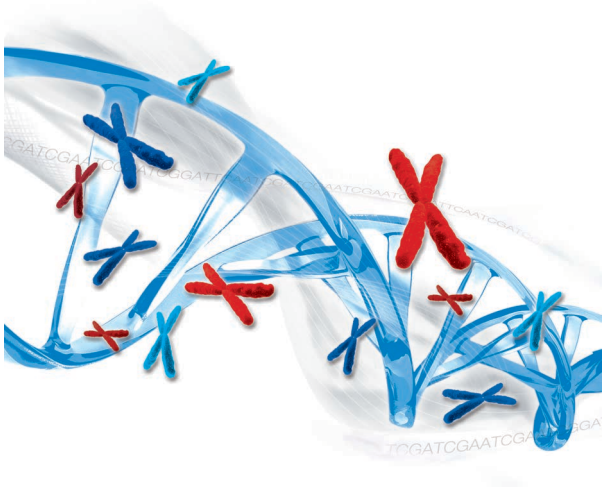
Oligonucleotide Array-Based CGH for Genomic DNA Analysis - Enzymatic Labeling

For Blood, Cells, or Tissues (with a High Throughput option)

Protocol

For Research Use Only. Not for use in diagnostic procedures.

Version 8.1, April 2025



Notices

Manual Part Number

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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 Agilent 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 enzymatically label DNA from blood, cells or frozen tissues. For processing FFPE samples, follow the *Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis (ULS Labeling for Blood, Cells, Tissues or FFPE) Protocol* (publication G4410-90020). FFPE samples are not supported for SurePrint G3 CGH+SNP microarray processing.

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 DNA Isolation

This chapter describes the method to isolate genomic DNA (gDNA) from blood, cells, or frozen tissues.

3 Sample Preparation

This chapter describes the standard method to process gDNA prior to labeling as well as the Whole Genome Amplification method for low input DNA projects.

4 Sample Labeling

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

5 Microarray Processing

This chapter describes the steps to hybridize, wash and scan Agilent CGH and CGH+SNP microarrays and to extract data using the Agilent Feature Extraction Software for use in Agilent CytoGenomics and Genomics 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 8.1

- Update to number of slides that can be processed with the Oligo aCGH/ChIP-on-chip Hybridization Kit. See **Table 18** on page 23.

What’s new in 8.0

- Updated document look and feel.
- Instructions on handling the newly redesigned “secure fit” slide boxes in which the microarray slides are shipped. Before opening the box for the first time, see **““Secure Fit” Slide Box Opening Instructions"** on page 100.
- Expanded instructions and new images in the **Microarray Processing** procedures to help avoid common problems and optimize hybridization of your sample to the microarray. Removed instructions on the use of heat blocks and water baths for sample incubations in favor of promoting the use of a thermal cycler.
- Part numbers for new GenetiSure Cyto CGH and CGH+SNP microarrays added to **“Agilent Oligo CGH Microarray Kit Contents"** on page 14.
- Updated web addresses for Agilent materials.
- Updated **Safety Notes**.
- Removed microarray scanning instructions for the Agilent B scanner.

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Content

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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 isolate gDNA from blood, cells, or frozen tissues, to increase the likelihood of a successful experiment. For processing FFPE samples, refer to the *Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis (ULS Labeling for Blood, Cells, Tissues, or FFPE) Protocol* (publication G4410-90020). FFPE samples are not supported when you process SurePrint G3 CGH+SNP microarrays.
- If the DNA isolation procedure described in this document cannot be followed, make sure that the DNA is free of RNA and protein contamination.
- 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.
- Acetonitrile is a flammable liquid and vapor. Harmful if inhaled, swallowed, or contacts skin. Target organs: liver, kidneys, cardiovascular system, and CNS.
- 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

- 1-inch × 3-inch microarray slides in quantities of:
  - 50 for 1-pack
  - 25 for 2-pack
  - 12 for 4-pack
  - 6 for 8-pack
- Sufficient reagents and consumables to process 50 (1-pack and 2-pack bundle) or 48 (4-pack and 8-pack bundle) 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

Table 1 SurePrint G3 CGH and CGH+SNP Bundles

Part Number	Description
G5920A, Option 1	SurePrint G3 Human CGH Bundle, 1×1M
G5921A, Option 1	SurePrint G3 Human CGH Bundle, 2×400K
G5921A, Option 2	SurePrint G3 CGH+SNP Bundle, 2×400K
G5922A, Option 1	SurePrint G3 Human CGH Bundle, 4×180K
G5922A, Option 2	SurePrint G3 ISCA CGH+SNP Bundle, 4×180K
G5923A, Option 1	SurePrint G3 Human CGH Bundle, 8×60K
G5982C	GenetiSure Cyto CGH Bundle, 8×60K

Table 1 SurePrint G3 CGH and CGH+SNP Bundles (continued)

Part Number	Description
G5983C	GenetiSure Cyto CGH Bundle, 4×180K
G5984C	GenetiSure Cyto CGH+SNP Bundle, 4×180K

Catalog SurePrint HD and G3 CGH Microarray Kits

- Five 1-inch × 3-inch, 1-pack and 2-pack microarray slides
- Three 1-inch × 3-inch, 4-pack and 8-pack microarray slides

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

See the tables that follow for available designs. For more information on CGH designs, go to **www.agilent.com**.

Table 2 Catalog SurePrint CGH+SNP Microarray Kits - Human

Part Number	Description
G4842A*	SurePrint G3 Human CGH+SNP Microarray Kit 2×400K
G4890A*	SurePrint G3 Human ISCA CGH+SNP Microarray Kit 4×180K
G4869A*	SurePrint G3 Human Cancer CGH+SNP Microarray Kit 4×180K
G5975A*	GenetiSure Cancer Research CGH+SNP, 2×400K†
G5974A*	GenetiSure Postnatal Research CGH+SNP, 2×400K†
G5984B*	GenetiSure Cyto CGH+SNP Microarray Slide 4×180K†
G5984A*	GenetiSure Cyto CGH+SNP Microarray Kit, 4×180K†

* These arrays can only be processed using the enzymatic labeling protocol.

† Requires Agilent CytoGenomics Software version 3.0.6 or higher for analysis

Table 3 Catalog CGH Microarray Kits - Human

Part Number	Description
G4447A	SurePrint G3 Human CGH Microarray Kit 1×1M (5 slides)
G4824A-021529	SurePrint G3 Human CGH Microarray Slide 1×1M
G4448A	SurePrint G3 Human CGH Microarray Kit 2×400K (5 slides)
G4825A-021850	SurePrint G3 Human CGH Microarray Slide 2×400K
G4449A	SurePrint G3 Human CGH Microarray Kit 4×180K (5 slides)

Table 3 Catalog CGH Microarray Kits - Human (continued)

Part Number	Description
G4826A-022060	SurePrint G3 Human CGH Microarray Slide 4×180K
G4450A	SurePrint G3 Human CGH Microarray Kit 8×60K (5 slides)
G4827A-021924	SurePrint G3 Human CGH Microarray Slide 8×60K
G4423B-016266	SurePrint G3 Human CGH 244A Supplemental Slide 1×244K
G5955A	SurePrint G3 Human CGH ISCA v2 Microarray Kit 8×60K (3 slides)
G4411B	Human Genome CGH 244A Microarray Kit 1×244K (5 slides)
G4423B-014693	Human Genome CGH 244A Microarray Slide 1×244K
G4412A	Human Genome CGH 105A Microarray Kit 2×105K
G4425B-014698	Human Genome CGH 105A Microarray Slide 2×105K
G4413A	Human Genome CGH Microarray Kit 4×44K (3 slides)
G4426B-014950	Human Genome CGH Microarray Slide 4×44K
G5982B-085590	GenetiSure Cyto CGH Microarray Slide 8×60K
G5983B-085589	GenetiSure Cyto CGH Microarray Slide 4×180K
G5982A	GenetiSure Cyto CGH Microarray Kit, 8×60K
G5983A	GenetiSure Cyto CGH Microarray Kit, 4×180K

Table 4 Catalog CNV Microarray Kits - Human

Part Number	Description
G4824A-023642	SurePrint G3 Human CNV Microarray Slide 1×1M
G4825A-021365	SurePrint G3 Human CNV Microarray Slide 2×400K
G4423B-018897	SurePrint G3 Human CNV Microarray Slide, Slide 1 of 2, 1×244K
G4423B-018898	SurePrint G3 Human CNV Microarray Slide, Slide 2 of 2, 1×244K
G4425B-022837	Human CNV Association Microarray Slide 2×105K

Table 5 Catalog CGH Microarrays- Mouse

Part Number	Description
G4824A-027414	SurePrint G3 Mouse CGH Microarray Slide 1×1M
G4839A	SurePrint G3 Mouse CGH Microarray Kit 4×180K (3 slides)

Table 5 Catalog CGH Microarrays- Mouse (continued)

Part Number	Description
G4826A-027411	SurePrint G3 Mouse CGH Microarray Kit 4×180K
G4423B-014695	Mouse Genome CGH Microarray Slide 1×244K
G4425B-014699	Mouse Genome CGH Microarray Slide 2×105K

Table 6 Catalog CGH Microarrays - Rat

Part Number	Description
G4824A-027065	SurePrint G3 Rat CGH Microarray Slide 1×1M
G4826A-027064	SurePrint G3 Rat CGH Microarray Slide 4×180K
G4423B-015223	Rat Genome CGH Microarray Slide 1×244K
G4425B-015235	Rat Genome CGH Microarray Slide 2×105K

Table 7 Catalog CGH Microarrays - Model Organism/Non-Human

Part Number	Description
G4826A-024419	SurePrint G3 Rhesus Macaque CGH Microarray Kit 4×180K (5 slides)
G4826A-024422	SurePrint G3 Chimpanzee CGH Microarray Kit 4×180K (5 slides)
G4826A-025242	SurePrint G3 Bovine CGH Microarray Kit 4×180K (5 slides)
G4826A-025522	SurePrint G3 Canine CGH Microarray Kit 4×180K (5 slides)
G4826A-025843	SurePrint G3 Rice CGH Microarray Slide 4×180K
G4423B-019553	Chicken Genome CGH Microarray, 1x244K

Unrestricted SurePrint HD and G3 CGH Microarrays

- One, two, four or eight microarrays printed on each 1-inch × 3-inch glass slide
- Number of microarray slides vary per kit and per order

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

See the tables that follow for available designs.

