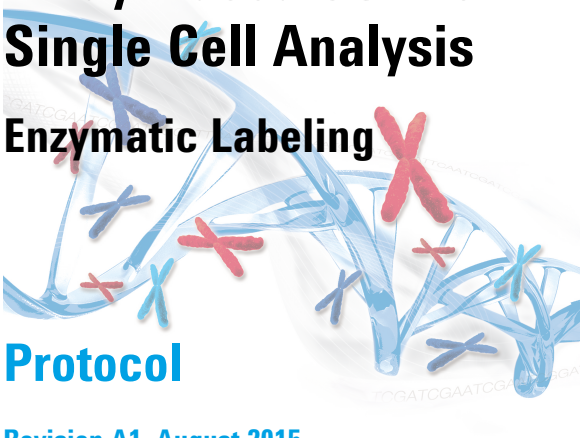
Agilent Oligonucleotide Array-Based CGH for Single Cell Analysis

Enzymatic Labeling



Protocol

Revision A1, August 2015

Before you begin, view hands-on videos of SurePrint procedures at
http://www.agilent.com/genomics/protocolvideos.

Agilent Technologies

Notices

Manual Part Number

G4410-90012

Edition

Revision A1, August 2015

Agilent Technologies, Inc.
5301 Stevens Creek Blvd.
Santa Clara, CA 95051 USA

Technical Support

Technical product support can be obtained by contacting your local Agilent Support Services representative. Agilent's worldwide sales and support center telephone numbers can be obtained at the following web site under **Contact Us**:
www.agilent.com/genomics

or send an e-mail to:
genomics@agilent.com

Warranty

The material contained in this document is provided "as is," and is subject to being changed, without notice, in future editions. Further, to the maximum extent permitted by applicable law, Agilent disclaims all warranties, either express or implied, with regard to this manual and any information contained herein, including but not limited to the implied warranties of merchantability and fitness for a particular purpose. Agilent shall not be liable for errors or for incidental or consequential damages in connection with the furnishing, use, or performance of this document or of any information contained herein. Should Agilent and the user have a separate written agreement with warranty terms covering the material in this document that conflict with these terms, the warranty terms in the separate agreement shall control.

Technology Licenses

The hardware and/or software described in this document are furnished under a license and may be used or copied only in accordance with the terms of such license.

Restricted Rights Legend

U.S. Government Restricted Rights. Software and technical data rights granted to the federal government include only those rights customarily provided to end user customers. Agilent provides this customary commercial license in Software and technical data pursuant to FAR 12.211 (Technical Data) and 12.212 (Computer Software) and, for the Department of Defense, DFARS 252.227-7015 (Technical Data - Commercial Items) and DFARS 227.7202-3 (Rights in Commercial Computer Software or Computer Software Documentation).

Safety Notices

CAUTION

A **CAUTION** notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in damage to the product or loss of important data. Do not proceed beyond a **CAUTION** notice until the indicated conditions are fully understood and met.

WARNING

A WARNING notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in personal injury or death. Do not proceed beyond a WARNING notice until the indicated conditions are fully understood and met.

In This Guide...

This guide describes the recommended operational procedures to analyze DNA copy number variations using Agilent 60-mer oligonucleotide microarrays for array-based comparative genomic hybridization (aCGH) analysis. This protocol is specifically developed and optimized to amplify and enzymatically label DNA from single cells, and to hybridize to 8×60K CGH microarrays. For processing DNA isolated from blood, cell culture, or frozen tissues, follow the *Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis (Enzymatic Labeling for Blood, Cells or Tissues (with a High Throughput option) Protocol* (p/n G4410-90010).

1 Before You Begin

This chapter contains information (such as procedural notes, safety information, required reagents and equipment) that you should read and understand before you start an experiment.

2 Sample Amplification

This chapter describes the standard method to process single cells and reference genomic DNA (gDNA) for Whole Genome Amplification prior to labeling.

3 Sample Labeling

This chapter describes the steps to differentially label the amplified DNA samples with fluorescent-labeled nucleotides.

4 Microarray Processing

This chapter describes the steps to hybridize, wash and scan Agilent CGH microarrays and to extract data using the Agilent Feature Extraction Software for use in Agilent CytoGenomics and Genomic Workbench.

6 Troubleshooting

This chapter contains possible causes for above-threshold DLRSD (Derivative Log Ratio Standard Deviation). A poor DLRSD score reflects high probe-to-probe log ratio noise.

7 Reference

This chapter contains reference information related to the amplification, labeling, hybridization and wash kits, and the protocol.

What's new in A1

- Instructions to calibrate hybridization oven for accuracy of the collected data is described.
- Added reference to compatibility matrix for non-Agilent scanners.
- Updated product labeling statement.

Content

1 Before You Begin

Make sure that you read and understand the information in this chapter and have the necessary equipment and reagents listed before you start an experiment.

Procedural Notes

- Follow the procedure described in this document to amplify DNA from single cells to increase the likelihood of a successful experiment.
- To prevent contamination of reagents by nucleases, always wear powder-free laboratory gloves, and use dedicated solutions and pipettors with nuclease-free aerosol-resistant tips.
- Maintain a clean work area.
- Do not mix stock solutions and reactions containing gDNA or enzymes on a vortex mixer. Instead, mix the solutions and reactions by gently tapping the tube with your finger.
- Avoid repeated freeze-thaw cycles of solutions containing gDNA or enzymes.
- When preparing frozen reagent stock solutions for use:
  1. Thaw the aliquot as quickly as possible without heating above room temperature.
  2. Mix briefly on a vortex mixer, and then spin in a microcentrifuge for 5 to 10 seconds to drive the contents off the walls and lid.
  3. Store on ice or in a cold block until use.
- 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 reagents are considered hazardous by the OSHA Hazard Communication Standard (29 CFR 1910.1200). Contains material that causes damage to the following organs: kidneys, liver, cardiovascular system, respiratory tract, skin, eye lens or cornea, stomach. May be harmful if swallowed. Avoid contact with eyes, skin and clothing.**
- **2× HI-RPM Hybridization Buffer is considered hazardous by the OSHA Hazard Communication Standard (29 CFR 1910.1200). Contains material that causes damage to the following organs: skin, central nervous system. May be harmful if swallowed. Avoid contact with eyes, skin and clothing.**
- **Triton is harmful if swallowed. Risk of serious damage to eyes. Wear suitable PPE. Triton is a component of the Agilent 2× HI-RPM Hybridization Buffer.**
- **Stabilization and Drying Solution is considered hazardous by the OSHA Hazard Communication Standard (29 CFR 1910.1200). Flammable liquid and vapor. Keep away from heat, sparks and flame. Keep container closed. Use only with adequate ventilation. This solution contains material which causes damage to the following organs: kidneys, liver, cardiovascular system, upper respiratory tract, skin, central nervous system (CNS), eye, lens or cornea.**

Agilent Oligo CGH Microarray Kit Contents

Store microarray kit at room temperature. After the microarray foil pouch is opened, store the microarray slides at room temperature (in the dark) under a vacuum desiccator or N_2 purge box. Do not store microarray slides in open air after breaking foil.

SurePrint G3 CGH Bundle

- Six 1-inch × 3-inch 8-pack microarray slides
- Sufficient reagents and consumables to process 48 samples:
  - SureTag Complete DNA Labeling Kit
  - Oligo aCGH/ChIP-on-chip Hybridization Kit
  - Hybridization gasket slides
  - Human Cot-1 DNA
  - Oligo aCGH/ChIP-on-chip Wash Buffer 1 and Oligo aCGH/ChIP-on-chip Wash Buffer 2
  - Agilent CytoGenomics Software License

Catalog SurePrint G3 CGH Microarray Kit

- Three 1-inch × 3-inch, 8-pack microarray slides

Unrestricted SurePrint G3 CGH Microarrays

- Eight microarrays printed on each 1-inch × 3-inch glass slide

Design files can be downloaded from http://www.agilent.com/genomics/suredesign.

