



**Agilent
Oligonucleotide
Array-Based CGH for
Single Cell Analysis
Enzymatic Labeling**

Protocol

Revision A0, June 2012



Agilent Technologies

Notices

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In This Guide...

This guide describes Agilent's 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 and Feature Extraction

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.

5 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.

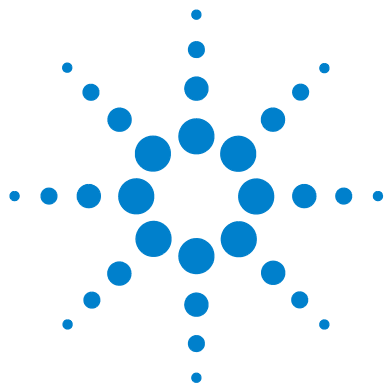
6 Reference

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

Content

1	Before You Begin	7
	Procedural Notes	8
	Safety Notes	9
	Agilent Oligo CGH Microarray Kit Contents	10
	Required Equipment	12
	Required Reagents	14
	Required Hardware and Software	15
2	Sample Amplification	17
	Step 1. Sample Preparation	20
	Step 2. Lysis and Fragmentation	22
	Step 3. Library Preparation	23
	Step 4. Amplification	24
	Step 5. Quality Analysis of the Amplified DNA	26
	Step 6. Preparation of Amplified DNA before Labeling	26
3	Sample Labeling	27
	Step 1. Fluorescent Labeling of Amplified DNA	28
	Step 2. Clean-up of Labeled Amplified DNA	31
	To determine yield, degree of labeling or specific activity	33
	Step 3. Preparation of Labeled Amplified DNA for Hybridization	34
4	Microarray Processing and Feature Extraction	37
	Hybridization	38
	Step 1. Prepare for hybridization	38
	Step 2. Hybridize	39
	Microarray Wash	40
	Step 1. Wash with Milli-Q ultrapure water	41
	Step 2. Clean with Acetonitrile (Wash Procedure B Only)	41

Step 3. Prewarm Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 (Overnight)	42
Step 4. Prewarm Stabilization and Drying Solution (Wash Procedure B Only)	42
Step 5. Wash microarrays	44
Step 6. Put slides in a slide holder	50
Scanning and Feature Extraction	52
Step 1. Scan the slides	52
Step 2. Extract data using the Feature Extraction program	53
5 Troubleshooting	61
If the DNA is not well mixed	62
If you have low specific activity or degree of labeling not due to poor sample quality	63
If you have low yield not due to poor sample quality	63
If you have post-labeling signal loss	64
If you have high BGNoise values	65
If you have poor reproducibility	65
6 Reference	67
Reagent Kit Components	68
Microarray Handling Tips	70
Agilent Microarray Layout and Orientation	71
Array/Sample tracking on microarray slides	74
Agilent Information Assets Access Agreement	75



1

Before You Begin

Procedural Notes	8
Safety Notes	9
Agilent Oligo CGH Microarray Kit Contents	10
Required Equipment	12
Required Reagents	14
Required Hardware and Software	15

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 N₂ purge box. Do not store microarray slides in open air after breaking foil.

SurePrint G3 CGH Bundle

- Six 1-inch × 3-inch slides
- Sufficient reagents and consumables to process 48 samples:
 - SureTag Complete DNA Labeling Kit
 - Oligo aCGH/ChIP-on-chip Hybridization Kit
 - Hybridization Chamber 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
- Disc that contains microarray design file in various file formats

Catalog SurePrint G3 CGH Microarray Kit

- Three 1-inch × 3-inch, 8-pack microarray slides
- Disc that contains microarray design file in various file formats

Unrestricted SurePrint G3 CGH Microarrays

- Eight microarrays printed on each 1-inch × 3-inch glass slide
- Number of microarrays varies per kit and per order
- Disc that contains microarray design file in various file formats

Design file can be downloaded from <http://e-array.com>.

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	Company and part no.
Thin-Wall Tube, 200 µL	Agilent p/n 410091 or equivalent
Agilent Microarray Scanner Bundle for 8×60K	Agilent p/n G4900DA or G2565CA
Hybridization Chamber, stainless	Agilent p/n G2534A
Hybridization Chamber 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 95 °C)	Ambion p/n AM12400 or equivalent
Magnetic stir bar (×2 or ×4) [‡]	Corning p/n 401435 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	Invitrogen p/n G5300-01
E-Gel PowerBase v.4	Invitrogen p/n G6200-04
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	Company and part no.
Vacuum Concentrator	Thermo Scientific p/n DNA120-115 or equivalent
250 mL capacity slide-staining dish, with slide rack (×3 or ×5) [‡]	Wheaton p/n 900200 <i>or</i> 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 dessicator 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	Company and part no.
10× Phosphate Buffered Saline, pH 7.4 (PBS)	Invitrogen p/n AM9624
Clear E-Gel (1.2% agarose, no stain), 18-pack	Invitrogen p/n G5518-01
SYBR Gold Nucleic Acid Gel Stain	Invitrogen p/n S11494
SYBR photographic filter	Invitrogen p/n S7569
TrackIt 1 Kb DNA Ladder	Invitrogen p/n 10488-072
DNase/RNase-free distilled water	Invitrogen p/n 10977-015
PicoPlex WGA Kit for Single-Cell (50 reactions)	Rubicon Genomics p/n R30050

Table 4 Required reagents for enzymatic sample labeling with the [SureTag Complete DNA Labeling Kit](#)

Description	Company and part no.
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 68.