Table 8 Unrestricted CGH Microarrays - Human

Part Number	Description
G4826A, AMADID 031748	Unrestricted SurePrint G3 CGH ISCA v2 Microarray, 4×180K
G4827A, AMADID 031746	Unrestricted SurePrint G3 CGH ISCA v2 Microarray, 8×60K
G4425B, AMADID 031750	Unrestricted HD CGH ISCA v2 Microarray, 2×105K
G4426B, AMADID 031747	Unrestricted HD CGH ISCA v2 Microarray, 4×44K

Custom SurePrint HD and G3 Microarrays

- One, two, four or eight microarray(s) printed on each 1-inch × 3-inch glass slide
- Number of microarrays varies per kit and per order

See the tables that follow for available formats.

Table 9 Custom SurePrint G3 CGH and CGH+SNP Microarrays

Part Number	Description
G4882A*	SurePrint G3 Custom CGH+SNP Microarray, 1×1M
G4883A*	SurePrint G3 Custom CGH+SNP Microarray, 2×400K
G4884A*	SurePrint G3 Custom CGH+SNP Microarray, 4×180K
G4885A*	SurePrint G3 Custom CGH+SNP Microarray, 8×60K
G4123A	SurePrint G3 Custom CGH Microarray, 1×1M
G4124A	SurePrint G3 Custom CGH Microarray, 2×400K
G4125A	SurePrint G3 Custom CGH Microarray, 4×180K
G4126A	SurePrint G3 Custom CGH Microarray, 8×60K

* These arrays can only be processed using the enzymatic labeling protocol.

Table 10 Custom SurePrint HD CGH Microarrays

Part Number	Description
G4423A	Custom HD-CGH Microarray, 1×244K
G4425A	Custom HD-CGH Microarray, 2×105K
G4426A	Custom HD-CGH Microarray, 4×44K
G4427A	Custom HD-CGH Microarray, 8×15K

Required Equipment

Table 11 Required equipment

Description	Vendor and part number
200-µL Thin-Wall Tube	Agilent p/n 410091 or equivalent
Agilent Microarray Scanner Bundle for 1×244K, 2×105K, 4×44K or 8×15K, *or* for 1×1M, 2×400K, 4×180K or 8×60K	 Agilent p/n G4900DA, G2565CA or G2565BA 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 1-pack microarrays *or* for 2-pack microarrays *or* for 4-pack microarrays *or* for 8-pack microarrays	 Agilent p/n G2534-60003 Agilent p/n G2534-60002 Agilent p/n G2534-60011 Agilent p/n G2534-60014
Hybridization oven; temperature set at 67°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)	Thermo Fisher Scientific 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 6795-420D or equivalent
Microcentrifuge	Eppendorf p/n 5430 or equivalent
E-Gel Opener‡	Thermo Fisher Scientific p/n G530001
E-Gel Simple Runner Electrophoresis Device‡	Thermo Fisher Scientific p/n G8000
Qubit 4 Fluorometer**	Thermo Fisher Scientific p/n Q33226
Thin wall, clear 0.5 mL PCR tubes**	Thermo Fisher Scientific p/n Q32856 *or* VWR p/n 10011-830
Sterile storage bottle	Nalgene 455-1000 or equivalent
UV-VIS spectrophotometer	NanoDrop 8000 or 2000, or equivalent

Table 11 Required equipment (continued)

Description	Vendor and part number
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
Vacuum Concentrator ††	Thermo Scientific Savant SpeedVac p/n DNA 130-115 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 Fisher Scientific p/n 121
Circulating water baths or heat blocks. For DNA extraction, set to 56°C. For restriction digestion and/or labeling, set to 37°C, 65°C and 98°C. For hybridization preparation, set to 37°C and 98°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 or G2565BA scanner in environments in which ozone levels are 5 ppb or higher.

‡ For use with Thermo Fisher Scientific E-gels.

** Optional.

†† Optional. Depends on microarray format and processing protocol used.

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

*** For optimal performance, Agilent recommends using a thermal cycler, rather than water baths or heat blocks, for restriction digestion, labeling, and hybridization preparation steps. In particular, the 98°C incubations benefit from the use of a thermal cycler in place of a water bath or heat block.

Table 12 Optional. Recommended when using the high throughput or amplification method for sample preparation.

Description	Vendor and part number
Thermal cycler with heated lid	Agilent p/n G8800A or equivalent
96-well PCR plate	Agilent p/n 401334 or equivalent
Centrifuge (for 96-well plate)	Eppendorf p/n 5810 or equivalent
Heat Sealer	Eppendorf p/n 5391000010 *or* Fisher Scientific p/n 05-412-610
Heat Sealing PCR Foil (removable)	Eppendorf p/n 0030127854 *or* Fisher Scientific p/n E0030127854

Table 13 Optional. Recommended when using high-throughput method on 2-pack microarrays.

Description	Vendor and part number
Tall Chimney PCR plate	Fisher Scientific p/n 14-230-242

Table 14 Optional equipment for DNA extraction from tissue.

Description	Vendor and part number
Thermal shaker	Eppendorf Thermomixer p/n 2231000574 or equivalent

Required Reagents

Table 15 Required reagents for gDNA isolation

Description	Vendor and part number
Phosphate Buffered Saline pH 7.4 (PBS)	VWR p/n 97062-818
E-Gel General Purpose Agarose Gels , 1.2%	Thermo Fisher Scientific p/n G501801
SYBR Gold Nucleic Acid Gel Stain	Thermo Fisher Scientific p/n S11494
SYBR photographic filter	Thermo Fisher Scientific p/n S7569
TrackIt 1 Kb Plus DNA Ladder	Thermo Fisher Scientific p/n 10488085
DNase/RNase-free distilled water	Thermo Fisher Scientific p/n 10977-015

Table 15 Required reagents for gDNA isolation (continued)

Description (continued)	Vendor and part number
Qubit dsDNA BR Assay Kit, for use with the Qubit fluorometer (100 assays)*	Thermo Fisher Scientific p/n Q32850
RNase A (100 mg/mL)	Qiagen p/n 19101
DNeasy Blood & Tissue Kit	Qiagen p/n 69504
Proteinase K (>600 mAU/mL, solution)	Qiagen p/n 19131
Ethanol (95% to 100% molecular biology grade)	Sigma-Aldrich p/n E7023-6×500ML

* Optional.

Table 16 Required reagents for enzymatic sample prep and labeling with the SureTag Complete DNA Labeling Kit (for Human Samples)

Description	Vendor and part number
SureTag Complete DNA Labeling Kit*†	Agilent p/n 5190-4240
Purification Columns‡ (50 units)	Agilent p/n 5190-3391
AutoScreen A, 96-well plates**	GE Healthcare Life Sciences p/n 25900598
1×TE (pH 8.0) , Molecular grade	Thermo Fisher Scientific p/n AM9849
GenElute PCR Clean-Up Kit††	Sigma-Aldrich p/n NA1020
GenomePlex Complete Whole Genome Amplification Kit††	Sigma-Aldrich p/n WGA2

* Kit content is listed in **"Reagent Kit Components"** on page 98.

† 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.

** Optional. Recommended if using the high-throughput protocol.

†† Optional. Recommended if using the Amplification Method for sample preparation.

Table 17 Required reagents for enzymatic sample prep and labeling with the SureTag DNA Labeling Kit (or when Human reference DNA is provided separately)

Description	Vendor and part number
SureTag DNA Labeling Kit*	Agilent p/n 5190-3400
Purification Column† (50 units)	Agilent p/n 5190-3391
AutoScreen A, 96-well plate‡	GE Healthcare p/n 25-9005-98
For possible use as a reference sample: • Human Genomic DNA *or* • Mouse Genomic DNA *or* • Rat Genomic DNA	 • For CGH microarrays: Promega p/n G1521 (female) or p/n G1471 (male) • For CGH+SNP microarrays: Coriell p/n NA18507, NA18517, NA12891, NA12878, or NA18579 • Jackson Labs p/n 000664 (female and male) • Harlan Sprague Dawley (custom)
1×TE (pH 8.0) , Molecular grade	Promega p/n V6231
GenElute PCR Clean-Up Kit**	Sigma-Aldrich p/n NA1020
GenomePlex Complete Whole Genome Amplification Kit**	Sigma-Aldrich p/n WGA2

* Kit content is listed in **"Reagent Kit Components"** on page 98.

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

‡ Optional. Recommended if using the high-throughput protocol.

** Optional. Recommended if using the Amplification Method for sample preparation.

Table 18 Required reagents for hybridization and wash

Description	Vendor and part number
Oligo aCGH/ChIP-on-chip Wash Buffer Kit *or* Oligo aCGH/ChIP-on-chip Wash Buffer 1 *and* Oligo aCGH/ChIP-on-chip Wash Buffer 2*	Agilent p/n 5188-5226 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 (minimum of 24 slides) or p/n 5188-5380 (minimum of 96 slides)
Cot-1 DNA (1.0 mg/mL) • Human Cot-1 DNA* *or* • Mouse Cot-1 DNA *or* • Rat Hybloc	 Agilent p/n 5190-3393 Thermo Fisher Scientific p/n 18440016 Applied Genetics p/n RHB

Table 18 Required reagents for hybridization and wash (continued)

Description (continued)	Vendor and part number
DNase/RNase-free distilled water	Thermo Fisher Scientific p/n 10977015
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 **www.agilent.com**.

2 DNA Isolation

This chapter describes the Agilent recommended procedure for isolating gDNA from blood, cells, or frozen tissues using the **DNeasy Blood & Tissue Kit**.

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), and copy-neutral Loss of Heterozygosity or Uniparental Disomy if CGH+SNP microarrays are used, in an experimental sample relative to a reference sample. The type of sample used as a reference is a matter of experimental choice; however, many experimenters use normal commercial gDNA as a reference sample, such as the **Human Reference DNA (Male and Female)** that is included in the **SureTag Complete DNA Labeling Kit**.

For processing FFPE samples, follow the *Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis (ULS Labeling for Blood, Cells, Tissues, or FFPE) Protocol* (publication G4410-90020). FFPE samples are not supported when you process SurePrint G3 CGH+SNP microarrays.

CGH+SNP Microarrays

When you process SurePrint G3 CGH+SNP microarrays, the reference needs to be DNA isolated from a single genotyped individual. You can use one of the following DNA samples as reference:

1. Human Reference DNA Male or Female, components of the **SureTag Complete DNA Labeling Kit**.
2. One of five supported HapMap samples: NA18507 (Yoruban Male), NA18517 (Yoruban Female), NA12891 (European Male), NA12878 (European Female), or NA18579 (Chinese Female). The HapMap samples can be ordered from the Coriell Institute for Medical Research.