Table 1 SurePrint G3 CGH Microarrays (8×60K)

Part Number	Description
G5923A Option 1	SurePrint G3 Human CGH Bundle, 8×60K
G4450A	Catalog SurePrint G3 Human CGH Microarray Kit 8×60K
G4827A, AMADID 021924	Unrestricted SurePrint G3 CGH Microarray, 8×60K, Human (same design as G4450A)

Required Equipment

Table 2 Required equipment

Description	Vendor and part number
200 µL Thin-Wall Tube	Agilent p/n 410091 or equivalent
Agilent Microarray Scanner Bundle for	Agilent p/n G4900DA or G2565CA
Hybridization Chamber, stainless	Agilent p/n G2534A
Hybridization gasket slides, 5-pack (20 and 100 packaging sizes are available)* for 8-pack microarrays	Agilent p/n G2534-60014
Hybridization oven; temperature set at 65°C	Agilent p/n G2545A
Hybridization oven rotator for Agilent Microarray Hybridization Chambers	Agilent p/n G2530-60029
Ozone-barrier slide covers (box of 20)†	Agilent p/n G2505-60550
Thermal cycler with heated lid	Agilent p/n G8800A or equivalent
UV-Transilluminator with SYBR photographic filter	Alpha Innotech p/n AlphaImager 2000 or equivalent
1.5 mL RNase-free Microfuge Tube (sustainable at 98°C)	Ambion p/n AM12400 or equivalent
Magnetic stir plate (×1 or ×3)‡	Corning p/n 6795-410 or equivalent
Magnetic stir plate with heating element	Corning p/n 6795-420 or equivalent
Microcentrifuge	Eppendorf p/n 5430 or equivalent
E-Gel Opener	Life Technologies p/n G5300-01
E-Gel PowerBase v.4	Life Technologies p/n G6200-04
Sterile storage bottle	Nalgene 455-1000 or equivalent
UV-VIS spectrophotometer	NanoDrop 8000 or 2000, or equivalent
P10, P20, P200 and P1000 pipettes	Pipetman P10, P20, P200, P1000 or equivalent
1.5 L glass dish	Pyrex p/n 213-R or equivalent

Table 2 Required equipment **(continued)**

Description	Vendor and part number
Vacuum Concentrator	Thermo Scientific Savant SpeedVac p/n DNA120-115 or equivalent
Magnetic stir bar, 7.9 × 38.1 mm (×2 or ×4)‡	VWR p/n 58948-150 or equivalent
250 mL capacity slide-staining dish, with slide rack (×3 or ×5)‡	Wheaton p/n 900200 *or* Thermo Shandon p/n 121
Circulating water baths or heat blocks set to 37°C, 65°C, and 95°C	
Ice bucket	
Clean forceps	
Powder-free gloves	
Sterile, nuclease-free aerosol barrier pipette tips	
Timer	
Vacuum desiccator or N_2 purge box for slide storage	
Vortex mixer	

* Included in the SurePrint G3 CGH Bundle.

† Optional. Recommended when processing arrays with a G2565CA scanner in environments in which ozone levels are 5 ppb or higher.

‡ The number varies depending on if wash procedure A or B is selected.

Required Reagents

Table 3 Required reagents for sample amplification

Description	Vendor and part number
10× Phosphate Buffered Saline, pH 7.4 (PBS)	Life Technologies p/n AM9624
Clear E-Gel (1.2% agarose, no stain), 18-pack	Life Technologies p/n G5518-01
SYBR Gold Nucleic Acid Gel Stain	Life Technologies p/n S11494
SYBR photographic filter	Life Technologies p/n S7569
TrackIt 1 Kb DNA Ladder	Life Technologies p/n 10488-072
DNase/RNase-free distilled water	Life Technologies p/n 10977-015
PicoPlex WGA Kit for Single-Cell (50 reactions)	Rubicon Genomics p/n R30050
Ethanol (95% to 100% molecular biology grade)	Sigma-Aldrich p/n E7023-6×500ML

Table 4 Required reagents for enzymatic sample labeling with the SureTag Complete DNA Labeling Kit

Description	Vendor and part number
SureTag Complete DNA Labeling Kit*†	Agilent p/n 5190-4240
Purification Columns‡ (50 units)	Agilent p/n 5190-3391
1×TE (pH 8.0), Molecular grade	Promega p/n V6231

* Kit content is listed in "Reagent Kit Components" on page 62.

† Included in the SurePrint G3 CGH Bundle.

‡ Included in the SureTag Complete DNA Labeling Kit. Order additional columns when processing more than 25 8-pack microarrays.

Table 5 Required reagents for hybridization and wash

Description	Vendor and part number
Oligo aCGH/ChIP-on-chip Wash Buffer Kit *or*	Agilent p/n 5188-5226
Oligo aCGH/ChIP-on-chip Wash Buffer 1 *and* Oligo aCGH/ChIP-on-chip Wash Buffer 2*	Agilent p/n 5188-5221 Agilent p/n 5188-5222
Stabilization and Drying Solution†	Agilent p/n 5185-5979
Oligo aCGH/ChIP-on-chip Hybridization Kit*	Agilent p/n 5188-5220 (25) or p/n 5188-5380 (100)
Human Cot-1 DNA*	Agilent p/n 5190-3393
DNase/RNase-free distilled water	Life Technologies p/n 10977-015
Milli-Q ultrapure water	Millipore
Acetonitrile†	Sigma-Aldrich p/n 271004-1L

* Included in the SurePrint G3 CGH Bundle.

† Optional components recommended if wash procedure B is selected.

Required Hardware and Software

- Refer to the Agilent Scanner manual and Agilent CytoGenomics or Feature Extraction manuals for minimum memory requirements and other specifications. Go to http://www.genomics.agilent.com.

2 Sample Amplification

NOTE Agilent cannot guarantee microarray performance and does not provide technical support to those who use non-Agilent protocols in processing Agilent microarrays.

The Agilent array-based Comparative Genomic Hybridization (aCGH) application uses a “two-color” process to measure DNA copy number changes (CNC) in an experimental sample relative to a reference sample.

This chapter describes the recommendations to process single cells and reference DNA prior to labeling. Single Cells are subjected to whole genome amplification to increase the amount of DNA while maintaining its genomic representation. For reference sample, the Human Reference DNA Male that is included in the SureTag Complete DNA Labeling Kit is diluted to single cell levels.

To minimize variations associated with the extreme dilution of gDNA and the amplification process, the diluted reference sample is amplified in 8 independent reactions and pooled prior to labeling. The pooled reference contains enough volume to process up to 42 single cell amplification reactions in single or multiple batches. Therefore, one amplification kit (50 reactions) contains sufficient reagents to amplify 42 single cells and associated references.

There is no need to re-determine the concentration of the Human Reference DNA Male. Its concentration is 200 ng/µL as measured by both spectrophotometer and fluorometer.

CAUTION Make sure that the Human Reference DNA Male is completely in solution by pipetting up and down. If needed, incubate at 37°C for 30 minutes.

Reference

PicoPlex WGA Kit for Single-Cell Whole Genome Amplification user manual, publication number R30050-09, v07.09.A, from Rubicon Genomics, Inc.

Genomic Amplification

The PicoPlex WGA Kit for Single-Cell allows you to generate a representative amplification of genomic DNA from a single cell.

Unstained and unfixed cells collected through flow sorting, micromanipulation and dilution can be used to amplify DNA to about 1 million fold, to yield 2 to 5 micrograms of amplified product.

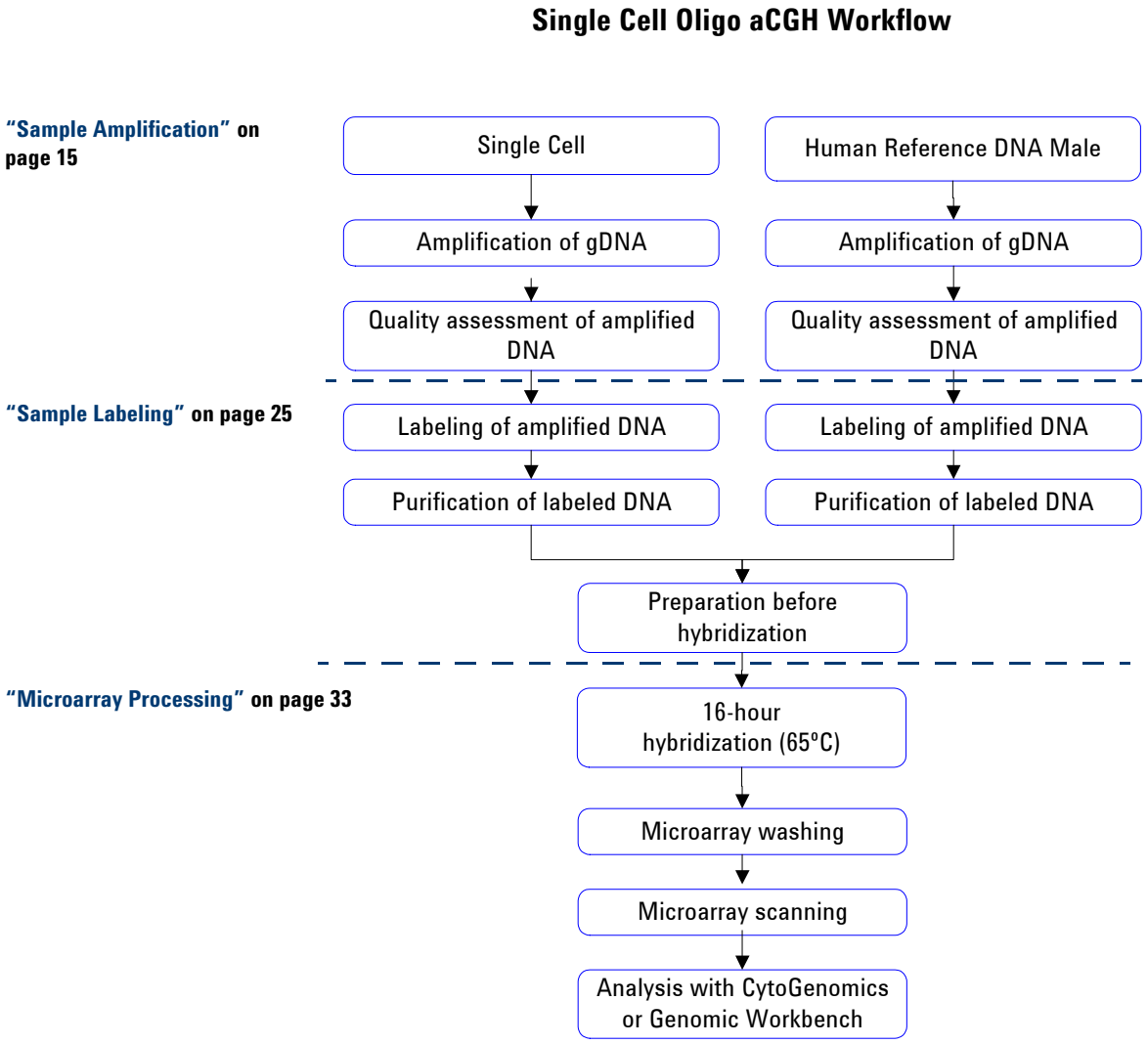


Figure 1 Amplification and labeling workflow for single cell analysis.