† 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	Company and part no.
Oligo aCGH/ChIP-on-chip Wash Buffer Kit <i>or</i>	Agilent p/n 5188-5226
Oligo aCGH/ChIP-on-chip Wash Buffer 1 <i>and</i>	Agilent p/n 5188-5221
Oligo aCGH/ChIP-on-chip Wash Buffer 2 [*]	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	Invitrogen p/n 10977-015
Milli-Q ultrapure water [†]	Millipore
Acetonitrile [†]	Sigma p/n 271004-1L

* Included in the [SurePrint G3 CGH Bundle](#).

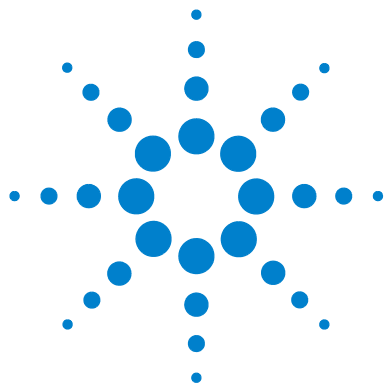
† Optional components recommended if wash procedure B is selected.

Required Hardware and Software

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

1 Before You Begin

Required Hardware and Software



2 Sample Amplification

- Step 1. Sample Preparation 20
- Step 2. Lysis and Fragmentation 22
- Step 3. Library Preparation 23
- Step 4. Amplification 24
- Step 5. Quality Analysis of the Amplified DNA 26
- Step 6. Preparation of Amplified DNA before Labeling 26

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.



2 Sample Amplification

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.

Single Cell Oligo aCGH Workflow

"Sample Amplification" on
page 17

"Sample Labeling" on page 27

**"Microarray Processing and Feature
Extraction"** on page 37

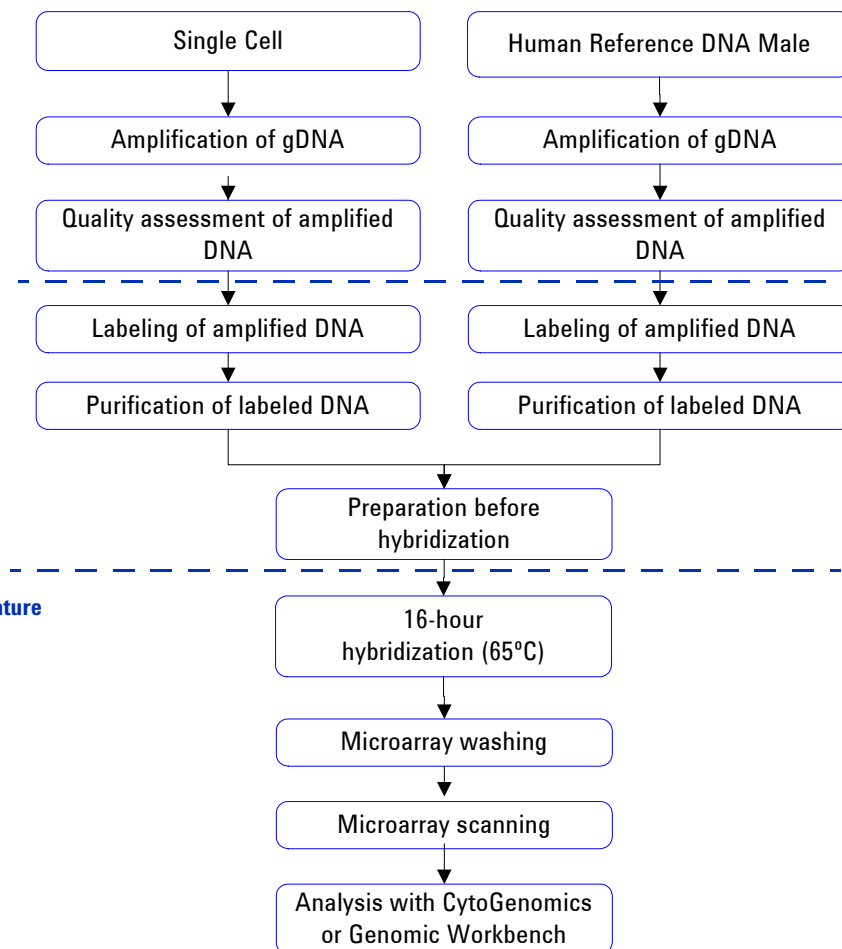


Figure 1 Amplification and labeling workflow for single cell analysis.