3 You can genotype your own reference DNA isolated from a single individual by hybridizing it against all 5 supported HapMap samples on the Agilent CGH+SNP microarrays. This experiment only needs to be done once.

The input amount of DNA for the experimental sample labeling reaction must be the same as for the reference sample labeling reaction. Inaccurate DNA quantitation can lead to different DNA inputs into the experimental and reference labeling reactions, which increases assay noise (as measured by the DLRSD QC metric). Different DNA isolation methods can also create quantitation artifacts. To minimize assay noise, Agilent recommends using a fluorometric method (such as Qubit) highly selective for double-stranded DNA. A Nanodrop spectrophotometer can be used to assess gDNA purity. There is no need to re-determine the concentration of the Agilent Human Reference DNA Male and Female, its concentration is 200 ng/µL as measured by both spectrophotometer and fluorometer.

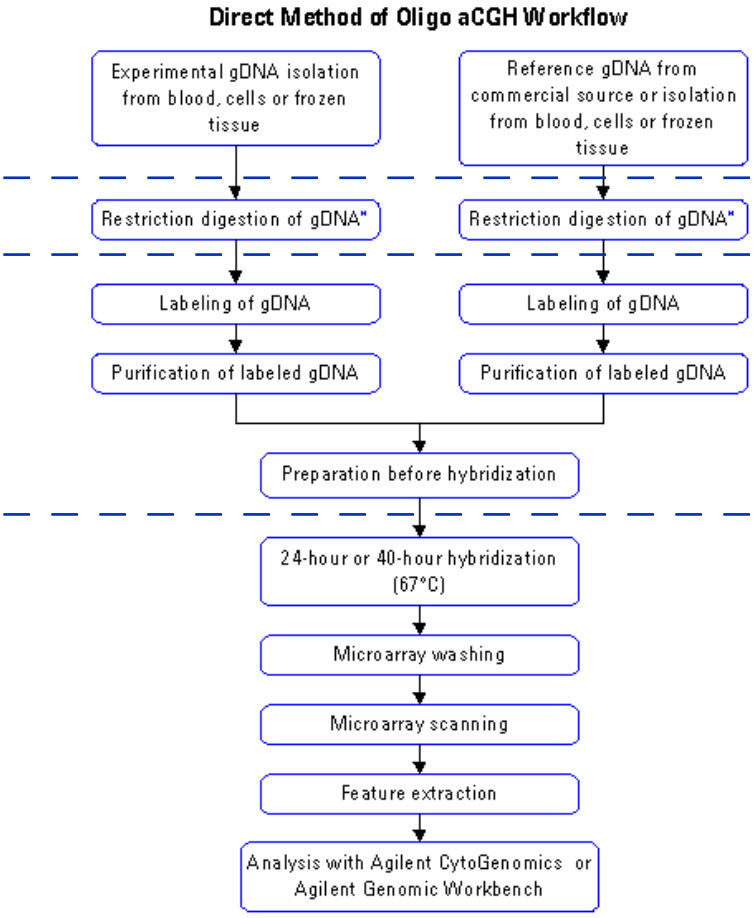
In addition, if you plan to run either the **GenetiSure Cancer Research CGH+SNP, 2×400K** or **GenetiSure Postnatal Research CGH+SNP, 2×400K†** microarrays, you must use Agilent Male/Female reference samples as Agilent has not validated any other reference samples with these two designs.

"DNA Isolation" on page 25

"Sample Preparation" on page 35

"Sample Labeling" on page 47

"Microarray Processing" on page 57



* Optional, if processing SurePrint HD and G3 CGH microarrays.
Required if processing SurePrint G3 CGH+SNP microarrays.

Figure 1. Direct workflow for sample preparation and microarray processing. Minimum of 0.5 µg (for 1-pack, 2-pack or 4-pack microarrays) or 0.2 µg (for 8-pack microarrays) starting gDNA per sample is required.

Step 1. gDNA Extraction

Use reagents from the **DNeasy Blood & Tissue Kit**.

1. Equilibrate a thermomixer and heat block or water bath to 56°C.
2. For blood with nonnucleated erythrocytes (mammals):
   a. Put 20 µL of **Proteinase K** into the bottom of a **1.5-mL RNase-free Microfuge Tube**.
   b. Add 50 to 100 µL of anticoagulated blood.
   c. Add enough **Phosphate Buffered Saline pH 7.4 (PBS)** to make a total volume of 220 µL.
   d. Go to **step 7**.
3. For blood with nucleated erythrocytes (such as chicken):
   a. Put 20 µL of **Proteinase K** into the bottom of a **1.5-mL RNase-free Microfuge Tube**.
   b. Add 5 to 10 µL of anticoagulant blood.
   c. Add enough **Phosphate Buffered Saline pH 7.4 (PBS)** to make a total volume of 220 µL.
   d. Go to **step 7**.
4. For cells:
   a. Spin a maximum of 5×10^6 cells in a centrifuge for 5 minutes at 300 × g. Resuspend the pellet in 200 µL of **Phosphate Buffered Saline pH 7.4 (PBS)**.
   b. Add 20 µL of **Proteinase K**.
   c. Go to **step 7**.
5. For frozen tissue:
   a. Cut up to 25 mg frozen tissue (up to 10 mg for spleen tissue) into small pieces and put into a **1.5-mL RNase-free Microfuge Tube**.
   b. Add 180 µL of **Buffer ATL**.
   c. Add 20 µL of **Proteinase K**.
   d. Mix well on a vortex mixer.
   e. Incubate in a thermomixer at 56°C shaking at 450 rpm until the tissue is completely lysed.

Lysis time varies depending on the type of tissue processed. Usually lysis is complete in 1 to 3 hours. If it is more convenient, samples can be lysed overnight.

f Let the sample cool to room temperature and spin in a microcentrifuge for 30 seconds at 6,000 × g to drive the contents off the walls and lid.

g Go to **step 7**.

6 For further purification of extracted DNA:

a Take a maximum 25 µg of DNA.

b Add enough **Phosphate Buffered Saline pH 7.4 (PBS)** to make a total volume of 220 µL.

c Add 20 µL of **Proteinase K**.

7 Add 4 µL of **RNase A (100 mg/mL)**, mix on a vortex mixer, and incubate for 2 minutes at room temperature. Spin in a microcentrifuge for 30 seconds at 6,000 × g to drive the contents off the walls and lid.

8 Add 200 µL of **Buffer AL** to each sample, mix thoroughly on a vortex mixer, and incubate at 56°C for 10 minutes in a heat block or water bath. Spin in a microcentrifuge for 30 seconds at 6,000 × g to drive the contents off the walls and lid.

9 Add 200 µL of 100% **Ethanol** to each sample, and mix thoroughly on a vortex mixer. Spin in a microcentrifuge for 30 seconds at 6,000 × g to drive the contents off the walls and lid.

10 Transfer the sample mixture onto a **DNeasy Mini Spin Column** in a **2 mL Collection Tube**. Spin in a centrifuge at 6,000 × g for 1 minute. Discard the flow-through and collection tube. Put the **DNeasy Mini Spin Column** in a new **2 mL Collection Tube**.

11 Before using for the first time, prepare **Buffer AW1** by adding 100% **Ethanol** to the **Buffer AW1** bottle (see bottle label for volume). Mark the appropriate check box to indicate that ethanol was added to the bottle.

12 Add 500 µL **Buffer AW1** onto the column, and spin in a microcentrifuge for 1 minute at 6,000 × g. Discard the flow-through and collection tube. Put the **DNeasy Mini Spin Column** in a new **2 mL Collection Tube**.

13 Before using for the first time, prepare **Buffer AW2** by adding 100% **Ethanol** to the **Buffer AW2** bottle (see bottle label for volume). Mark the appropriate check box to indicate that ethanol was added to the bottle.

14 Add 500 µL of **Buffer AW2** onto the column, and spin in a centrifuge for 3 minutes at 20,000 × g to dry the DNeasy membrane. Discard the flow-through and collection tube.

15 Put the **DNeasy Mini Spin Column** in a clean **1.5-mL RNase-free Microfuge Tube**, and pipette 200 µL of **Buffer AE** directly onto the center of the DNeasy column membrane.

16 Incubate at room temperature for 1 minute, and then spin in a microcentrifuge for 1 minute at 6,000 × g to elute the DNA.

17 Repeat elution with **Buffer AE** once as described in **step 15** and **step 16**. Combine the duplicate samples in one microcentrifuge tube for a final volume of 400 µL.

Step 2. gDNA Quantitation and Quality Analysis

Accurate assessment of gDNA quantity and quality are crucial to the success of an Agilent Oligo aCGH experiment. High quality gDNA should be free of contaminants such as carbohydrates, proteins, and traces of organic solvents, and should also be intact with minimal degradation.

See "FFPE Tissues" in the *Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis (ULS Labeling for Blood, Cells, Tissues or FFPE) Protocol* (publication G4410-90020) for details on how to isolate gDNA from FFPE tissues. FFPE samples are not supported when you process SurePrint G3 CGH+SNP microarrays.

Use Quant-iT dsDNA Broad-Range Assay Kit to measure the concentration of double-strand DNA by fluorometry. Use the NanoDrop ND-1000 UV-VIS Spectrophotometer (or equivalent) to assess gDNA concentration and purity. Use agarose gel electrophoresis to assess gDNA intactness and the average molecular weight for each sample.

NOTE

Agilent recommends the use of a fluorometric quantitation method for the highest quality data.

Fluorometry

Use the **Qubit dsDNA BR Assay Kit** at room temperature (22°C to 28°C). Temperature fluctuations can affect the accuracy of the assay.

1 Set up **Thin wall, clear 0.5 mL PCR tubes**** for the two standards plus the number of samples you are processing.

2 Make a **Qubit working solution**.

For each standard and sample to be quantified, mix the components in **Table 19** together on a vortex mixer for 2 to 3 seconds.

Table 19 Qubit working solution

Component	Amount
Qubit dsDNA BR reagent	1 µL
Qubit dsDNA BR buffer	199 µL

3 Load 190 µL of **Qubit working solution** into the two **Thin wall, clear 0.5 mL PCR tubes**** labeled for the standards.