This section describes the recommended procedure to amplify DNA from single cells and from the Human Reference DNA Male using the PicoPlex WGA Kit for Single-Cell.

Step 1. Sample Preparation

Do these steps in a (sterile) vertical laminar flow hood.

1 Prepare a **1× dilution** of 10× Phosphate Buffered Saline, pH 7.4 (PBS). Use DNase/RNase-free distilled water to dilute.

2 Label sterile 200 µL Thin-Wall Tube for 8 reference sample reactions and for a negative control, according to Table 6. Cap the tube after adding each sample to prevent cross-contamination.

Table 6

Description	Material Amount	PCR tube
Negative control	2.5 µL of 1× PBS	#1
Human Reference DNA Male	30 pg gDNA in 2.5 µL of 1× PBS	#2 through #9
Single Cell	collected in 2.5 µL of 1× PBS	#10 and onward

3 Prepare negative control:

   a Add 2.5 µL of **1× PBS** to PCR tube #1.

4 Prepare reference sample:

   a Prepare 3 × 1.5 mL RNase-free Microfuge Tube. Label as #1, #2, and #3.

   b Into each tube, add the volume of **1× PBS** indicated in Table 7.

   c Into tube #1, add 3 µL of Human Reference DNA Male. Mix well.

   d Into tube #2, add 3 µL of the content of tube #1. Mix well.

   e Into tube #3, add 3 µL of the content of tube #2. Mix well.

   f Add 2.5 µL (30 pg) of tube #3 into each of PCR tubes #2 through #9.

5 Prepare single cells:

   a Isolate and wash cells in **1× PBS** to minimize carryover of external DNA contaminants from the preparation.

   b Transfer washed single cells in a maximum of 2.5 µL of **1× PBS** into 200 µL Thin-Wall Tube (#10 and onward).

Table 7

Sample tube	Sample Dilution	Volume of 1× PBS	gDNA volume
1	5 ng/µL	117 µL	3 µL of Human Reference DNA Male (200 ng/µL)
2	100 pg/µL	147 µL	3 µL of 5 ng/µL dilution
3	12 pg/µL	22 µL	3 µL of the 100 pg/µL dilution

Keep all samples in 200 µL Thin-Wall Tube on ice until required.

Step 2. Lysis and Fragmentation

Use the reagents in the PicoPlex WGA Kit for Single-Cell.

1 Add 2.5 µL of Cell Extraction Buffer to each 200 µL Thin-Wall Tube that contains the single cell, the diluted reference gDNA or the negative control to make a total volume of 5 µL. Mix well by pipetting up and down.

2 Mix the components in Table 8 on ice to prepare the Extraction Master Mix.

Table 8 Extraction Master Mix

	Volume (µL)	×17 rxns* (µL) including excess	×25 rxns† (µL) including excess
Extraction Enzyme Dilution Buffer	4.8	86.4	129.6
Cell Extraction Enzyme	0.2	3.6	5.4
Final Volume	**5**	**90**	**135**

* Includes 8 Reference DNA reactions, 8 single cell reactions, and a negative control reaction

† Includes 8 Reference DNA reactions, 16 single cell reactions, and a negative control reaction

3 Add 5 µL of Extraction Master Mix to each 5 µL reaction from step 1 for a total volume of 10 µL.

4 Mix gently by pipetting up and down. Spin briefly in a centrifuge to drive the contents off the walls and lid.

5 Place the samples in a thermal cycler with heated lid. Run the program in Table 9.

Table 9 Cell lysis and DNA fragmentation using thermal cycler (total time approximately 15 minutes)

Step	Temperature	Time
Step 1	75°C	10 minutes
Step 2	95°C	4 minutes
Step 3	Room Temperature	Hold

Step 3. Library Preparation

1 Mix the components in Table 10 on ice to prepare the Pre-amplification Master Mix.

Table 10 Pre-amplification Master Mix

	Volume (µL)	×17 rxns* (µL) including excess	×25 rxns† (µL) including excess
PicoPlex Pre-Amp Buffer	4.8	86.4	129.6
PicoPlex Pre-Amp Enzyme	0.2	3.6	5.4
Final Volume	**5**	**90**	**135**

* Includes 8 Reference DNA reactions, 8 single cell reactions, and a negative control reaction

† Includes 8 Reference DNA reactions, 16 single cell reactions, and a negative control reaction

2 Add 5 µL of Pre-amplification Master Mix to each reaction tube for a total of 15 µL.

3 Gently mix by pipetting up and down. Spin briefly in a centrifuge to drive the contents off the walls and lid.

4 Place the samples in a thermal cycler with heated lid. Run the program in Table 11.

Table 11 Library Preparation using thermal cycler (total time approximately 50 minutes)

Step	Temperature	Time
Step 1	95°C	2 minutes
Step 2 (12 cycles)	95°C	15 seconds
	15°C	50 seconds
	25°C	40 seconds
	35°C	30 seconds
	65°C	40 seconds
	75°C	40 seconds
Step 3	4°C	Hold

5 Remove samples from the thermal cycler and spin briefly in a centrifuge to drive the contents off the walls and lid.

Step 4. Amplification

1 Mix the components in Table 12 on ice to prepare the Amplification Master Mix.

Table 12 Amplification Master Mix

	Volume (µL)	×17 rxns* (µL) including excess	×25 rxns† (µL) including excess
Nuclease-Free Water	34.2	615.6	889.2
PicoPlex Amplification Buffer	25	450	650
PicoPlex Amplification Enzyme	0.8	14.4	20.8
Final volume	**60**	**1,080**	**1,560**

* Includes 8 Reference DNA reactions, 8 single cell reactions, and a negative control reaction

† Includes 8 Reference DNA reactions, 16 single cell reactions, and a negative control reaction

2 Add 60 µL of Amplification Master Mix to each 15 µL reaction from the previous step to make a total volume of 75 µL.

3 Mix gently by pipetting up and down. Spin briefly in a centrifuge to drive the contents off the walls and lid.

4 Place the samples in a thermal cycler with heated lid. Run the program in Table 13.

Table 13 PCR Amplification (total time approximately 40 minutes)

Step	Temperature	Time
Step 1	95°C	2 minutes
Step 2 (14 cycles)	95°C	15 seconds
	65°C	1 minute
	75°C	1 minute
Step 3	4°C	Hold

5 Maintain the reactions at 4°C or store at -20°C for up to three days until ready for labeling.

Step 5. Quality Analysis of the Amplified DNA

1. Load 5 µL of amplified DNA of each sample with 5 µL of DNase/RNase-free distilled water in the well of a single-comb Clear E-Gel (1.2% agarose, no stain). (You do not need to add loading buffer in this system).
2. As a control, load 20 ng of unamplified Human Reference DNA Male in 10 µL of DNase/RNase-free distilled water in one of the wells of the E-Gel.
3. Mix 5 µL of TrackIt 1 Kb DNA Ladder with 95 µL of deionized water and load 10 µL of the diluted ladder in one of the wells of the E-Gel.
4. Run the gel for 30 minutes as described in Invitrogen's instructions.
5. Open the gel cassette with E-Gel Opener as described in Invitrogen's instructions.
6. Stain the gel with SYBR Gold Nucleic Acid Gel Stain (diluted 1:10,000 by adding 10 µL of SYBR Gold Nucleic Acid Gel Stain to 100 mL of DNase/RNase-free distilled water) in a plastic tray for 15 minutes.
7. Visualize the gel on the UV-transilluminator using a SYBR photographic filter.

Step 6. Preparation of Amplified DNA before Labeling

1. In a 1.5 mL RNase-free Microfuge Tube, combine the 8 reactions of the amplified reference DNA.

   The pooled reference can be used to process up to 42 single cell amplification reactions in single or multiple batches. Use this pooled reference to process single cells amplified using the same PicoPlex WGA Kit for Single-Cell as the references.
2. For each single cell sample to be labeled, transfer 13 µL of pooled reference DNA to a 1.5 mL RNase-free Microfuge Tube or 200 µL Thin-Wall Tube.
3. Transfer 13 µL of each single cell amplified DNA to a new 1.5 mL RNase-free Microfuge Tube or 200 µL Thin-Wall Tube.

Proceed directly to "Sample Labeling" on page 25, or store amplified DNA at -20°C.

3 Sample Labeling

The SureTag Complete DNA Labeling Kit contains sufficient two-color labeling reaction reagents for 50 8-pack microarrays (50 reactions of each color). You do not need to label the negative control reactions from the amplification step.