This section describes Agilent's 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 **Thin-Wall Tube, 200 µL** 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 **Thin-Wall Tube, 200 µL** (#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 Thin-Wall Tube, 200 μL 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 [Thin-Wall Tube](#), 200 μL 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)	$\times 17$ rxns [*] (μL) including excess	$\times 25$ 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

2 Sample Amplification
Step 4. Amplification

- 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

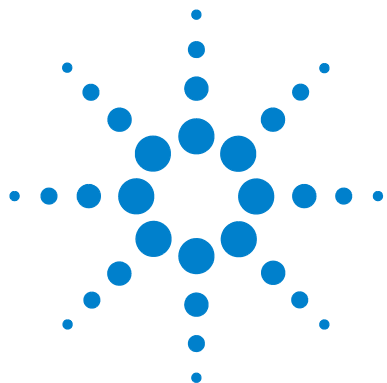
- 1** Load 5 μ L of amplified DNA of each sample with 5 μ L of [DNase/RNase-free distilled water](#) in the well of a single-comb [Clear E-Gel \(1.2% agarose, no stain\)](#). (You do not need to add loading buffer in this system).
- 2** As a control, load 20 ng of unamplified [Human Reference DNA Male](#) in 10 μ L of [DNase/RNase-free distilled water](#) in one of the wells of the E-Gel.
- 3** Mix 5 μ L of [TrackIt 1 Kb DNA Ladder](#) with 95 μ L of deionized water and load 10 μ L 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 μ L 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 μ L of pooled reference DNA to a [1.5 mL RNase-free Microfuge Tube](#) or [Thin-Wall Tube, 200 \$\mu\$ L](#).
- 3** Transfer 13 μ L of each single cell amplified DNA to a new [1.5 mL RNase-free Microfuge Tube](#) or [Thin-Wall Tube, 200 \$\mu\$ L](#).

Proceed directly to “[Sample Labeling](#)” on page 27, or store amplified DNA at -20°C.



3 Sample Labeling

- Step 1. Fluorescent Labeling of Amplified DNA 28
- Step 2. Clean-up of Labeled Amplified DNA 31
- To determine yield, degree of labeling or specific activity 33
- Step 3. Preparation of Labeled Amplified DNA for Hybridization 34

The [SureTag Complete DNA Labeling Kit](#) contains sufficient two-color labeling reaction reagents for 50 8-pack arrays (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.

- 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 <i>or</i> 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.

3 Sample Labeling

Step 1. Fluorescent Labeling of Amplified DNA

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. Clean-up 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 $6,000 \times g$ to drive the contents off the walls and lid.
- 2 Add 430 μL of [1 \$\times\$ TE \(pH 8.0\)](#), [Molecular grade](#) 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 $14,000 \times g$ 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 \$\times\$ TE \(pH 8.0\)](#), [Molecular grade](#) to each [column](#). Spin for 10 minutes at $14,000 \times g$ 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 $1,000 \times g$ 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 \$\times\$ TE \(pH 8.0\)](#), [Molecular grade](#). Do not excessively dry the DNA because the pellets will become difficult to resuspend.
- 8 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, degree of labeling or specific activity”](#) on page 33. Refer to [Table 17](#) on page 33 for expected yield of labeled amplified DNA and specific activity after labeling and clean-up.

3 Sample Labeling

Step 2. Clean-up of Labeled Amplified DNA

- 10 In a fresh 1.5 mL RNase-free Microfuge Tube or Thin-Wall Tube, 200 μL , 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 $-20\text{ }^{\circ}\text{C}$ in the dark.

To determine yield, degree of labeling or specific activity

Use the NanoDrop 8000 or 2000 UV-VIS Spectrophotometer to measure yield, 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 \times TE (pH 8.0), Molecular grade** to blank the instrument.
- 3 Use 1.5 μL of purified labeled DNA for quantitation. Measure the absorbance at $A_{260\text{nm}}$ (DNA), $A_{550\text{nm}}$ (cyanine 3), and $A_{650\text{nm}}$ (cyanine 5).
- 4 Calculate the Degree of Labeling or Specific Activity of the labeled DNA:

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

$$\text{Specific Activity}^* = \frac{\text{pmol per } \mu\text{L of dye}}{\mu\text{g per } \mu\text{L DNA}}$$

*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

$$\text{Yield } (\mu\text{g}) = \frac{\text{DNA Concentration (ng/}\mu\text{L)} \cdot \text{Sample Volume } (\mu\text{L})}{1000 \text{ ng/}\mu\text{g}}$$

Refer to [Table 17](#) for expected yield of labeled amplified DNA and specific activity after labeling and clean-up.

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 61.

Step 3. Preparation of Labeled Amplified DNA for 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.

1 Prepare the 10× Blocking Agent:

- a 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).
- b 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.

2 Prepare the samples for hybridization:

- a Equilibrate water baths or heat blocks to 95 °C and 37 °C or use a thermal cycler.
- b Mix the components in Table 18 to prepare the Hybridization Master Mix.

Step 3. Preparation of Labeled Amplified DNA for Hybridization

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](#)

- c Add 29 μL of the Hybridization Master Mix to the [1.5 mL RNase-free Microfuge Tube](#) or [Thin-Wall Tube](#), 200 μL that contains the labeled amplified DNA for a total volume of 45 μL.
- d Mix the sample by pipetting up and down, then quickly spin in a centrifuge to drive contents to the bottom of the reaction tube.
- e 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

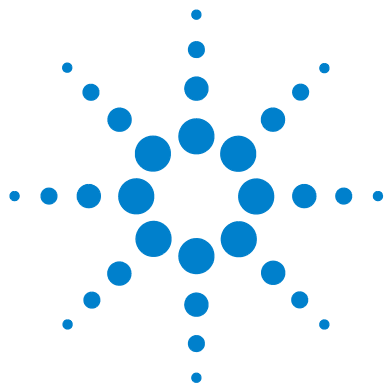
Step	Temperature	Time
Step 1	95 °C	3 minutes exactly
Step 2	37 °C	30 minutes

- f 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.