4 Load 180 to 199 µL of **Qubit working solution** into the tubes labeled for your samples.

5 Add 10 µL of **Qubit dsDNA BR standard #1** or **Qubit dsDNA BR standard #2** to the appropriate tube.

6 Add 1 to 20 µL of your DNA sample to the appropriate tubes.

7 Mix the content of all the tubes on a vortex mixer for 2 to 3 seconds. Be careful not to create bubbles.

8 Incubate the tubes at room temperature for 2 minutes.

- To calibrate the Qubit:

  a On the home screen of the Qubit 1.0, use the up or down arrow to select **dsDNA Broad Range Assay** as assay type, and then press **GO**. The standard screen is automatically displayed.

  b Select **Run new calibration**, and then press **GO**.

  c Insert the tube with the first standard into the Qubit Fluorometer, close the lid and press **GO**. After the reading is done, remove the standard.

  d Insert the tube with the second standard into the Qubit Fluorometer, close the lid, and press **GO**. After the reading is done remove the standard.

  The calibration is complete after the second standard has been read.

- To measure sample concentration:

  a After the calibration is complete, insert a sample and press **GO**.

  b When the measurement is complete (approximately 5 seconds later), make a note of the reading.

  c The result is displayed on the screen. The number displayed is the concentration of the nucleic acid in the assay tube.

  d Remove the sample from the instrument, insert the next sample, and press **GO**.

e Repeat sample readings until all samples have been read.

f Calculate the concentration of your original sample.

The Qubit Fluorometer gives a value for the Qubit dsDNA BR assay in µg/mL. This value corresponds to the concentration after your samples were diluted into the assay tube. To calculate the concentration of your sample, use this equation:

Sample concentration = *QF value* × (200/*y*)

where

QF value = the value given by the Qubit Fluorometer

y = the volume of sample you added to the assay tube.

UV-VIS Spectrophotometry

1 In the Nanodrop program menu, select **Nucleic Acid Measurement**, and then select **Sample Type** to be **DNA-50**.

2 Use 1.5 µL of **Buffer AE** to blank the instrument.

3 Use 1.5 µL of each gDNA sample to measure DNA concentration. Record the gDNA concentration (ng/µL) for each sample. Calculate the yield as

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

4 Record the $\mathbf{A_{260}/A_{280}}$ and $\mathbf{A_{260}/A_{230}}$ ratios. High-quality gDNA samples have an $\mathbf{A_{260}/A_{280}}$ ratio of 1.8 to 2.0, which indicates the absence of contaminating proteins. Scanning the absorbance from 220-320 nm will show whether contaminants exist that affect absorbance at 260 nm. Check the absorbance scans for a peak at 260 nm and an overall smooth shape as shown in **Figure 2**. The ideal 260/230 ratio for pure DNA is >1.0.

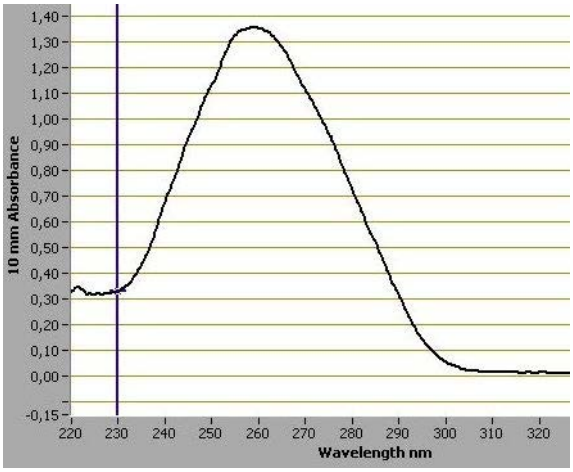


Figure 2. Typical spectrum of pure DNA

Agarose Gel Electrophoresis

1. Load 20 ng of gDNA for each sample in 10 µL of **DNase/RNase-free distilled water** in the well of a single-comb **E-Gel General Purpose Agarose Gels , 1.2%**. (You do not need to add loading buffer in this system).
2. As a control, load 20 ng of **Human Reference DNA (Male and Female)** or commercial **Human Genomic DNA** 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 Plus 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**.

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3 Sample Preparation

This chapter describes the two Agilent recommended options to process gDNA prior to labeling.

You can choose between two methods for sample preparation prior to labeling: **"Direct Method"** on page 36 and **"Amplification Method"** on page 40. **Figure 1** on page 27 and **Figure 3** on page 41 show the respective workflows.

CAUTION

The amplification method is not an option when you process SurePrint G3 CGH+SNP microarrays.

CAUTION

Agilent recommends using a thermal cycler for all sample incubation steps during restriction digestion, labeling, and hybridization preparation. Heat blocks may increase sample-to-sample variability and water baths may fluctuate in temperature, especially at high temperatures. The 98°C incubations, in particular, benefit from the use of a thermal cycler in place of a water bath or heat block.

Direct Method

CAUTION

***For optimal performance, use high quality, intact template gDNA*. If the DNA isolation procedure described in this protocol cannot be followed, make sure that the DNA is free of RNA and protein contamination. If needed, repurify already isolated DNA and start from step 6 on page 29 in the previous chapter.**

Make sure that the gDNA is completely in solution by pipetting up and down. If needed, incubate at 37°C for 30 minutes. If the gDNA concentration is greater than 350 ng/µL, dilute 1:2 in Buffer AE or 1×TE (pH 8.0) and requantitate to make sure quantitation is accurate.

Use the Direct Method if you have at least 0.5 µg (for 1-, 2-, or 4-pack microarrays) or 0.2 µg (for 8-pack microarrays) of starting gDNA. You must use equal amounts of gDNA for both the experimental and reference channels. The required gDNA input amount and volume depends on the microarray format used and whether a restriction digestion is done before the labeling reaction (see **Table 20**).

NOTE

For a wide variety of samples, high quality CGH microarray data is achieved when a restriction digestion step is used before the labeling step. But you can also achieve high quality data if you replace the restriction digestion step by a longer incubation at 98°C after you add the random primers in the labeling reaction step. See **"Step 1. Fluorescent Labeling of gDNA"** on page 48.

If you have 50 ng to <0.5 µg (for 1-, 2- or 4-pack microarrays) or <0.2 µg (for 8-pack microarrays) gDNA, see **"Amplification Method"** on page 40.

Table 20 Requirement of gDNA Input Amount and Volume per Microarray*

Microarray format	gDNA input amount requirement (µg)	Volume of gDNA with restriction digestion (µL)	Volume of gDNA without restriction digestion (µL)
1-pack	0.5 to 1.0	20.2	26
2-pack or 4-pack	0.5 to 1.0	20.2	26
8-pack	0.2 to 0.5	10.1	13

* The gDNA requirement is the same for HD and G3 microarrays (e.g. 0.5 to 1.5 µg for both 4×44K and 4×180K microarrays).

Restriction Digestion with the SureTag or SureTag Complete DNA Labeling Kit

Both the **SureTag Complete DNA Labeling Kit** and the **SureTag DNA Labeling Kit** contain **10× Restriction Enzyme Buffer**, **BSA**, **Alu I** and **Rsa I**.

CAUTION

If a DNA concentration step is required before the restriction digestion, you must avoid carrying over high amounts of salt, EDTA, and contaminants to the restriction digestion reaction.

1 Thaw **10× Restriction Enzyme Buffer** and **BSA** (included in the **SureTag Complete DNA Labeling Kit** and the **SureTag DNA Labeling Kit**). Flick the tube to briefly mix, and spin in a microcentrifuge.

   Store all reagents on ice while in use and return promptly to -20°C.

2 For each reaction, add the amount of gDNA to the appropriate nuclease-free tube or well in the PCR plate and add enough **DNase/RNase-free distilled water** to bring to the final volume listed in **Table 20** on page 36.

3 Prepare the Digestion Master Mix by mixing the components in **Table 21** or **Table 22**, based on the microarray format used, on ice in the order indicated. Mix well by pipetting up and down.

Table 21 Digestion Master Mix (for 1-pack, 2-pack and 4-pack microarrays)

Component	Per reaction (µL)	× 16 rxns (µL) (including excess)	× 48 rxns (µL) (including excess)	× 96 rxns (µL) (including excess)
Nuclease-Free Water	2.0	34	100	200
10× Restriction Enzyme Buffer	2.6	44.2	130	260
BSA	0.2	3.4	10	20
Alu I	0.5	8.5	25	50
Rsa I	0.5	8.5	25	50
Final volume of Digestion Master Mix	**5.8**	**98.6**	**290**	**580**

Table 22 Digestion Master Mix (for 8-pack microarrays)

Component	Per reaction (µL)	× 16 rxns (µL) (including excess)	× 48 rxns (µL) (including excess)	× 96 rxns (µL) (including excess)
Nuclease-Free Water	1	17	50	100
10× Restriction Enzyme Buffer	1.3	22.1	65	130
BSA	0.1	1.7	5	10
Alu I	0.25	4.25	12.5	25
Rsa I	0.25	4.25	12.5	25
Final volume of Digestion Master Mix	**2.9**	**49.3**	**145**	**290**

4 Add 5.8 µL (for 1-, 2-, or 4-pack microarrays) or 2.9 µL (for 8-pack microarrays) of Digestion Master Mix to each reaction tube containing the gDNA to make a total volume of 26 µL (for 1-, 2-, or 4-pack microarrays) or 13 µL (for 8-pack microarrays). Mix well by pipetting up and down.

5 Set the thermal cycler to run the program in **Table 23**. Pre-heat the thermal cycler by starting the program and then pausing it as soon as the thermal block reaches the starting temperature.

Table 23 Restriction digestion of the DNA using a thermal cycler

Step	Temperature	Time
Step 1	37°C	2 hours
Step 2	65°C	20 minutes
Step 3	4°C	hold

6 Transfer the plate or tubes into the thermal cycler, and then start the program.

7 At the end of the thermal cycler program, remove the samples from the thermal cycler. Spin them briefly in a centrifuge to drive the contents off the walls and lid. Put samples on ice.

8 *Optional*. Take 2 µL of the digested gDNA and run on a 0.8% agarose gel stained with SYBR Gold to assess the completeness of the digestion. The majority of the digested products should be between 200 bp and 500 bp.

9 Proceed directly to **"Sample Labeling"** on page 47, or store digested gDNA for up to a month at -20°C.

Do not do the restriction digestion steps in the next section, **"Amplification Method"**.

Amplification Method

CAUTION

For optimal performance, use high quality, intact template gDNA. If the DNA isolation procedure described in this protocol cannot be followed, make sure that the DNA is free of RNA and protein contamination. If needed, repurify already isolated DNA and start from step 6 on page 29 in the previous chapter.