It also contains clean-up columns for 25 reactions of each color. Order additional columns when processing more than 25 8-pack microarrays.

The kit uses random primers and the exo-Klenow fragment to differentially label DNA samples with fluorescent-labeled nucleotides. For the Agilent Oligo aCGH application, the experimental sample is labeled with one dye while the reference sample is labeled with the other dye. The “polarity” of the sample labeling is a matter of experimental choice. Typically, the test sample is labeled with cyanine 5 and the reference with cyanine 3.

Step 1. Fluorescent Labeling of Amplified DNA

NOTE Cyanine 3-dUTP and cyanine 5-dUTP are light sensitive and are subject to degradation by multiple freeze thaw cycles. Minimize light exposure throughout the labeling procedure.

CAUTION The test/reference sample pairs must be treated identically when they are processed, or else the quality of your data can be adversely affected. The best way to ensure that the sample pairs are exposed to the same temperature during the denaturation step is to use a water bath.

1 Equilibrate heat blocks or water baths to 95°C, 37°C and 65°C, or use a thermal cycler.

2 Spin the samples in a centrifuge for 1 minute at 6,000 × g to drive the contents off the walls and lid.

3 Add 2.5 µL of Random Primer to each reaction tube that contains 13 µL of amplified DNA to make a total volume of 15.5 µL. Mix well by pipetting up and down gently.

4 Transfer sample tubes to a circulating water bath or heat block at 95°C. Incubate at 95°C for 3 minutes, then move to ice and incubate on ice for 5 minutes.

or

Transfer sample tubes to a thermal cycler. Program the thermal cycler according to Table 14 and run the program.

Table 14 DNA denaturation using a thermal cycler

Step	Temperature	Time
Step 1	95 °C	3 minutes
Step 2	4 °C	hold

5 Spin the samples in a centrifuge for 1 minute at 6,000 × g to drive the contents off the walls and lid.

6 Prepare and add Labeling Master Mix:

a Mix the components in Table 15 on ice in the order indicated to prepare one cyanine-3 and one cyanine-5 Labeling Master Mix.

Table 15 Labeling Master Mix

Component	Per reaction (µL)	× 8 rxns (µL) (including excess)	× 16 rxns (µL) (including excess)
5× Reaction Buffer	5.0	42.5	85
10× dNTPs	2.5	21.25	42.5
Cyanine 3-dUTP *or* Cyanine 5-dUTP	1.5	12.75	25.5
Exo (-) Klenow	0.5	4.25	8.5
Final volume of Labeling Master Mix	**9.5**	**80.75**	**161.5**

b Add 9.5 µL of Labeling Master Mix to each reaction tube that contains the amplified DNA to make a total volume of 25 µL. Mix well by gently pipetting up and down.

7 Incubate the samples:

a Transfer sample tubes to a circulating water bath or heat block at 37°C. Incubate at 37°C for 2 hours.

b Transfer sample tubes to a circulating water bath or heat block at 65°C. Incubate at 65°C for 10 minutes to inactivate the enzyme.

c Move the sample tubes to ice.

or

Transfer sample tubes to a thermal cycler. Program the thermal cycler according to Table 16 and run the program.

Table 16 DNA labeling using a thermal cycler

Step	Temperature	Time
Step 1	37°C	2 hours
Step 2	65°C	10 minutes
Step 3	4°C	hold

Reactions can be stored up to a month at -20°C in the dark.

Step 2. Purification of Labeled Amplified DNA

Labeled amplified DNA is purified using the reaction Purification Column provided with the SureTag Complete DNA Labeling Kit. The Purification Column includes:

- column
- 2-mL collection tube

NOTE Keep cyanine-3 and cyanine-5 labeled amplified DNA samples separated throughout this clean-up step.

1. Spin the labeled amplified DNA samples in a centrifuge for 1 minute at 6,000 × g to drive the contents off the walls and lid.
2. Add 430 µL of 1×TE (pH 8.0) to each reaction tube.
3. For each DNA sample to be purified, place a column into a 2-mL collection tube and label the column appropriately. Load each labeled DNA onto a column.
4. Cover the column with a cap and spin for 10 minutes at 14,000 × g in a microcentrifuge at room temperature. Discard the flow-through and place the column back in the 2-mL collection tube.
5. Add 480 µL of 1×TE (pH 8.0) to each column. Spin for 10 minutes at 14,000 × g in a microcentrifuge at room temperature. Discard the flow-through.
6. Invert the column into a fresh 2-mL collection tube that has been appropriately labeled. Spin for 1 minute at 1,000 × g in a microcentrifuge at room temperature to collect purified sample.

   The volume per sample will be approximately 20 to 32 µL.
7. Concentrate the labeled DNA sample to dryness and resuspend in 9.5 µL of 1×TE (pH 8.0). Do not excessively dry the DNA because the pellets will become difficult to resuspend.
8. Mix thoroughly. If the sample has dried or precipitated after concentration, incubate the tube that contains labeled DNA sample on ice for 5 minutes, and then pipette the solution up and down 10 times.
9. Take 1.5 µL of each sample to determine yield and specific activity. See "To determine yield, and degree of labeling or specific activity" on page 31.

Refer to Table 17 on page 31 for expected yield of labeled amplified DNA and specific activity after labeling and clean-up.

10 In a fresh 1.5 mL RNase-free Microfuge Tube or 200 µL Thin-Wall Tube, combine test and reference sample using the appropriate cyanine-5-labeled sample and cyanine-3-labeled sample for a total mixture volume of 16 µL.

Labeled DNA can be stored up to one month at -20°C in the dark.

To determine yield, and degree of labeling or specific activity

Use the NanoDrop 8000 or 2000 UV-VIS Spectrophotometer to measure yield, and degree of labeling or specific activity.

1 From the main menu, select **MicroArray Measurement**, then from the **Sample Type** menu, select **DNA-50**.

2 Use 1.5 µL of 1×TE (pH 8.0) to blank the instrument.

3 Use 1.5 µL of purified labeled DNA for quantitation. Measure the absorbance at A_{260nm} (DNA), A_{550nm} (cyanine 3), and A_{650nm} (cyanine 5).

4 Calculate the Degree of Labeling or Specific Activity of the labeled DNA:

$$\text{Degree of Labeling} = \frac{340 \times \text{pmol per µL dye}}{\text{ng per µL DNA} \times 1000} \times 100\%$$

$$\text{Specific Activity*} = \frac{\text{pmol per µL of dye}}{\text{µg per µL DNA}}$$

*pmol dyes per µg DNA

The Specific Activity is Degree of Labeling divided by 0.034.

5 Record the DNA concentration (ng/µL) for each sample. Calculate the yield as

$$\text{Yield (µg)} = \frac{\text{DNA Concentration (ng/µL)} \times \text{Sample Volume (µL)}}{1000 \text{ ng/µg}}$$

Refer to Table 17 for expected yield of labeled amplified DNA and specific activity after labeling and purification.

Table 17 Expected Yield and Specific Activity after Labeling and Clean-up of the amplified DNA

Yield (µg)	Specific Activity of Cyanine 3 Labeled Sample (pmol/µg)	Specific Activity of Cyanine 5 Labeled Sample (pmol/µg)
9 to 14	20 to 30	15 to 25

The cyanine-3 and cyanine-5 yield after labeling should be the same. If not, refer to "Troubleshooting" on page 55.

This page intentionally left blank.

4 Microarray Processing

Microarray processing consists of hybridization, washing, and scanning.

Hybridization

If you are new to microarray processing, refer to the “Running a microarray experiment” training presentation, which you can find when you go to http://www.genomics.agilent.com and search on the title of the presentation (“Running a microarray experiment”). This presentation shows you how to hybridize, wash and scan microarray slides.

To practice hybridization, prepare a 1:1 2× HI-RPM Hybridization Buffer and water mix and use a microscope slide or used microarray slide, and a gasket slide. You can use the same slide to practice wash and placement of slide in the slide holder.

Before you begin, make sure you read and understand “Microarray Handling Tips” on page 64.

Step 1. Prepare the 10× Blocking Agent

1 Add 1,350 µL of DNase/RNase-free distilled water to the vial containing lyophilized 10× aCGH Blocking Agent (included in the Oligo aCGH/ChIP-on-chip Hybridization Kit).

2 Leave at room temperature for 60 minutes and mix on a vortex mixer to reconstitute sample before use or storage.

NOTE The 10× Blocking Agent can be prepared in advance and stored at -20°C.

Step 2. Prepare labeled amplified DNA for hybridization

1 Equilibrate water baths or heat blocks to 95°C and 37°C or use a thermal cycler.

2 Mix the components in Table 18 to prepare the Hybridization Master Mix.

Table 18 Hybridization Master Mix

Component	Volume (µL) per hybridization	× 8 rxns (µL) (including excess)	× 16 rxns (µL) (including excess)
Human Cot-1 DNA (1.0 mg/mL)	2	17	34
10× aCGH Blocking Agent*	4.5	38.25	76.5
2× HI-RPM Hybridization Buffer*	22.5	191.25	382.5
Final Volume of Hybridization Master Mix	29	246.5	493

* Included in the Oligo aCGH/ChIP-on-chip Hybridization Kit

3 Add 29 µL of the Hybridization Master Mix to the 1.5 mL RNase-free Microfuge Tube or 200 µL Thin-Wall Tube that contains the labeled amplified DNA for a total volume of 45 µL.