3 Sample Labeling

Step 3. Preparation of Labeled Amplified DNA for Hybridization



4 Microarray Processing and Feature Extraction

Hybridization 38

Microarray Wash 40

Scanning and Feature Extraction 52

Microarray processing consists of hybridization, washing, and scanning.

Feature Extraction is the process by which data is extracted from the scanned microarray image (.tif) and translated into log ratios, allowing researchers to measure DNA copy number changes in their experiments in conjunction with Agilent CytoGenomics or Genomic Workbench Software.



Hybridization

Familiarize yourself with the assembly and disassembly instructions for use with the Agilent microarray hybridization chamber and gasket slides. Please 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 70.

Step 1. Prepare for hybridization

- 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 71.

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 2. Hybridize

- 1 Put assembled slide chamber in the rotator rack in a hybridization oven set to 65 °C. 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.

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.

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](#).

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 44	No
> 5 ppb < 10 ppb	"Wash Procedure A (without Stabilization and Drying Solution)" on page 44	Yes
> 10 ppb	"Wash Procedure B (with Stabilization and Drying Solution)" on page 46	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. 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 2. 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 46.

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 3. 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 disposable plastic bottle and warm overnight in an incubator or circulating water bath set to 37 °C.
- 2 Put a slide-staining dish into a 1.5 L glass dish three-fourths filled with Milli-Q ultrapure water and warm to 37 °C by storing overnight in an incubator set to 37 °C.

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 Put a slide rack into slide-staining dish #2. 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. Put this dish on a magnetic stir plate.
- 3 Put the prewarmed 1.5 L glass dish filled with water and containing slide-staining dish #3 on a magnetic stir plate with heating element. Fill the slide-staining dish #3 approximately three-fourths full with Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 (warmed to 37 °C). Add a magnetic stir bar. Turn on the heating element and maintain temperature of Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 at 37 °C; monitor using 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. Do this by slipping one of the blunt ends of the forceps between the slides and then gently twist the forceps to separate the slides. Let the gasket slide drop to the bottom of the staining dish. 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 using setting 4 for 5 minutes. Adjust the setting to get good but not vigorous mixing.
- 9 Transfer slide rack to slide-staining dish #3 containing [Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2](#) at 37 °C, and stir using setting 4 for 1 minute.
- 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](#) pre-warmed to 37 °C.

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 Completely fill slide-staining dish #1 with [Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1](#) at room temperature.
- 2 Put a slide rack into slide-staining dish #2. 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. Put this dish on a magnetic stir plate.
- 3 Put the prewarmed [1.5 L glass dish](#) filled with water and containing slide-staining dish #3 on a magnetic stir plate with heating element. Fill the slide-staining dish #3 approximately three-fourths full with [Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2](#) (warmed to 37 °C). Add a magnetic stir bar. Turn on the heating element and maintain temperature of [Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2](#) at 37 °C; monitor using a thermometer.
- 4 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.
- 5 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.
- 6 Remove one hybridization chamber from incubator and resume rotation of the others. Record whether bubbles formed during hybridization, and if all bubbles are rotating freely.

4 Microarray Processing and Feature Extraction

Step 5. Wash microarrays

- 7 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](#).
- 8 With the sandwich completely submerged in [Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1](#), pry the sandwich open from the barcode end only. Do this by slipping one of the blunt ends of the forceps between the slides and then gently twist the forceps to separate the slides. Let the gasket slide drop to the bottom of the staining dish. 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!*
- 9 Repeat [step 6](#) through [step 8](#) 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.
- 10 When all slides in the group are placed into the slide rack in slide-staining dish #2, stir using setting 4 for 5 minutes. Adjust the setting to get good but not vigorous mixing.
- 11 Transfer slide rack to slide-staining dish #3 containing [Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2](#) at 37 °C, and stir using setting 4 for 1 minute.
- 12 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 using setting 4 for 10 seconds.
- 13 Transfer slide rack to slide-staining dish #5 filled with [Stabilization and Drying Solution](#), and stir using setting 4 for 30 seconds.

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

15 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.

16 Repeat step 1 through step 15 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.

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

Step 6. Put slides in a slide holder

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 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 Agilent 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.

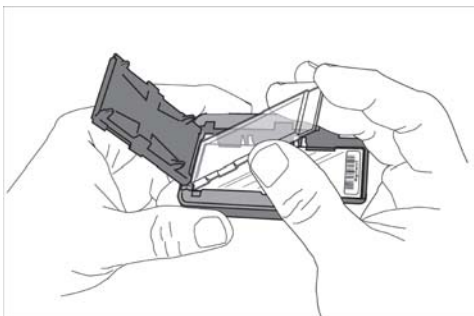


Figure 3 Inserting the ozone-barrier slide cover

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

Scan slides immediately to minimize impact of environmental oxidants on signal intensities. If necessary, store slides in the original slide boxes in a N₂ purge box, in the dark.