If you choose the amplification method, also amplify the reference DNA and make sure it is at a similar degree of intactness.

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

GenomePlex can be used on degraded samples if the extracted DNA is 500 bp or greater in size. However, greater quantities (up to 100 ng) of damaged DNA are required to get acceptable yield of final product. DNA isolated from FFPE samples is often severely degraded and damaged and is not always suitable for GenomePlex amplification.

Do not use the amplification method to process SurePrint G3 CGH+SNP microarrays.

Use the Amplification Method if you have limited amounts of gDNA. If you have 0.5 µg (for 1-, 2-, or 4-pack microarrays) or 0.2 µg (for 8-pack microarrays) or more gDNA, see **"Direct Method"** on page 36.

Reference

GenomePlex Whole Genome Amplification (WGA) Kit. Technical Bulletin. Sigma-Aldrich. 2006. TR/PHC 06/05-1

Genomic Amplification

The Sigma GenomePlex Whole Genome Amplification (WGA) kit allows you to generate a representative amplification of gDNA. The kit uses a linker mediated primer PCR amplification technology based upon random fragmentation of gDNA and conversion of the resulting small fragments to PCR-amplifiable OmniPlex Library molecules flanked by universal priming sites. The OmniPlex library is then PCR amplified using universal oligonucleotide primers and a limited number of cycles. It is suitable to use with purified gDNA from a variety of sources including fresh frozen tissues and cultured cell lines.

"DNA Isolation" on page 25

"Amplification Method" on page 40

"Sample Labeling" on page 47

"Microarray Processing" on page 57

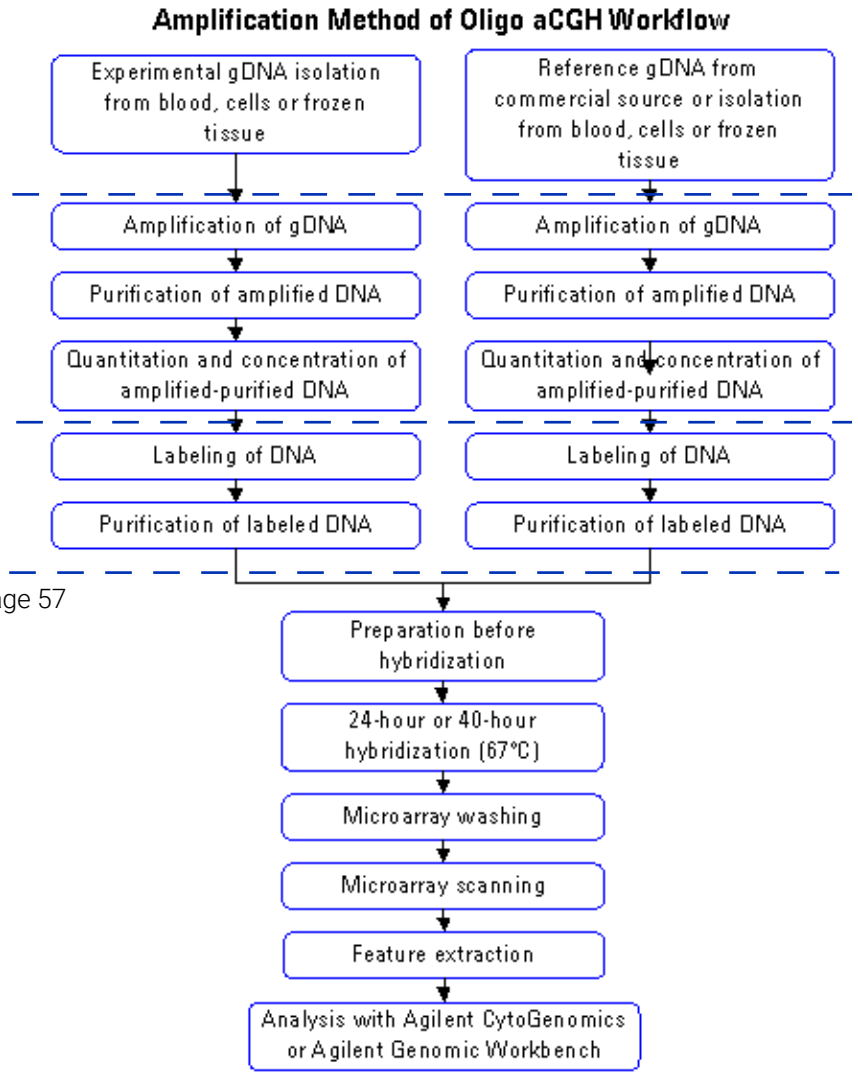


Figure 3. Amplification workflow for sample preparation and microarray processing. Minimum of 50 ng of starting gDNA per sample is required.

This section describes the Agilent recommended procedure to amplify gDNA using the Sigma GenomePlex Whole Genome Amplification (WGA) Kit (p/n WGA2).

Step 1. Fragmentation

1 Add 50 ng of gDNA to a **200-µL Thin-Wall Tube** or plate. Add **DNase/RNase-free distilled water** to bring to a final volume of 10 µL.

2 Add 1 µL of **10× Fragmentation Buffer** to each reaction tube containing the gDNA to make a total volume of 11 µL and mix well by pipetting up and down.

3 Place the tube or plate in a thermal cycler with heated lid at 95°C for *exactly* 4 minutes.

CAUTION **The incubation is very time sensitive. Any deviation may alter results.**

4 Immediately cool the sample on ice, then spin briefly in a centrifuge to drive the contents off the walls and lid.

CAUTION **You must continue to "Step 2. Library Preparation" without interruption. The ends of the library DNA can degrade.**

Step 2. Library Preparation

1 Add 2 µL of **1× Library Preparation Buffer** to each reaction tube.

2 Add 1 µL of **Library Stabilization Solution** to each reaction tube.

3 Mix thoroughly, spin briefly in a centrifuge to drive the contents off the walls and lid and place in a thermal cycler with heated lid at 95°C for 2 minutes.

4 Cool the sample on ice, spin briefly in a centrifuge to drive the contents off the walls and lid, and return to ice.

5 Add 1 µL **Library Preparation Enzyme** to make a total volume of 15 µL. Mix thoroughly, and spin briefly in a centrifuge to drive the contents off the walls and lid.

6 Set the thermal cycler to run the program in **Table 24**.

Table 24 Library Preparation Isothermal Reaction using thermal cycler (total time approximately 1 hour)

Temperature	Time (minutes)
16°C	20
24°C	20
37°C	20
75°C	5
4°C	Hold

7 At the end of the thermal cycler program, remove the samples from the thermal cycler. Spin them briefly in a centrifuge to drive the contents off the walls and lid. Samples may be amplified immediately or stored at -20°C for up to three days.

Step 3. Amplification

1 Prepare the **Amplification Master Mix** by mixing the components in **Table 25** on ice.

Table 25 Amplification Master Mix

	Volume (µL)	x16 rxns (µL) including excess	x48 rxns (µL) including excess	×96 rxns (µL) including excess
10× Amplification Master Mix	7.5	127.5	375	750
Nuclease-Free Water	47.5	807.5	2,375	4,750
WGA DNA Polymerase	5	85	250	500
Final volume of Amplification Master Mix	**60**	**1,020**	**3,000**	**6,000**

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 thoroughly, spin briefly in a centrifuge to drive the contents off the walls and lid, and place the samples in a thermal cycler with heated lid.

4 Set the thermal cycler to run the program in **Table 26**.

Table 26 PCR Amplification (total time approximately 2 hours)

Step	Temperature	Time
Initial Denaturation	95°C	3 minutes
Do 14 cycles as follows:		
Denature	94°C	15 seconds
Anneal/Extend	65°C	5 minutes
After cycling	4°C	hold

5 At the end of the thermal cycler program, remove the samples from the thermal cycler. Spin them briefly in a centrifuge to drive the contents off the walls and lid.

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

Step 4. Purification of PCR products

Use **GenElute PCR Clean-Up Kit** for the purification of amplified DNA.

1 Before using for the first time, dilute the **Wash Solution Concentrate** with 48 ml of 100% **Ethanol**.

2 Insert a **GenElute plasmid mini spin column** (with a blue O-ring) into a **2 mL Collection Tube**, if not already assembled. Add 0.5 mL of the **Column Preparation Solution** to each **GenElute plasmid mini spin column** and spin in a centrifuge at 12,000 × g for 30 seconds to 1 minute. Discard the eluate, but keep the **2 mL Collection Tube**.

   The **Column Preparation Solution** maximizes binding of the DNA to the membrane resulting in more consistent yields.

3 Add 375 µL of **Binding Solution** to each 75 µL sample. Transfer the solution into the **GenElute plasmid mini spin column**. Spin the column in a centrifuge at maximum speed (12,000 to 16,000 × g) for 1 minute. Discard the eluate, but keep the **2 mL Collection Tube**.

4 Place the binding column into the same collection tube. Apply 0.5 mL of diluted **Wash Solution Concentrate** to the column and spin in a centrifuge at

maximum speed for 1 minute. Discard the eluate, but keep the **2 mL Collection Tube**.

5 Place the column into the same collection tube. Spin the column in a centrifuge at maximum speed for 2 minutes, without any additional wash solution, to remove excess ethanol. Discard any residual eluate as well as the **2 mL Collection Tube**.

6 Transfer the column to a fresh **2 mL Collection Tube**. Apply 50 µL of **Elution Solution** to the center of each **GenElute plasmid mini spin column**. Incubate at room temperature for 1 minute.

7 To elute the DNA, spin the **GenElute plasmid mini spin column** in a centrifuge at maximum speed for 1 minute.

The PCR amplification product is now present in the eluate and is ready for quantitation and labeling without restriction enzyme digestion. The final amplified DNA can be stored at -20°C.

Step 5. Quantitation of Amplified-Purified DNA

Quantitate amplified-purified DNA using the NanoDrop ND-1000 UV-VIS Spectrophotometer or equivalent.

1 Select **Nucleic Acid Measurement**, then select **Sample Type** to be **DNA-50**.

2 Use 1.5 µL of **Elution Solution** to blank the instrument.

3 Use 1.5 µL of each purified DNA to measure DNA concentration. Record the DNA concentration (ng/µL) for each sample.

4 Calculate the amplification yield (µg) as

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

Step 6. Preparation of Amplified-Purified DNA before Labeling

1 Add 2 µg of amplified-purified DNA to a **1.5-mL RNase-free Microfuge Tube** or well in the PCR plate and bring to a final volume of 26 µL (1-, 2-, or 4-pack

microarrays) or 13 µL (8-pack microarrays) with **DNase/RNase-free distilled water**.