4 Mix the sample by pipetting up and down, then quickly spin in a centrifuge to drive contents to the bottom of the reaction tube.

5 Transfer sample tubes to a circulating water bath or heat block at 95°C. Incubate at 95°C for 3 minutes, then immediately transfer sample tubes to a circulating water bath or heat block at 37°C. Incubate at 37°C for 30 minutes.

or

Transfer sample tubes to a thermal cycler. Program the thermal cycler according to the following table and run the program:

Table 19 Thermal cycler program

Step	Temperature	Time
Step 1	95°C	3 minutes exactly
Step 2	37°C	30 minutes

6 Remove sample tubes from the water bath, heat block, or thermal cycler. Spin 1 minute at 6000 × g in a centrifuge to collect the sample at the bottom of the tube.

The samples are ready to be hybridized.

CAUTION The samples must be hybridized immediately. If not, keep the temperature of hybridization sample mixtures as close to 37°C as possible on a heat block, thermal cycler or in an oven.

Step 3. Prepare the hybridization assembly

Refer to the *Agilent Microarray Hybridization Chamber User Guide* (G2534-90001) for in-depth instructions on how to load samples, assemble and disassemble chambers, as well as other helpful tips. This user guide can be downloaded from the Agilent Web site at www.agilent.com/chem/dnamanuals-protocols.

Before you begin, make sure you read and understand "Microarray Handling Tips" on page 64.

1. Load a clean gasket slide into the Agilent SureHyb chamber base with the gasket 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 40 µL of hybridization sample mixture onto the gasket well in a "drag and dispense" manner.

   Load all gasket wells before you load the microarray slide. Refer to "Agilent Microarray Layout and Orientation" on page 65.

CAUTION Keep the temperature of hybridization sample mixtures as close to 37°C as possible. To do this, process them in small batches and/or put them on a heat block, thermal cycler or in an oven.

3. Put a microarray slide "active side" down onto the gasket slide, so the numeric barcode side is facing up and the "Agilent"-labeled barcode is facing down. Assess that the sandwich-pair is properly aligned.
4. Put the SureHyb chamber cover onto the sandwiched slides and slide the clamp assembly onto both pieces.
5. Hand-tighten the clamp firmly onto the chamber.
6. Vertically rotate the assembled chamber to wet the slides and assess the mobility of the bubbles. Tap the assembly on a hard surface if necessary to move stationary bubbles.

Step 4. Hybridize

1 Load each assembled chamber into the oven rotator rack. Start from the center of the rack (position 3 or 4 when counting from the left). Set your hybridization rotator to rotate at 20 rpm.

2 Hybridize at 65°C for 16 hours.

CAUTION If you are not loading all the available positions on the hybridization rotator rack, be sure to *balance* the loaded hybridization chambers on the rack similar to a centrifuge to prevent unnecessary strain on the oven motor.

CAUTION You must calibrate the hybridization oven regularly for accuracy of the collected data. Refer to *Agilent G2545A Hybridization Calibration Procedure* (p/n G2545-90002) for more information.

For more information on the effects of hybridization temperature and time, as well as the rotation speed on the final microarray results, please refer to the application note titled "60-mer Oligo-Based Comparative Genomic Hybridization" (publication 5989-4848EN) from the Agilent Web site at www.agilent.com/chem/dnaapplications.

NOTE The Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 that is used in the microarray wash procedure needs to be warmed overnight. While you are waiting for the microarray slides to hybridize, do the steps in "Step 1. Prewarm Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 (overnight)" on page 40.

Microarray Wash

NOTE

The microarray wash procedure must be done in environments where ozone levels are 5 ppb or less. For Scanner C, if ozone levels are between 5 to 10 in your laboratory, use the Agilent Ozone Barrier Slide Cover. SureScan microarray scanner uses a slide holder with a built-in ozone barrier. If ozone levels exceed 10 ppb, use the Stabilization and Drying Solution together with the ozone barrier.

You can also use Carbon Loaded Non-woven Filters to remove ozone from the air. These filters can be installed in either your HVAC system, or as part of small Ozone Controlled Enclosures. These free-standing enclosures can be installed either on a lab bench or as a walk-in room within your lab. These products are available through filter suppliers listed in Agilent Technical Note 5989-0875EN.

Before you begin, determine which wash procedure to use:

Table 20 Wash procedure to follow

Ozone level in your lab	Wash Procedure	Ozone-Barrier Slide Cover
< 5 ppb	"Wash Procedure A (without Stabilization and Drying Solution)" on page 43	No
> 5 ppb < 10 ppb	"Wash Procedure A (without Stabilization and Drying Solution)" on page 43	Yes
> 10 ppb	"Wash Procedure B (with Stabilization and Drying Solution)" on page 45	Yes

CAUTION

Do not use detergent to wash the staining dishes as some detergents may leave fluorescent residue on the dishes. If you do, you must ensure that all traces are removed by thoroughly rinsing with Milli-Q ultrapure water.

- Always use clean equipment when conducting the wash procedures.
- Use only dishes that are designated and dedicated for use in Agilent oligo aCGH experiments.

Step 1. Prewarm Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 (overnight)

The temperature of Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 must be at 37°C for optimal performance.

1 Add the volume of buffer required to a Sterile storage bottle and warm overnight in an incubator or circulating water bath set to 37°C.

2 Put a slide-staining dish with a lid, a 1.5 L glass dish, and one to two liters of Milli-Q ultrapure water in an incubator or water bath set at 37°C to warm overnight.

Step 2. Wash with Milli-Q ultrapure water

Rinse slide-staining dishes, slide racks and stir bars thoroughly with high-quality Milli-Q ultrapure water before use and in between washing groups.

1 Run copious amounts of Milli-Q ultrapure water through the slide-staining dishes, slide racks and stir bars.

2 Empty out the water collected in the dishes at least five times.

3 Repeat step 1 and step 2 until all traces of contaminating material are removed.

Step 3. Clean with Acetonitrile (Wash Procedure B Only)

Acetonitrile wash removes any remaining residue of Stabilization and Drying Solution from slide-staining dishes, slide racks and stir bars that were used in previous experiments with "Wash Procedure B (with Stabilization and Drying Solution)" on page 45.

WARNING **Do Acetonitrile washes in a vented fume hood. Acetonitrile is highly flammable and toxic.**

1. Add the slide rack and stir bar to the slide-staining dish, and transfer to a magnetic stir plate.
2. Fill the slide-staining dish with 100% Acetonitrile.
3. Turn on the magnetic stir plate and adjust the speed to a setting of 4 (medium speed).
4. Wash for 5 minutes at room temperature.
5. Discard the Acetonitrile as is appropriate for your site.
6. Repeat step 1 through step 5.
7. Air dry everything in the vented fume hood.
8. Continue with the Milli-Q ultrapure water wash as previously instructed.

Step 4. Prewarm Stabilization and Drying Solution (Wash Procedure B Only)

The Stabilization and Drying Solution contains an ozone scavenging compound dissolved in Acetonitrile. The compound in solution is present in saturating amounts and may precipitate from the solution under normal storage conditions. If the solution shows visible precipitation, warming of the solution will be necessary to redissolve the compound. Washing slides using Stabilization and Drying Solution showing visible precipitation will have profound adverse affects on microarray performance.

WARNING **The Stabilization and Drying Solution is a flammable liquid. Warming the solution will increase the generation of ignitable vapors. Use gloves and eye/face protection in every step of the warming procedures.**

WARNING **Do not use a hot plate, oven, an open flame or a microwave. Do not increase temperature rapidly. Warm and mix the material away from ignition sources.**

WARNING **Failure to follow the outlined process will increase the potential for fire, explosion, and possible personal injury.**

1. Put a clean magnetic stir bar into the Stabilization and Drying Solution bottle and recap.
2. Partially fill a plastic bucket with hot water at approximately 40°C to 45°C (for example from a hot water tap).
3. Put the Stabilization and Drying Solution bottle into the hot water in the plastic bucket.
4. Put the plastic bucket on a magnetic stirrer (*not a hot-plate*) and stir.
5. The hot water cools to room temperature. If the precipitate has not all dissolved replenish the cold water with hot water.
6. Repeat step 5 until the solution is clear.
7. After the precipitate is completely dissolved, allow the solution to equilibrate to room temperature prior to use.

CAUTION Do not filter the Stabilization and Drying Solution, or the concentration of the ozone scavenger may vary.

Step 5. Wash microarrays

Wash Procedure A (*without* Stabilization and Drying Solution)

Always use fresh Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1 and Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 for each wash group (up to five slides).

Table 21 lists the wash conditions for the Wash Procedure A without Stabilization and Drying Solution.

Table 21 Wash conditions

	Dish	Wash buffer	Temperature	Time
Disassembly	#1	Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1	Room temperature	
1st wash	#2	Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1	Room temperature	5 minutes
2nd wash	#3	Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2	37°C	1 minute

1 Completely fill slide-staining dish #1 with Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1 at room temperature.

2 Prepare dish #2:

   a Put a slide rack into slide-staining dish #2.

   b Add a magnetic stir bar. Fill slide-staining dish #2 with enough Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1 at room temperature to cover the slide rack.

   c Put this dish on a magnetic stir plate.