Scanning and Feature Extraction

Step 1. Scan the slides

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

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:

Table 23 C Scanner Scan Settings

For G3 Microarray Formats	
Dye channel	R+G (<i>red and green</i>)
Scan region	Agilent HD (61 x 21.6 mm)
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. Extract data using the Feature Extraction program

The Feature Extraction software v10.5 or higher supports extraction of microarray TIFF images (.tif) of Agilent CGH microarrays scanned on the Agilent SureScan or C Scanner.

Feature Extraction version 10.7 or higher can automatically download Grid Templates, protocols and QC metrics (QCMT) from eArray if configured appropriately. See [“Automatic Download from eArray” on page 57](#) for configuration.

[Figure 4](#) shows an example of an Agilent SurePrint G3 8×60K CGH microarray image opened in the Feature Extraction software.

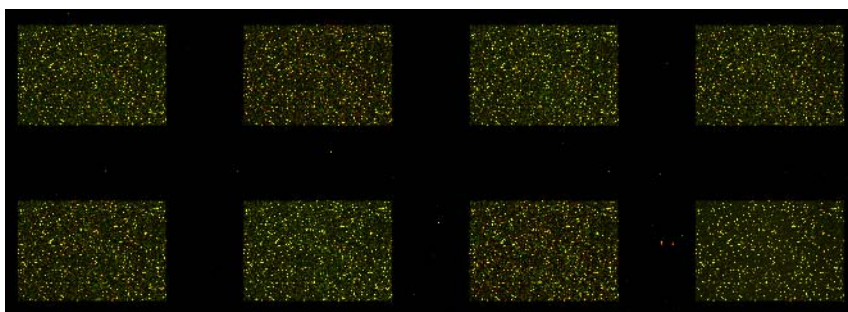


Figure 4 Agilent SurePrint G3 8×60K CGH microarray shown in red and green channels

4 Microarray Processing and Feature Extraction

Step 2. Extract data using the Feature Extraction program

- 1 Open the Agilent Feature Extraction program.
- 2 Add the images (.tif) to be extracted to the Feature Extraction Project.
 - a Click **Add New Extraction Set(s)** icon on the toolbar or right-click the Project Explorer and select **Add Extraction...**

You can also drag the image (.tif) from the desktop to the Feature Extraction project pane.
 - b Browse to the location of the .tif files, select the .tif file(s) and click **Open**. To select multiple files, use the **Shift** or **Ctrl** key when selecting.

The Feature Extraction program automatically assigns a default grid template and protocol for each extraction set, if the following conditions are met:

 - As of v10.5, the Feature Extraction program automatically associates the protocol for a given microarray based on the application specified in the design file and the number of channels present in the image. If you need to use a protocol other than the Agilent default protocol, specify it in the Grid Template properties.
 - For auto assignment of the CGH Feature Extraction protocol, the **default CGH protocol** must be specified in the Feature Extraction Grid Template properties.

To access the Feature Extraction Grid Template properties, double-click on the grid template in the Grid Template Browser.
- 3 Set Feature Extraction Project Properties.
 - a Select the **Project Properties** tab.
 - b In the **General** section, enter your name in the Operator field.
 - c In all other sections, verify that at least the following default settings as shown in [Figure 5](#) below are selected.

For Feature Extraction 10.5 or higher, the metric sets are part of the protocol, and there is no need to set them.

QC metrics updates are available automatically from eArray if configured appropriately. See [“Automatic Download from eArray” on page 57](#) for configuration.

Step 2. Extract data using the Feature Extraction program

Project Properties Extraction Set Configuration	
General	
Operator	Unknown
Input	
Number of Extraction Sets Included	0
Output and Data Transfer	
Outputs	
MAGE	None
JPEG	None
TEXT	Local file only
Output Package	Compact
Visual Results	Local file only
Grid	None
QC Report	Local PDF file only
FTP Send Tiff File	False
Local File Folder	
Same As Image	True
Results Folder	
FTP Setting	
Automatic Protocol Assignment	
Highest Priority Default Protocol	Grid Template Default
Project Default Protocol	
Automatic Grid Template Assignment	
Use Grid file if available	False
External DyeNorm List File	
Overwrite Previous Results	False

Figure 5 Default settings in Feature Extraction 10.5

4 Check the Extraction Set Configuration.

a Select the **Extraction Set Configuration** tab.

b Verify that the correct grid template is assigned to each extraction set in the **Grid Name** column. To assign a different grid template to an extraction set, select one from the pull down menu.

If a grid template is not available to select from the pull down menu, you must add it to the Grid Template Browser. To add, right-click inside the Grid Template Browser, select **Add**. Look for the design file (.xml) and click **Open** to load grid template into the Feature Extraction database.

To update to the latest grid templates via Online Update, right-click **Grid Template Browser** and select **Online Update**. You can also download the latest grid templates from Agilent Web site at <http://e-array.com>. After downloading, add the grid template to the Grid Template Browser.

c Verify that the most recent protocol is assigned to each extraction set in the **Protocol Name** column.