Both the experimental and reference channels require equal amounts of amplified-purified DNA for the subsequent labeling reaction.

2 If the DNA sample volume exceeds 26 µL (for 1-pack, 2-pack, or 4-pack microarrays) or 13 µL (for 8-pack microarrays), concentrate the amplified-purified DNA using a vacuum concentrator (such as a Speed Vac).

You can concentrate the DNA to dryness and resuspend in **DNase/RNase-free distilled water**. Do not excessively dry the DNA because the pellets will become difficult to resuspend.

Proceed directly to **"Sample Labeling"** on page 47 or store amplified-purified DNA at -20°C. No restriction digestion step is needed.

4 Sample Labeling

The **SureTag Complete DNA Labeling Kit** and **SureTag DNA Labeling Kit** contain sufficient two-color labeling reaction reagents for:

- 25 1-pack, 2-pack, or 4-pack microarrays *or*
- 50 8-pack microarrays

Both the **SureTag Complete DNA Labeling Kit** and the **SureTag DNA Labeling Kit** contain 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 gDNA 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 gDNA

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 thermal cycler.**

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

2 Add **Random Primers**:

- For 1-pack, 2-pack, and 4-pack microarrays, add 5 µL of **Random Primers** to each reaction tube containing 26 µL of gDNA to make a total volume of 31 µL (or 24 µL of gDNA to make a total volume of 29 µL if the optional agarose gel step on **page 38** was done). Mix well by pipetting up and down gently.
- For 8-pack microarrays, add 2.5 µL of **Random Primers** to each reaction tube that contains 13 µL of gDNA to make a total volume of 15.5 µL (or 11 µL of gDNA to make a total volume of 13.5 µL if the optional agarose gel step on **page 38** was done). Mix well by pipetting up and down gently.

3 Set the thermal cycler to run the program in **Table 27**. Pre-heat the thermal cycler by starting the program and then pausing it as soon as the thermal block reaches the starting temperature.

Table 27 DNA denaturation and fragmentation using a thermal cycler

Step	Temperature	Time (with restriction digestion)	Time (without restriction digestion)
Step 1	98°C	3 minutes	10 minutes
Step 2	4°C	hold	hold

4 Transfer the plate or tubes into the thermal cycler, and then start the program.

5 At the end of the thermal cycler program, remove the samples from the thermal cycler. Spin the samples in a centrifuge for 1 minute at 6,000 × g to drive the contents off the walls and lid.

6 For 1-pack, 2-pack and 4-pack microarrays:

a Mix the components in **Table 28** on ice in the order indicated to prepare one cyanine-3 and one cyanine-5 **Labeling Master Mix**.

Table 28 Labeling Master Mix

Component	Per reaction (µL)	× 8 rxns (µL) (including excess)	× 24 rxns (µL) (including excess)	× 48 rxns (µL) (including excess)
Nuclease-Free Water	2.0*	17*	50*	100*
5× Reaction Buffer	10.0	85	250	500
10× dNTPs	5.0	42.5	125	250
Cyanine 3-dUTP *or* **Cyanine 5-dUTP**	3.0	25.5	75	150
Exo (-) Klenow	1.0	8.5	25	50
Final volume of Labeling Master Mix	**19.0 or 21.0***	**161.5 or 178.5***	**475 or 525***	**950 or 1050***

* Do *not* add **Nuclease-Free Water** if you skipped the optional agarose gel step (**step 8** on **page 38**).

b Add 19 µL (or 21 µL) of **Labeling Master Mix** to each reaction tube containing the gDNA to make a total volume of 50 µL. Mix well by gently pipetting up and down.

7 For 8-pack microarrays:

a Mix the components in **Table 29** on ice in the order indicated to prepare one cyanine-3 and one cyanine-5 **Labeling Master Mix**.

Table 29 Labeling Master Mix (for 8-pack microarrays)

Component	Per reaction (µL)	× 8 rxns (µL) (including excess)	× 24 rxns (µL) (including excess)	× 48 rxns (µL) (including excess)
Nuclease-Free Water*	2.0*	17*	50*	100*
5× Reaction Buffer	5.0	42.5	125	250
10× dNTPs	2.5	21.25	62.5	125
Cyanine 3-dUTP *or* **Cyanine 5-dUTP**	1.5	12.75	37.5	75

Table 29 **Labeling Master Mix** (for 8-pack microarrays)

Component	Per reaction (µL)	× 8 rxns (µL) (including excess)	× 24 rxns (µL) (including excess)	× 48 rxns (µL) (including excess)
Exo (-) Klenow	0.5	4.25	12.5	25
Final volume of Labeling Master Mix	**9.5 or 11.5***	**80.75 or 97.75***	**237.5 or 287.5***	**475 or 575***

* Do *not* add **Nuclease-Free Water** if you skipped the optional agarose gel step (**step 8** on **page 38**).

b Add 9.5 µL (or 11.5 µL) of **Labeling Master Mix** to each reaction tube that contains the gDNA to make a total volume of 25 µL. Mix well by gently pipetting up and down.

8 Set the thermal cycler to run the program in **Table 30**. Pre-heat the thermal cycler by starting the program and then pausing it as soon as the thermal block reaches the starting temperature.

Table 30 **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

9 Transfer the plate or tubes into the thermal cycler, and then start the program.

10 At the end of the thermal cycler program, remove the samples from the thermal cycler. Spin them briefly in a centrifuge to drive the contents off the walls and lid. Put samples on ice.

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

Step 2. Purification of Labeled gDNA

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

- column
- 2-mL collection tube

For high-throughput, labeled gDNA can be purified using individual **AutoScreen A, 96-well plate** from GE Healthcare. See **"GE Healthcare 96-Well Plates High-Throughput Option"** on page 53.

NOTE

Keep cyanine-3 and cyanine-5 labeled gDNA samples separated throughout this clean-up step.

Agilent Purification Columns

1. Spin the labeled gDNA 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 gDNA sample to be purified, place a **column** into a **2-mL collection tube** and label the **column** appropriately. Load each labeled gDNA 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. Add **1×TE (pH 8.0)**, or use a concentrator to bring the sample volume to that listed in **Table 31**. Do not excessively dry the gDNA 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 gDNA 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 55. Refer to **Table 33** on page 56 for expected yield of labeled gDNA and specific activity after labeling and clean-up, when starting with high quality gDNA.

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 listed in **Table 31**. Use the appropriate container listed in **Table 31**.

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

Table 31 Sample volume and total mixture volumes

Microarray	Cyanine 3 or Cyanine 5 sample volume after purification	Total mixture volume after Nanodrop and combining	Container
1-pack	80.5 µL	158 µL	**1.5-mL RNase-free Microfuge Tube** or **200-µL Thin-Wall Tube**
2-pack	41 µL	79 µL	**1.5-mL RNase-free Microfuge Tube**, **200-µL Thin-Wall Tube**, or **Tall Chimney PCR plate**
4-pack	21 µL	39 µL	**1.5-mL RNase-free Microfuge Tube**, **200-µL Thin-Wall Tube**, **Tall Chimney PCR plate**, or **96-well PCR plate**
8-pack	9.5 µL	16 µL	**1.5-mL RNase-free Microfuge Tube** **200-µL Thin-Wall Tube**, **Tall Chimney PCR plate**, or **96-well PCR plate**

GE Healthcare 96-Well Plates High-Throughput Option

NOTE

Use the same centrifuge speed and length for all three spinning steps (**step 4**, **step 7** and **step 11**). If you spin only one plate, make sure that you counterbalance.

For 1-pack, 2-pack, and 4-pack microarray samples, you can use two wells of the **AutoScreen A, 96-well plate** per sample, or concentrate the samples down to 25 µL with a vacuum concentrator (such as a Speed Vac).

1 Get two **96-well PCR plate**s. Label one each as:
   - wash plate
   - collection plate

   The **wash plate** can be reused in next experiments.

1 Remove the **AutoScreen A, 96-well plate** from the foil storage pouch.

   If the **AutoScreen A, 96-well plate** were stored at 4°C, allow them to equilibrate to ambient temperature before use (approximately two hours).

2 Carefully remove the top and bottom seal of the **AutoScreen A, 96-well plate**.

   Once the bottom seal is removed, keep the **AutoScreen A, 96-well plate** on top of a **wash plate**. Do not allow the bottom surface to come in contact with laboratory bench top liners, wipes, or other materials.

3 Place the purification plates in reusable **wash plate**s.

4 Spin the purification plates in a centrifuge for 5 minutes at 910 × g.

5 Discard the flow-through from the **wash plate**s, and place the **AutoScreen A, 96-well plate** back to the same **wash plate**s.

6 Add 150 µL of **DNase/RNase-free distilled water** to the **AutoScreen A, 96-well plate**.

7 Spin again in a centrifuge for 5 minutes at 910 × g.

8 Discard the flow-through.

9 Transfer the **AutoScreen A, 96-well plate** to the sample **collection plate**.

10 Add labeled gDNA to the **AutoScreen A, 96-well plate**:
   - For 1-pack, 2-pack, 4-pack microarray samples that were not concentrated to 25 µL, add 2×25 µL labeled gDNA to two separate wells.
   - For 1-pack, 2-pack, 4-pack microarray samples that were concentrated to 25 µL with concentrator and for 8-pack microarray samples, add 1×25 µL labeled gDNA to one well.

11 Spin in a centrifuge for 5 minutes at 910 × g to collect the purified labeled gDNA in the sample collection plate. The volume per sample will be approximately 20 µL.

12 For 1-pack, 2-pack and 4-pack microarray samples that were not concentrated prior to purification, combine the duplicate samples for a total volume of approximately 40 µL.

13 Take 1.5 µL of each sample to determine the yield and specific activity. See **"To determine yield, and degree of labeling or specific activity"** on page 55.

Refer to **Table 33** on page 56 for expected yield of labeled gDNA and specific activity after labeling and clean-up, when starting with high quality gDNA.

14 Combine the test and reference sample using the appropriate cyanine 5-labeled sample and cyanine-3-labeled sample. Use the appropriate container listed in **Table 32**. Add **1×TE (pH 8.0)** or use a concentrator to bring to the Total Mixture Volume in **Table 32**.