3 Prepare dish #3:

   a Put the prewarmed 1.5 L glass dish on a magnetic stir plate with heating element.

   b Put the slide-staining dish #3 into the 1.5 L glass dish.

   c Fill the 1.5 L glass dish with pre-warmed Milli-Q ultrapure water.

   d Fill the slide-staining dish #3 approximately three-fourths full with Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 (warmed to 37°C).

   e Add a magnetic stir bar.

   f Turn on the heating element and maintain temperature of Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 at 37°C. Monitor with a thermometer.

4 Remove one hybridization chamber from the incubator and resume rotation of the others. Record whether bubbles formed during hybridization and if all bubbles are rotating freely.

5 Prepare the hybridization chamber disassembly.

   a Put 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 microarray-gasket sandwich from the chamber base by lifting one end and then grasping in the middle of the long sides. Keep the microarray slide numeric barcode facing up as you quickly transfer the sandwich to slide-staining dish #1.

   d Without letting go of the slides, submerge the microarray-gasket sandwich into slide-staining dish #1 containing Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1.

6 With the sandwich completely submerged in Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1, pry the sandwich open from the barcode end only:

   a Slip one of the blunt ends of the forceps between the slides.

   b Gently twist the forceps to separate the slides.

   c Let the gasket slide drop to the bottom of the staining dish.

   d Remove the microarray slide, grasp it from the upper corners with thumb and forefinger, and quickly put into slide rack in the slide-staining dish #2 containing Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1 at room temperature. Minimize exposure of the slide to air. *Touch only the barcode portion of the microarray slide or its edges!*

7 Repeat step 4 through step 6 for up to four additional slides in the group. A maximum of five disassembly procedures yielding five microarray slides is advised at one time in order to facilitate uniform washing.

8 When all slides in the group are put into the slide rack in slide-staining dish #2, stir at 350 rpm for 5 minutes. Adjust the setting to get good but not vigorous mixing.

9 Wash the slides in Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2:

   a Transfer slide rack to slide-staining dish #3, which contains Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 at 37°C:

   a Activate the magnetic stirrer.

   b Wash microarray slides for at least 1 minute and no more than 2 minutes.

   Adjust the setting to get thorough mixing without disturbing the microarray slides.

10 Slowly remove the slide rack trying to minimize droplets on the slides. It should take 5 to 10 seconds to remove the slide rack.

11 Discard used Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1 and Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2.

12 Repeat step 1 through step 11 for the next group of five slides using fresh Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1 and Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 warmed to 37°C.

13 Scan slides immediately to minimize the impact of environmental oxidants on signal intensities. If necessary, store slides in orange slide boxes in a N_2 purge box, in the dark.

Wash Procedure B (*with* Stabilization and Drying Solution)

Cyanine reagents are susceptible to degradation by ozone. Use this wash procedure if the ozone level exceeds 10 ppb in your laboratory.

Always use fresh Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1 and Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 for each wash group (up to five slides).

The Acetonitrile (dish #4) and Stabilization and Drying Solution (dish #5) below may be reused for washing up to 4 batches of 5 slides (total 20 slides) in one experiment. Do not pour the Stabilization and Drying Solution back in the bottle.

WARNING **The Stabilization and Drying Solution must be set-up in a fume hood. Put the Wash Buffer 1 and Wash Buffer 2 set-up areas close to, or preferably in, the same fume hood. Use gloves and eye/face protection in every step of the washing procedure.**

Table 22 lists the wash conditions for the Wash Procedure B with Stabilization and Drying Solution.

Table 22 Wash conditions

	Dish	Wash Buffer	Temperature	Time
Disassembly	#1	Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1	Room temperature	
1st wash	#2	Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1	Room temperature	5 minutes
2nd wash	#3	Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2	37°C	1 minute
Acetonitrile wash	#4	Acetonitrile	Room temperature	10 seconds
3rd wash	#5	Stabilization and Drying Solution	Room temperature	30 seconds

1 In the fume hood, fill slide-staining dish #4 approximately three-fourths full with Acetonitrile. Add a magnetic stir bar and put this dish on a magnetic stir plate.

2 In the fume hood, fill slide-staining dish #5 approximately three-fourths full with Stabilization and Drying Solution. Add a magnetic stir bar and put this dish on a magnetic stir plate.

3 Do step 1 through step 9 in "Wash Procedure A (without Stabilization and Drying Solution)" on page 43.

4 Remove the slide rack from Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 and tilt the rack slightly to minimize wash buffer carry-over. Quickly transfer the slide rack to slide-staining dish #4 containing Acetonitrile, and stir at 350 rpm for 10 seconds.

5 Transfer slide rack to slide-staining dish #5 filled with Stabilization and Drying Solution, and stir at 350 rpm for 30 seconds.

6 Slowly remove the slide rack trying to minimize droplets on the slides. It should take 5 to 10 seconds to remove the slide rack.

7 Discard used Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1 and Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2.

NOTE

The Acetonitrile and the Stabilization and Drying Solution may be reused for washing of up to four batches of five slides (that is, total 20 microarray slides) in one experiment. Pour the Stabilization and Drying Solution to a different marked bottle, and protect from light with other flammables. After each use, rinse the slide rack and the slide-staining dish that were in contact with the Stabilization and Drying Solution with Acetonitrile followed by a rinse in Milli-Q ultrapure water.

8 Repeat step 1 through step 7 for the next group of five slides using fresh Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1 and Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 prewarmed to 37°C.

9 Dispose of Acetonitrile and Stabilization and Drying Solution as flammable solvents.

Step 6. Put slides in a slide holder

Scan slides immediately to minimize impact of environmental oxidants on signal intensities. If necessary, store slides in the original slide boxes in a N_2 purge box, in the dark.

For SureScan microarray scanner

1. Carefully place the end of the slide without the barcode label onto the slide ledge.
2. Gently lower the microarray slide into the slide holder. Make sure that the active microarray surface (with "Agilent"-labeled barcode) faces up, toward the slide cover.
3. Close the plastic slide cover, pushing on the tab end until you hear it click.

For more detailed instruction, refer to the *Agilent G4900DA SureScan Microarray Scanner System User Guide*.

Figure 2 Slide in slide holder for SureScan microarray scanner

For Agilent Scanner C

- In environments in which the ozone level exceeds 5 ppb, immediately put the slides with active microarray surface ("Agilent"-labeled barcode) facing up in a slide holder. Make sure that the slide is not caught up on any corner. Put an ozone-barrier slide cover on top of the array as shown in Figure 3. Refer to the *Agilent Ozone-Barrier Slide Cover User Guide* (p/n G2505-90550), included with the slide cover, for more information.

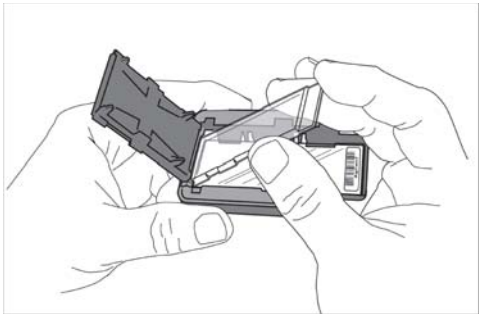


Figure 3 Inserting the ozone-barrier slide cover

- In environments in which the ozone level is below 5 ppb, put the slides with Agilent barcode facing up in a slide holder.

Microarray Scanning and Analysis

Step 1. Scan the microarray slides

A SureScan or Agilent C microarray scanner is required for G3 microarrays.

Agilent provides support for Agilent microarrays scanned on select non-Agilent scanners. Please see “Feature Extraction Compatibility Matrix for Non Agilent scanners” for scanner compatibility and settings (http://www.chem.agilent.com/Library/usermanuals/Public/G1662-90043_ScannerCompatibilityMatrix.pdf).

However, Agilent can guarantee the quality of data only if the data comes from Agilent microarrays scanned on Agilent scanners.

Agilent SureScan Microarray Scanner

1. Put assembled slide holders into the scanner cassette.
2. Select **Protocol AgilentG3_CGH**.
3. Verify that the Scanner status in the main window says Scanner Ready.
4. Click **Start Scan**.

Agilent C Scanner Settings

1. Put assembled slide holders with or without the ozone-barrier slide cover into scanner carousel.
2. Select Start Slot *m* End Slot *n* 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.
3. Select **Profile AgilentG3_CGH**.
4. Verify scan settings. See Table 23.

Table 23 C Scanner Scan Settings

	For G3 Microarray Formats
Dye channel	R+G (*red and green*)
Scan region	Agilent HD (61 x 21.6 mm)

Table 23 C Scanner Scan Settings

	For G3 Microarray Formats
Scan resolution	3 µm
Tiff file dynamic range	16 bit
Red PMT gain	100%
Green PMT gain	100%
XDR	<No XDR>

5 Check that **Output Path Browse** is set for desired location.

6 Verify that the Scanner status in the main window says Scanner Ready.

7 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.

Step 2. Analyze microarray image

- After scanning is completed, extract features and analyze.