4 Microarray Processing and Feature Extraction

Step 2. Extract data using the Feature Extraction program

If a protocol is not available to select from the pull down menu, you must import it to the Feature Extraction Protocol Browser. To import, right-click the **Feature Extraction Protocol Browser**, select **Import**. Browse for the Feature Extraction protocol (.xml) and click **Open** to load the protocol into the Feature Extraction database. Visit Agilent Web site at www.agilent.com/chem/feprotocols to download the latest protocols.

Protocols are also available automatically from eArray if configured appropriately. See “[Automatic Download from eArray](#)” on page 57 for configuration.

- 5 Save the Feature Extraction Project (.fep) by selecting **File > Save As** and browse for desired location.
- 6 Verify that the icons for the image files in the Feature Extraction Project Window no longer have a red X through them. A red X through the icon indicates that an extraction protocol was not selected or that the Grid Template is not in the database. If needed, reselect the extraction protocol for that image file.
- 7 Select **Project > Start Extracting**.
- 8 After the extraction is completed successfully, view the QC report for each extraction set by double-clicking the QC Report link in the **Summary Report** tab. Determine whether the grid has been properly placed by inspecting **Spot Finding of the Four Corners of the Array**. See [Figure 7](#) and [Figure](#) .

Automatic Download from eArray

Feature Extraction version 10.7 or higher can automatically download Grid Templates, protocols and QC metrics (QCM or QCMT). To set this up, in the eArray Login Setting dialog box, under **Advanced Options**, click **Use eArray server during extraction**. See Figure 6.

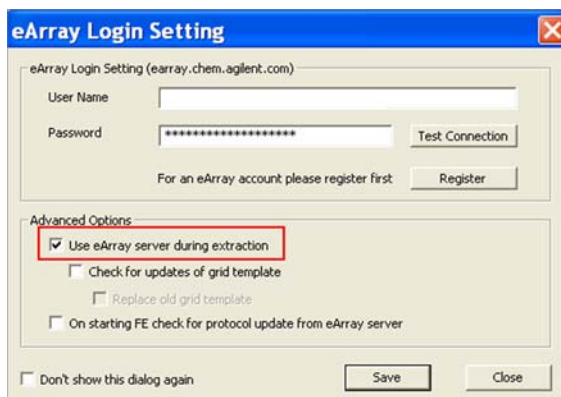


Figure 6 eArray Login Setting. You can mark the other two check boxes under Advanced Options if you want to get update of grid templates already in the database or to get protocol updates. See the Feature Extraction user guide for more information.

Microarray QC Metrics

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 and in Agilent CytoGenomics and Genomic Workbench. 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