If needed, you can concentrate the combined cyanine 5- and cyanine 3-labeled gDNA mixture to dryness and resuspend in **1×TE (pH 8.0)** to the final volume listed in **Table 32**. Do not excessively dry the samples because the pellets will become difficult to resuspend.

Table 32 Total mixture volumes

Microarray	Treatment prior to purification	Cy3 or Cy5 sample volume after purification	Volume after NanoDrop and combining	1× TE volume	Total mixture volume	Container
1-pack	without vacuum concentration*	40 µL	77 µL	81 µL	158 µL	**1.5-mL RNase-free Microfuge Tube**
	with vacuum concentration	20 µL	37 µL	121 µL	158 µL	**1.5-mL RNase-free Microfuge Tube**
2-pack	without vacuum concentration*	40 µL	77 µL	2 µL	79 µL	**1.5-mL RNase-free Microfuge Tube** or **Tall Chimney PCR plate**
	with vacuum concentration	20 µL	37 µL	42 µL	79 µL	**1.5-mL RNase-free Microfuge Tube** or **Tall Chimney PCR plate**
4-pack	without vacuum concentration*	40 µL	77 µL	0 µL†	39 µL	**1.5-mL RNase-free Microfuge Tube**, **Tall Chimney PCR plate**, or **96-well PCR plate**

Table 32 Total mixture volumes (continued)

Microarray	Treatment prior to purification	Cy3 or Cy5 sample volume after purification	Volume after NanoDrop and combining	1× TE volume	Total mixture volume	Container
	with vacuum concentration	20 µL	37 µL	2 µL	39 µL	**1.5-mL RNase-free Microfuge Tube**, **Tall Chimney PCR plate**, or **96-well PCR plate**
8-pack	without vacuum concentration	20 µL	37 µL	0 µL†	16 µL	**1.5-mL RNase-free Microfuge Tube**, **Tall Chimney PCR plate**, or **96-well PCR plate**

* You will use 2 wells of the purification plate per sample.

† Concentrate the sample to the volume indicated in the Total Mixture Volume column.

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 gDNA 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 gDNA:

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

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

*pmol dyes per µg gDNA

The Specific Activity is Degree of Labeling divided by 0.034.

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

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

Refer to **Table 33** for expected yield of labeled gDNA and specific activity after labeling and purification, when starting with high quality gDNA.

Table 33 Expected Yield and Specific Activity after Labeling and Clean-up

Input gDNA (µg)	Yield (µg)	Specific Activity of Cyanine 3 and Cyanine 5 Labeled Sample (pmol/µg)
0.2*	3 to 6	15 to 50
0.5	8 to 13	20 to 60
1	9 to 14	20 to 60

* Half labeling reaction for 8-pack microarrays (half the amount of random primers, dye, enzyme, and dNTPs)

If you replaced the restriction digestion step by a longer incubation at 98°C, the specific activity of cyanine 3 and -5 labeled sample will be about 5 pmol/µg lower than the values indicated in **Table 33**. The Yield after labeling and the signal to noise of the microarrays will be the same.

The cyanine-3 and cyanine-5 yield after labeling should be the same. If not, refer to **"Troubleshooting"** on page 89.

5 Microarray Processing

Microarray processing consists of hybridization, washing, scanning, and analysis.

Microarray Hybridization

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 **""Secure Fit" Slide Box Opening Instructions"** on page 100 and **"Microarray Handling Tips"** on page 102.

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

Reconstituted 10× Blocking Agent is stable for 120 days when stored at -20°C and can be frozen and thawed up to three times.

Step 2. Prepare labeled gDNA for hybridization

1 Mix the components according to the microarray format to prepare the Hybridization Master Mix.

Table 34 Hybridization Master Mix for 1-pack microarray

Component	Volume (µL) per hybridization	× 8 rxns (µL) (including excess)	× 24 rxns (µL) (including excess)	× 48 rxns (µL) (including excess)
Cot-1 DNA (1.0 mg/mL)*	50	425	1,250	2,500
10× aCGH Blocking Agent†	52	442	1,300	2,600
2× HI-RPM Hybridization Buffer†	260	2,210	6,500	13,000
Final Volume of Hybridization Master Mix	362	3,077	9,050	18,100

* Use **Cot-1 DNA (1.0 mg/mL)** from the appropriate species.

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

Table 35 Hybridization Master Mix for 2-pack microarray

Component	Volume (µL) per hybridization	× 8 rxns (µL) (including excess)	× 24 rxns (µL) (including excess)	× 48 rxns (µL) (including excess)
Cot-1 DNA (1.0 mg/mL)*	25	212.5	625	1,250
10× aCGH Blocking Agent†	26	221	650	1,300
2× HI-RPM Hybridization Buffer†	130	1,105	3,250	6,500
Final Volume of Hybridization Master Mix	181	1,538.5	4,525	9,050

* Use **Cot-1 DNA (1.0 mg/mL)** from the appropriate species.

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

Table 36 Hybridization Master Mix for 4-pack microarray

Component	Volume (µL) per hybridization	× 8 rxns (µL) (including excess)	× 24 rxns (µL) (including excess)	× 48 rxns (µL) (including excess)
Cot-1 DNA (1.0 mg/mL)*	5	42.5	125	250
10× aCGH Blocking Agent†	11	93.5	275	550
2× HI-RPM Hybridization Buffer†	55	467.5	1,375	2,750
Final Volume of Hybridization Master Mix	71	603.5	1,775	3,550

* Use **Cot-1 DNA (1.0 mg/mL)** from the appropriate species.

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

Table 37 Hybridization Master Mix for 8-pack microarray

Component	Volume (µL) per hybridization	× 8 rxns (µL) (including excess)	× 24 rxns (µL) (including excess)	× 48 rxns (µL) (including excess)
Cot-1 DNA (1.0 mg/mL)*	2	17	50	100
10× aCGH Blocking Agent†	4.5	38.25	112.5	225
2× HI-RPM Hybridization Buffer†	22.5	191.25	562.5	1,125
Final Volume of Hybridization Master Mix	29	246.5	725	1,450

* Use **Cot-1 DNA (1.0 mg/mL)** from the appropriate species.

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

2 Add the appropriate volume of the Hybridization Master Mix to the **1.5-mL RNase-free Microfuge Tube**, **200-µL Thin-Wall Tube**, **Tall Chimney PCR plate** well or **96-well PCR plate** well that contains the labeled gDNA to make the total volume listed in **Table 38**.

Table 38 Volume of Hybridization Master Mix per hybridization

Microarray format	Volume of Hybridization Master Mix	Total volume
1-pack	362 µL	520 µL
2-pack	181 µL	260 µL

Table 38 Volume of Hybridization Master Mix per hybridization (continued)

Microarray format	Volume of Hybridization Master Mix	Total volume
4-pack	71 µL	110 µL
8-pack	29 µL	45 µL

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

4 Set the thermal cycler to run the program in **Table 39**. Pre-heat the thermal cycler by starting the program and then pausing it as soon as the thermal block reaches the starting temperature.

Table 39 Thermal cycler program

Step	Temperature	Time
Step 1	98°C	3 minutes exactly
Step 2	37°C	30 minutes

5 Transfer the plate or tubes into the thermal cycler, and then start the program.

6 At the end of the thermal cycler program, remove the samples from the 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 by extending the 37°C incubation step on the thermal cycler or by keeping the samples in a 37°C preheated oven until ready for hybridization.

Step 3. Prepare the hybridization assembly

Refer to the *Agilent Microarray Hybridization Chamber User Guide* (publication G2534-90004) 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**.

Before you begin, make sure you read and understand **""Secure Fit" Slide Box Opening Instructions"** on page 100 and **"Microarray Handling Tips"** on page 102.

Remove gasket slide from its packaging

NOTE

- Do not remove gasket slide from protective sleeve until ready for use.
- Do not slice or cut open the gasket slide protective packaging.
- Handle only the edges of the gasket slide.
- Prior to use, inspect gasket slides for visible gaps or cuts through the gaskets or any debris within the hybridization areas as these are indications of instability. Do not use gasket slides that have these features.

1 With tweezers, carefully lift up the corner of the clear plastic covering and slowly pull back the protective film.

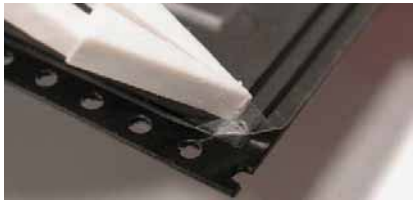


Figure 4. Removal of clear plastic covering

2 With clean, powder-free gloved fingers, remove the gasket slide from its package. Handle the slide only on its edges.

To avoid any potential contamination from surrounding surface materials, immediately insert the gasket slide in the chamber base using the instructions below.

Insert the gasket slide into the chamber base

1 Hold the gasket slide so that the barcode label is facing towards you. This side of the slide is the gasket side.

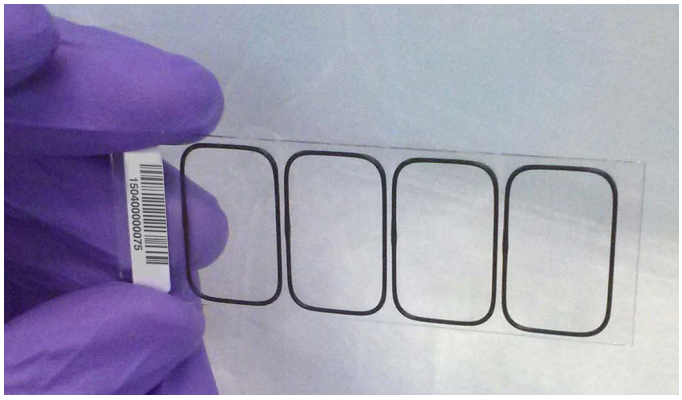


Figure 5. Gasket slide, gasket side

2 Locate the four chamber base guideposts and rectangular barcode guide in the chamber base.

3 Position the gasket slide between the 4 chamber base guide posts (see **Figure 6**) with the barcode label resting over the base's rectangular barcode guide.