Feature extraction is the process by which data is extracted from the scanned microarray image (.tif) and translated into log ratios, allowing researchers to identify aberrations in their samples.

Use the Agilent Feature Extraction software.

Agilent provides Feature Extraction software as a standalone program and as an integral part of CytoGenomics software (Windows version only).

- Use the Windows version of Agilent CytoGenomics for automated and streamlined analysis of human samples. During the extraction and analysis process, Agilent CytoGenomics generates feature extraction files, QC and aberration reports.
- To use Agilent CytoGenomics on a Mac computer, first use Feature Extraction on a computer that is running Windows to extract features. Feature Extraction does not run on Mac computers.

- For non-human samples, use Feature Extraction (available for Windows only) to extract features, and then use Agilent Genomic Workbench to run an analysis workflow on the extracted features.

Microarray QC Metrics for high DNA quality samples

These metrics are only appropriate for samples analyzed with Agilent CGH microarrays by following the standard operational procedures provided in this user guide. These metrics are reported in the Feature Extraction QC report generated by Feature Extraction (standalone or as included in the Agilent CytoGenomics software). They can be used to assess the relative data quality from a set of microarrays in an experiment. In some cases, they can indicate potential processing errors that have occurred or suggest that the data from particular microarrays might be compromised. Many factors can influence the range of these metrics, including biological sample, experimental processing, scanner sensitivity, and image processing. The value guidelines presented below represent the thresholds that Agilent has observed when analyzing samples using this protocol.

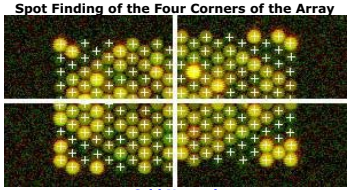
Table 24 QC metric thresholds for Single Cells Enzymatic labeling

Metric	Recommended
BGNoise	< 10
Signal Intensity	> 50
Signal to Noise	> 30
Reproducibility	< 0.2
DLRSD	< 1

QC Report - Agilent Technologies : 2 Color CGH

Date	Wednesday, May 02, 2012 - 16:31	Sample(red/green)	
User Name	Administrator	FE Version	10.7.3.1
Image	US23502418_252192416531_S01 [1_4]	BG Method	Detrend on (NegC)
Protocol	CGH_107_Sep09 (Read Only)	Multiplicative Detrend	True
Grid	021924_D_F_20111015	Dye Norm	Linear
Saturation Value	65510 (r), 65510 (g)		

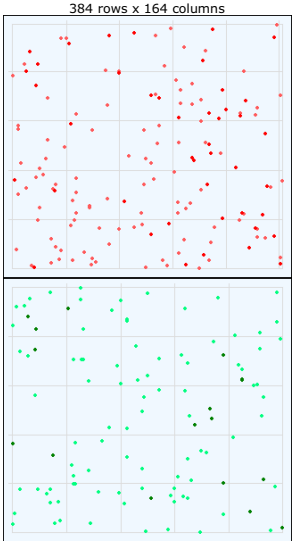
Spot Finding of the Four Corners of the Array



Grid Normal

Outlier Numbers with Spatial Distribution

384 rows x 164 columns



Red FeaturePopulation Red Feature NonUniform
Green FeaturePopulation Green Feature NonUniform

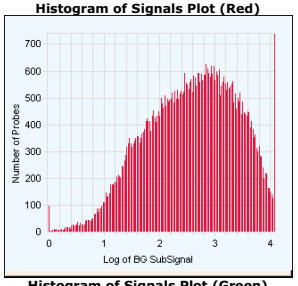
Feature	Red	Green	Any	% Outlier
Non Uniform	44	17	45	0.07
Population	107	91	183	0.29

Evaluation Metrics for CGH_QCMT_Sep09 :
Excellent (4) ; Good (6) ; Evaluate (1)

Metric Name	Value	Excellent	Good	Evaluate
IsGoodGrid	1.00	>1	NA	<1
AnyColorPrcntFeatNonUn...	0.07	<1	1 to 5	>5
DerivativeLR_Spread	0.78	<0.20	0.20 to 0.30	>0.30
gRepro	0.08	0 to 0.05	0.05 to 0.20	<0 or >0.20
g_BGNoise	6.12	<5	5 to 10	>10
g_Signal2Noise	53.07	>100	30 to 100	<30
g_SignalIntensity	324.62	>150	50 to 150	<50
rRepro	0.08	0 to 0.05	0.05 to 0.20	<0 or >0.20
r_BGNoise	7.68	<5	5 to 10	>10
r_Signal2Noise	48.55	>100	30 to 100	<30
r_SignalIntensity	372.85	>150	50 to 150	<50

♦ Excellent ♦ Good ♦ Evaluate

Histogram of Signals Plot (Red)



Histogram of Signals Plot (Green)

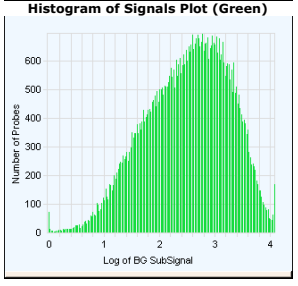


Figure 4 Feature Extraction QC Report, page 1

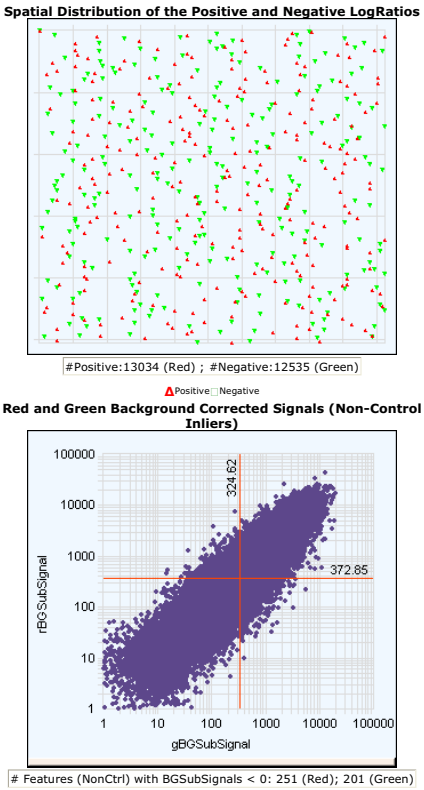


Figure 5 Feature Extraction QC Report, page 2

6 Troubleshooting

This chapter contains potential causes for above-threshold DLRSD (Derivative Log Ratio Standard Deviation). A poor DLRSD score reflects high probe-to-probe log ratio noise.

If the DNA is not well mixed

High DLRSD values can be caused by imbalances in the DNA input.

- ✔ Before diluting the Agilent Human Reference DNA Male, make sure that the gDNA is completely in solution by pipetting up and down. If needed, incubate at 37°C for 30 minutes.
- ✔ Mix the gDNA well between dilutions and when preparing the tube reactions for the whole genome amplification.
- ✔ Due to variations inherent to the extreme dilution of gDNA and the amplification process, make sure to amplify 8 independent reactions of the Agilent Human Reference DNA Male. Prior to labeling, pool the amplified reference samples in one single tube.
- ✔ Post-amplification, check the efficiency of the whole genome amplification reaction in a 1 to 1.5% agarose gel.

If you have low specific activity or degree of labeling not due to poor sample quality

Low specific activity or degree of labeling can result from sub-optimal labeling conditions such as Cyanine dUTP with too many freeze thaws, enzyme degradation due to being left warm for too long, wrong temperatures or times, volume mistakes, or too much exposure to light or air.

- ✔ Store Cyanine dUTP at -20°C. Keep enzymes on ice and return to -20°C as quickly as possible.
- ✔ Double check incubation times and temperatures (use a calibrated thermometer), and use a thermal cycler with heated lid.
- ✔ Evaporation can be a problem when you process samples at high temperatures. Make sure that sample tubes are well closed.
- ✔ Make sure that the pipettors are not out of calibration.
- ✔ Make sure that the DNA, reagents, and master mixes are well mixed. Tap the tube with your finger or use a pipette to move the entire volume up and down. Then spin in a microcentrifuge for 5 to 10 seconds to drive the contents off the walls and lid. Do not mix the stock solutions and reactions that contain DNA or enzymes on a vortex mixer.

If you have low yield not due to poor sample quality

Possible sample loss during clean-up after labeling.

- ✔ See “Step 2. Purification of Labeled Amplified DNA” on page 29 to remove unreacted dye. Many other columns result in the loss of shorter fragments.

If you have post-labeling signal loss

Signal loss can be due to wash or hybridization conditions that are too stringent, or degradation of the Cyanine 5 signal.

Cyanine 5 signal degradation can be caused by ozone or NOx compounds coming from pollution and/or compressors and centrifuges. Cyanine 5 signal degradation can result in less red signal around the edges of the features, a visible gradient of significant Cy5/Cy3 positive ratios and more significant Cy5/Cy3 negative ratios – especially on the left side of the slide and on slides scanned later in a batch.