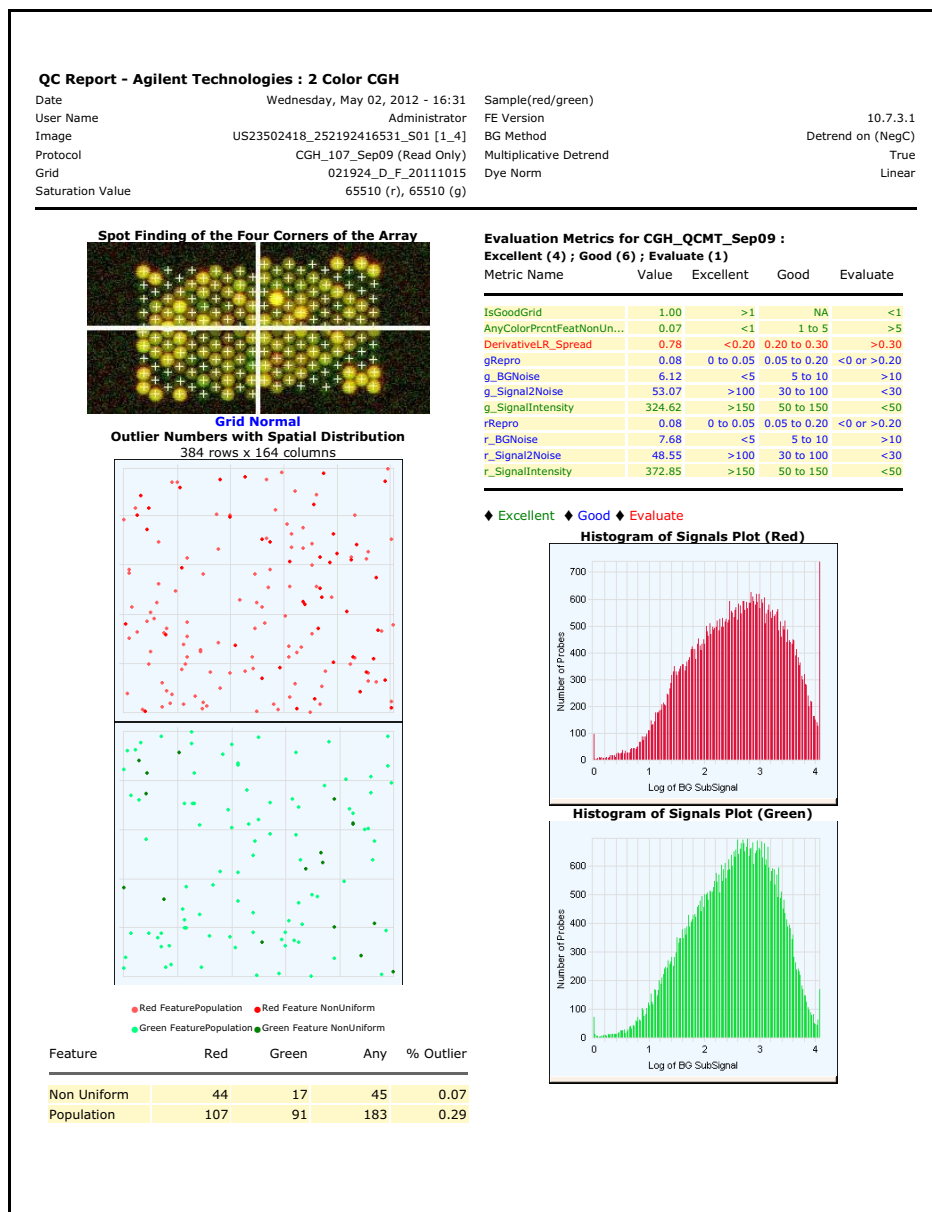
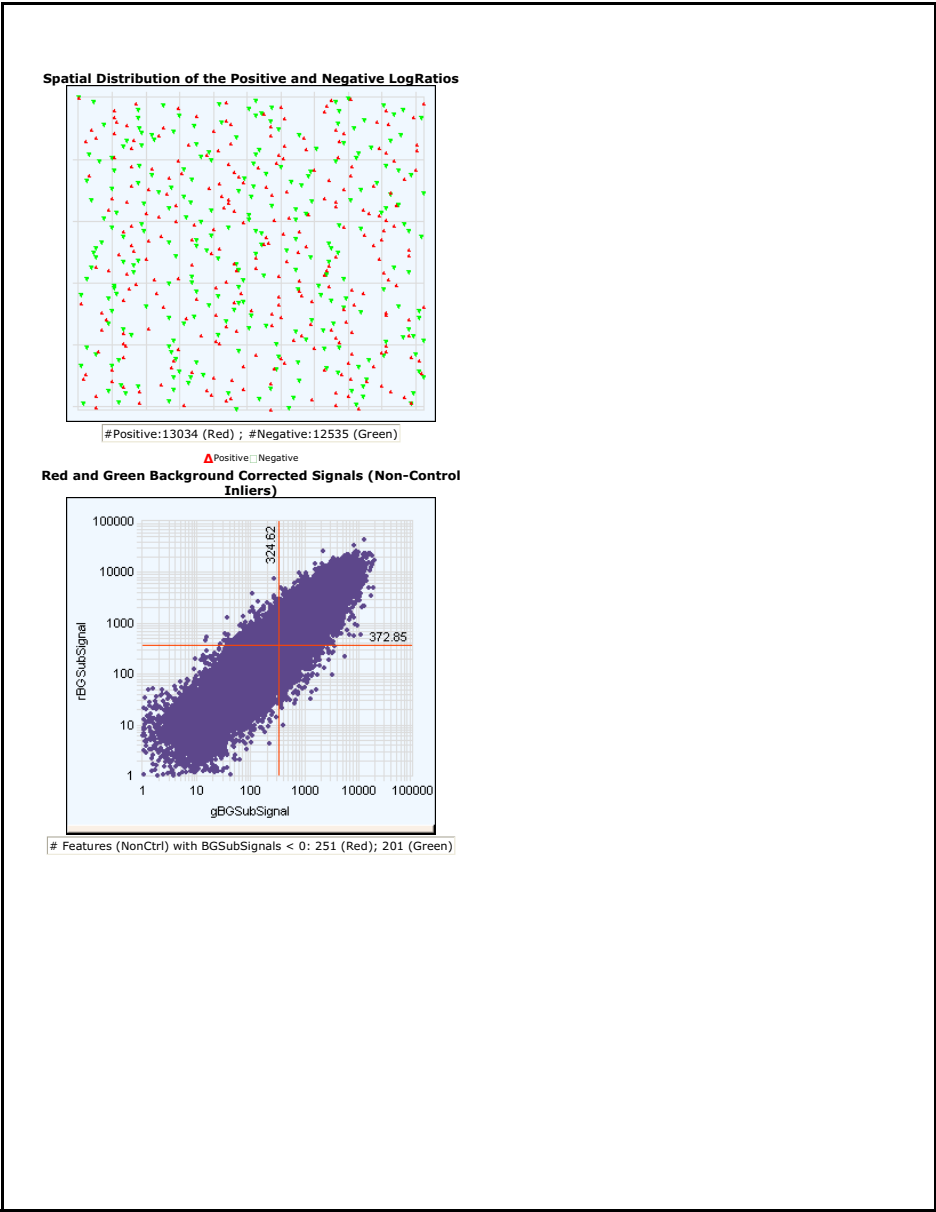


Figure 7 CGH QC report from single cell generated from Feature Extraction 10.7, page 1

4 **Microarray Processing and Feature Extraction**
Step 2. Extract data using the Feature Extraction program



CGH QC report from single cell generated from Feature Extraction v10.7, page 2



5 Troubleshooting

If the DNA is not well mixed [62](#)

If you have low specific activity or degree of labeling not due to poor sample quality [63](#)

If you have low yield not due to poor sample quality [63](#)

If you have post-labeling signal loss [64](#)

If you have high BGNoise values [65](#)

If you have poor reproducibility [65](#)

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

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 4 °C or -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. Clean-up of Labeled Amplified DNA](#)” on page 31 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 NO_x 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 oven and Wash 2 temperature.
- ✓ 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 46.