- ✔ Check that the oven temperature is 65°C. If needed, recalibrate the hybridization oven. Follow the steps in *Agilent G2545A Hybridization Calibration Procedure* (p/n G2545-90002).
- ✔ Check that the temperature of Wash 2 is 37°C.
- ✔ Check that Wash 2 was not accidentally used instead of Wash 1.
- ✔ Wash and scan slides in an ozone controlled environment (<5 ppb), such as an ozone tent.
- ✔ Use small batches that can be washed and scanned in about 40 minutes to minimize exposure to air.
- ✔ For Agilent Scanner C, use the Agilent Ozone-Barrier Slide Cover (p/n G2505-60550). The SureScan scanner has built-in ozone protection.
- ✔ Use the Stabilization and Drying Solution as described in "Wash Procedure B (with Stabilization and Drying Solution)" on page 45.

If you have high BGNoise values

High BGNoise can cause lower signal-to-noise values (see Table 24 for thresholds) and higher DLRSD values. BGNoise is defined as the standard deviation of the signals on the negative controls. If the BGNoise is high, examine the microarray image for visible non-uniformities. High BGNoise is often introduced during hybridization steps or washes.

✔ Make sure that the oven is calibrated. Follow the steps in *Agilent G2545A Hybridization Calibration Procedure* (p/n G2545-90002).

Sample hybridization at incorrect temperatures affects the stringency of the hybridization.

✔ Make sure that wash dishes, racks and stir bars are clean. Do not use tap water or detergents to clean wash equipment. If needed, rinse wash equipment with acetonitrile followed by rinses with MilliQ water.

✔ If needed, wash the slides with acetonitrile:

1. In the fume hood, fill a slide-staining dish approximately three-fourths full with acetonitrile.
2. Add a magnetic stir bar and put this dish on a magnetic stir plate.
3. Put the slides in a slide rack and transfer the slide rack to the slide-staining dish containing acetonitrile, and stir at 350 rpm for 1 minute.
4. Slowly remove the slide rack and scan the slides immediately.

If you have poor reproducibility

Poor reproducibility (see Table 24 for thresholds), defined as high CVs of signals of replicated probes may indicate that the hybridization volume was too low or that the oven stopped rotating during the hybridization. Only very high scores on this metric will affect the DLRSD.

✔ When setting up the gasket-slide hybridization sandwich dispense the hybridization sample mixture slowly in a “drag and dispense” manner to prevent spills.

✔ Check that the oven is rotating.

This page intentionally left blank.

7 Reference

This chapter contains reference information that pertains to this protocol.

Reagent Kit Components

The contents of the reagent kits used in this protocol are listed here.

Table 25 SureTag Complete DNA Labeling Kit

Component
Human Reference DNA Male
Human Reference DNA Female*
10× Restriction Enzyme Buffer*
BSA*
Alu I*
Rsa I*
Purification Column
Nuclease-Free Water
Exo (-) Klenow
5× Reaction Buffer
Cyanine 5-dUTP
Cyanine 3-dUTP
10× dNTPs
Random Primer

* Not used in this protocol.

Table 26 PicoPlex WGA Kit for Single-Cell

Component
Cell Extraction Buffer
Extraction Enzyme Dilution Buffer
Cell Extraction Enzyme
PicoPlex Pre-Amp Buffer
PicoPlex Pre-Amp Enzyme
PicoPlex Amplification Buffer
PicoPlex Amplification Enzyme
Nuclease-Free Water

Table 27 Oligo aCGH/ChIP-on-chip Hybridization Kit

Component
2× HI-RPM Hybridization Buffer
10× aCGH Blocking Agent

Table 28 Oligo aCGH/ChIP-on-chip Wash Buffer Kit

Component
Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1
Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2

Microarray Handling Tips

Each microarray is printed on the side of the glass slide containing the “Agilent”-labeled barcode. This side is called the “active” side. The numeric barcode is on the inactive side of the slide.

CAUTION You must familiarize yourself with the assembly and disassembly instructions for use with the Agilent Microarray Hybridization Chamber (G2534A) and gasket slides. Practice slide kits are available.

In this “processing and hybridization” procedure, the hybridization mixture is applied directly to the gasket slide, and not to the active side of the oligo microarray. Instead, the active side of the oligo microarray is placed on top of the gasket slide to form a “sandwich slide” pair.

To avoid damaging the microarray, always handle glass slides carefully by their edges. Wear powder-free gloves. Never touch the surfaces of the slides. If you do, you may cause irreparable damage to the microarray.

Never allow the microarray surface to dry out during the hybridization process and washing steps.

Agilent Microarray Layout and Orientation

Agilent oligo microarray (1 microarray/slide format) as imaged on the Agilent microarray scanner

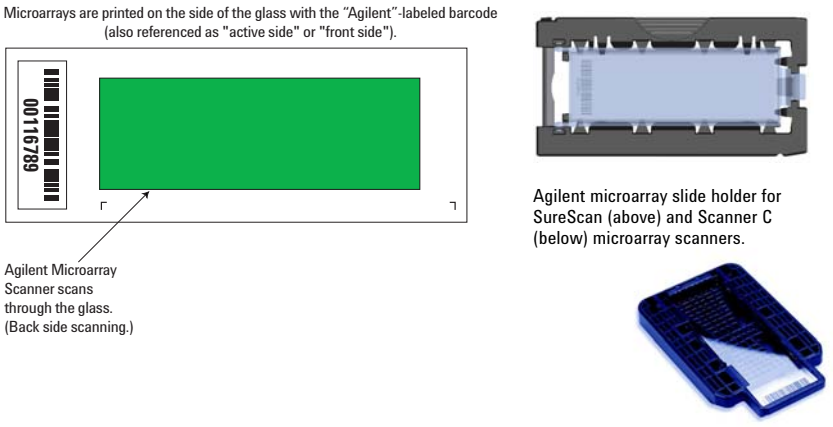


Figure 6 Agilent microarray slide and slide holder

Agilent oligo microarrays formats and the resulting "microarray design files" are based on how the Agilent microarray scanner images 1-inch x 3-inch 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 microarray slide holder with the "Agilent" labeled barcode facing the opening of the slide holder (on SureScan Microarray Scanner) or facing the inside of the slide holder (Scanner C). In this orientation, the "active side" containing the microarrays is protected from potential damage by fingerprints and other elements. Once securely placed, the numeric barcode, non-active side of the slide, is visible from the outside of the slide holder.

Figure 6 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". 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.

Non-Agilent Front Side Microarray Scanners

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

- If the scanner images the microarrays by reading them on the “front side” of the glass slide (“Agilent”-labeled barcode side of the slide) and
- If the microarray image produced by the non-Agilent scanner is oriented in a “portrait” or “landscape” mode, and “Agilent”-labeled barcode is on the left-side, right-side, up or down, as viewed as an image in the imaging software (see Figure 7).

This changes the feature numbering and location as it relates to the “microarray design files”.

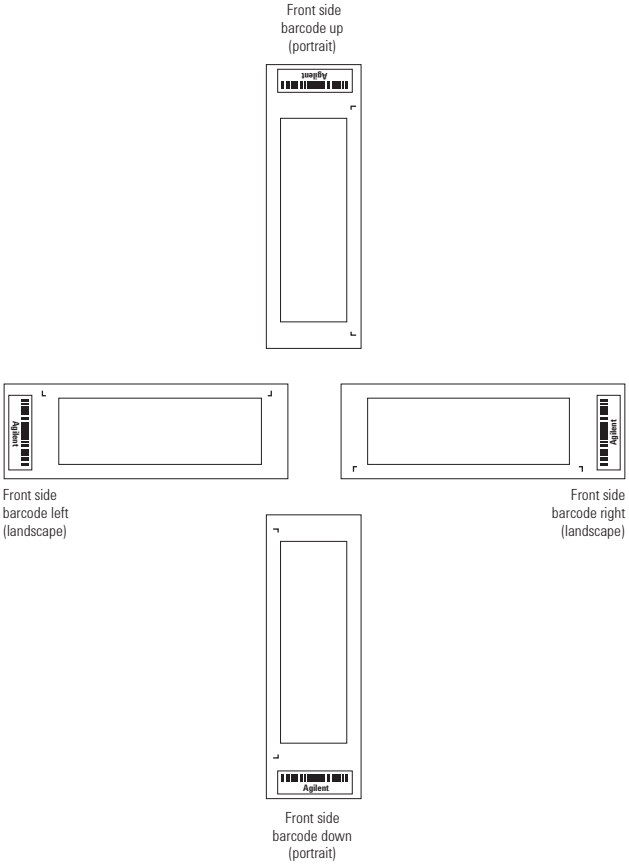


Figure 7 Microarray slide orientation

Array/Sample tracking on microarray slides

Use the forms below to make notes to track your samples on microarray slides.

Position the gasket slide in the SureHyb chamber base with the label to the left and load the samples: top row, left to right, then lower row, left to right. The array suffix assignments from Feature Extraction will then occur in the order shown.

Arrays

	Array 1_1	Array 1_2	Array 1_3	Array 1_4
BARCODE	Sample:	Sample:	Sample:	Sample:
	Sample:	Sample:	Sample:	Sample:
	Array 2_1	Array 2_2	Array 2_3	Array 2_4

Barcode Number _____

Figure 8 8-pack microarray slide

www.agilent.com

In This Book

This guide contains information to run the Oligonucleotide Array-Based CGH for Single Cell Analysis – Enzymatic Labeling protocol.

Revision A1, August 2015

G4410-90012
Revision A1

Agilent Technologies