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 the washes.

- ✓ 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 using setting 4 for 1 minute.
 - 4 Slowly remove the slide rack and scan the slides immediately.

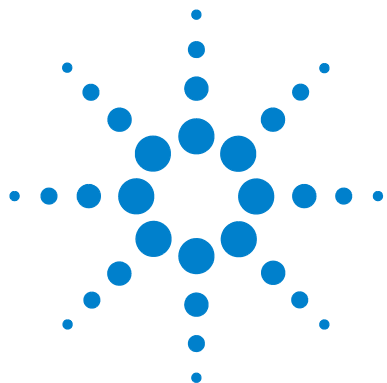
If you have poor reproducibility

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.

5 Troubleshooting

If you have poor reproducibility



6 Reference

Reagent Kit Components	68
Microarray Handling Tips	70
Agilent Microarray Layout and Orientation	71
Array/Sample tracking on microarray slides	74
Agilent Information Assets Access Agreement	75

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

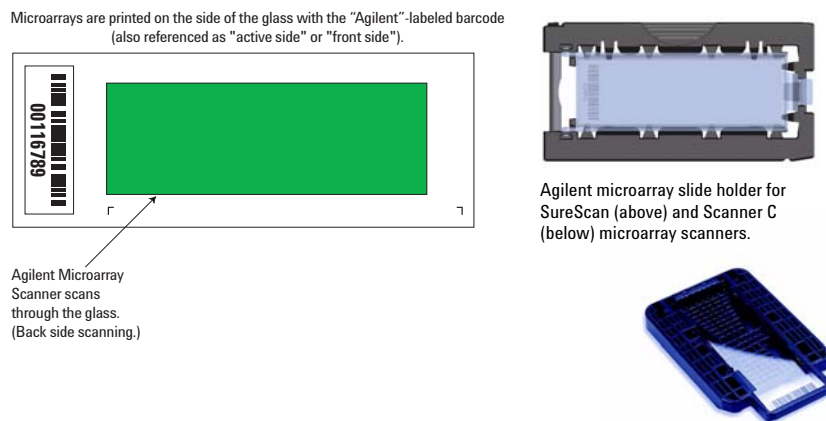


Figure 8 Agilent microarray slide and slide holder

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 8 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" included in 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.

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 9](#)).

This changes the feature numbering and location as it relates to the “microarray design files” found on the disc in each Agilent oligo microarray kit.

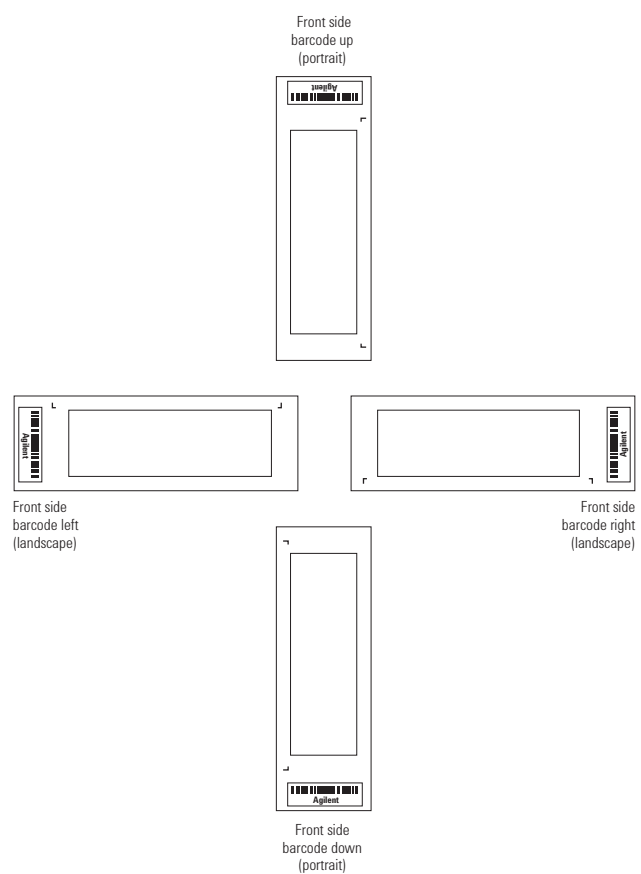


Figure 9 Microarray slide orientation

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
B A R C O D E	Sample:	Sample:	Sample:	Sample:
	Sample:	Sample:	Sample:	Sample:
	Array 2_1	Array 2_2	Array 2_3	Array 2_4
Barcode Number _____				

Figure 10 8-pack microarray slide

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- 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.

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In This Book

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

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