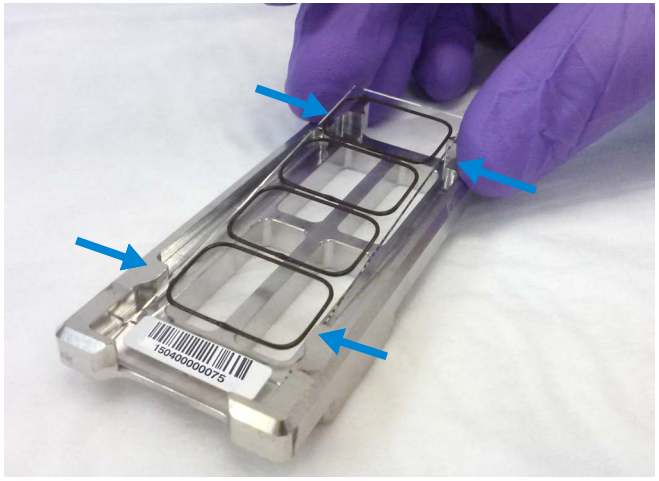


Figure 6. Chamber base, guide posts denoted with arrows

4 Gently place the gasket slide into the chamber base.

5 Make sure the gasket slide rests flush against the chamber base. Re-adjust to a flush position against the chamber base if needed.

Figure 7. Correct positioning of gasket slide in chamber base

Load the sample

1 Slowly dispense the appropriate volume of hybridization sample mixture onto the gasket well in a "drag and dispense" manner (described below). For multi-pack microarray formats (2-pack, 4-pack or 8-pack microarray), load all gasket wells before you add the microarray slide.

- 490 µL (for 1-pack microarray)
- 245 µL (for 2-pack microarray)
- 100 µL (for 4-pack microarray)
- 40 µL (for 8-pack microarray)

The "drag and dispense" method helps to distribute the sample evenly across the surface of the well and avoids spillover of sample over the gasket edge. Start with the pipette tip near the top edge of the well. *Do not directly touch the gasket or the glass with the pipette tip*. Then, dispense the mixture while you move your pipette tip to the opposite end of the well so that the sample is distributed across the well space. Avoid creating large air bubbles as you dispense the mixture as they could lead to spillover.

This image is for demonstration purposes only. Always put the gasket slide in the chamber base before you dispense the hybridization sample mixture.

Figure 8. Drag and dispense method – Start dispensing when the pipette tip is near the top of the well. Finish dispensing when the pipette tip is near the bottom of the well.

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.**

Add the microarray slide

1 Remove a microarray slide from the slide storage box between your thumb and index finger, *numeric barcode side facing up and Agilent label facing down*.

2 Use the four chamber base guideposts and rectangular end of the base to position the microarray slide as you lower it to within 3 mm (1/8”) above the gasket slide, making sure the microarray slide is not tilted with respect to the gasket slide. Barcode ends of both the gasket slide and the microarray slide must line up at the corners of the chamber base. Once positioned, gently rest the microarray slide on the lower gasket slide. Refer to **Figure 9** for proper technique on holding the microarray slide with both hands.

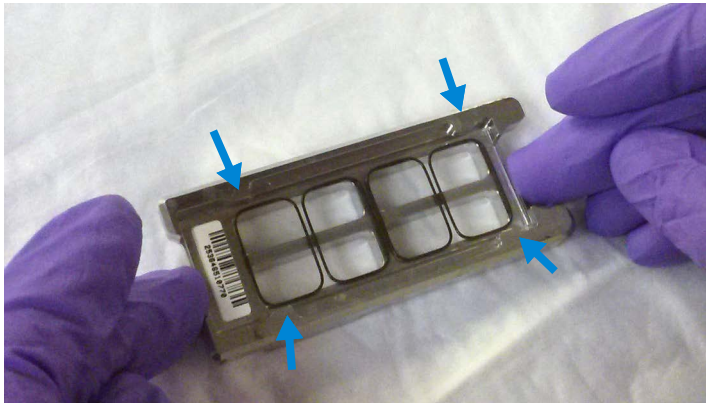


Figure 9. Chamber base with gasket and microarray slide applied, guide posts denoted with arrows

CAUTION **Do not drop the microarray slide onto the gasket slide as this increases the chances of sample mixing between gasket wells.**

Once placed, do not attempt to move the chamber and sandwiched slides as this can cause leakage of the hybridization solution.

Assemble the chamber

1 Place the chamber cover, correct side facing up, onto the chamber base which contains the “sandwiched” slides.

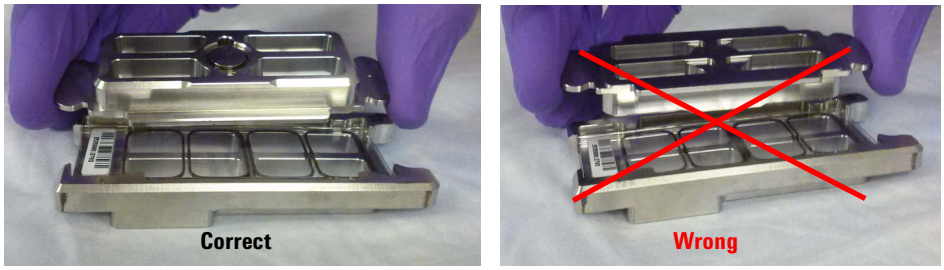


Figure 10. Chamber cover in correct (left) and incorrect (right) orientations

2 From the rounded corner of the chamber base, slip the clamp onto the chamber base and cover until it stops firmly in place, resting at the center of the two pieces.

Keep the chamber assembly flat on the lab bench to avoid spilling the hybridization solution.

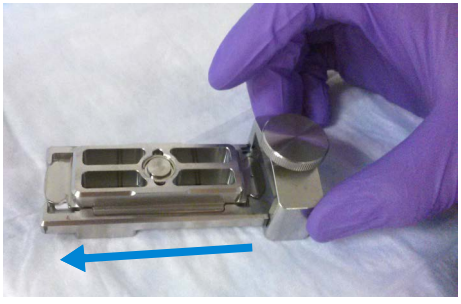


Figure 11. Slipping the clamp onto the chamber base

3 Firmly tighten the thumbscrew fully.

The slides will not be harmed by hand-tightening.

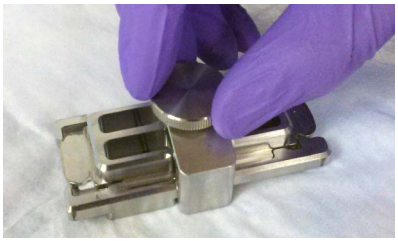


Figure 12. Tightening of the thumbscrew on the clamp

CAUTION

If you do not completely tighten the thumbscrew, hybridization solution can leak out during hybridization.

Do not use tools to tighten the thumbscrew. The use of pliers or other tools can damage the parts and will void the warranty.

4 Rotate the final assembled chamber in a *vertical orientation*, clockwise, 2 to 3 times to wet the gaskets (see **Figure 13**).

Rotation helps ensure that the hybridization solution will coat the entire surface of the microarray during the incubation process.

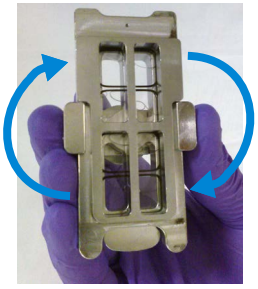


Figure 13. Rotation of the final assembled chamber

5 Inspect for good bubble formation.

- Hold the chamber vertically and inspect for stray or small bubbles that do not move as you rotate the chamber.
- Use the "large mixing bubble" to dislodge small stray or stationary bubbles.
- If the small stray or stationary bubbles persist, gently tap the assembled chamber on a firm surface. Rotate the chamber on its sides as you tap. Inspect again and repeat if needed until the small stray or stationary bubbles dissipate.

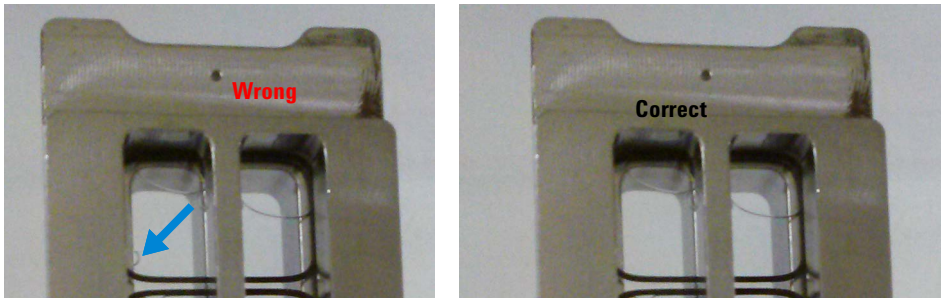


Figure 14. The slide on the left shows a stray, stationary bubble (denoted with arrow), which must be removed before hybridization. The slide on the right shows only large mixing bubbles, which move freely around the chamber when rotated. Bubbles are acceptable, as long as they move freely when you rotate the chamber.

Step 4. Hybridize

1 Load the assembled chamber into the oven rotator rack, starting from the center of the rack (position 3 or 4 when counting from left to right). Refer to the figure below for correct and incorrect orientations.

Figure 15. Assembled chambers in correct (left) and incorrect (middle and right) orientations

2 Close the door and set the rotator speed to 20 rpm.

3 Hybridize at 67°C for:

- 24 hours (4-pack and 8-pack microarrays)
- 40 hours (1-pack and 2-pack microarrays)

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* (publication G2545-90002) for more information.**

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 71.

Microarray Wash

NOTE

The microarray wash procedure must be done in environments where ozone levels are 5 ppb or less. For Scanner C and Scanner B, 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.

Before you begin, determine which wash procedure to use:

Table 40 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 73	No
> 5 ppb < 10 ppb	**"Wash Procedure A (without Stabilization and Drying Solution)"** on page 73	Yes
> 10 ppb	**"Wash Procedure B (with Stabilization and Drying Solution)"** on page 77	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 77.

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 41 lists the wash conditions for the Wash Procedure A without **Stabilization and Drying Solution**.

Table 41 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.

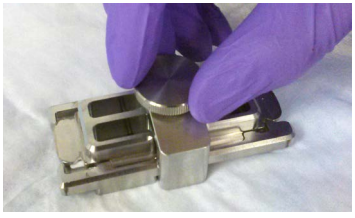


Figure 16. Loosening of the thumbscrew

   b Slide off the clamp assembly and remove the chamber cover.

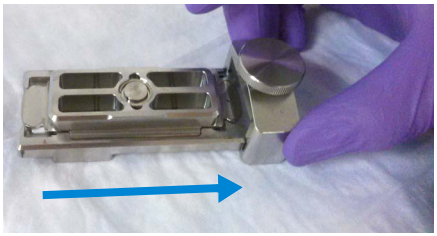


Figure 17. Removal of the clamp

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!*

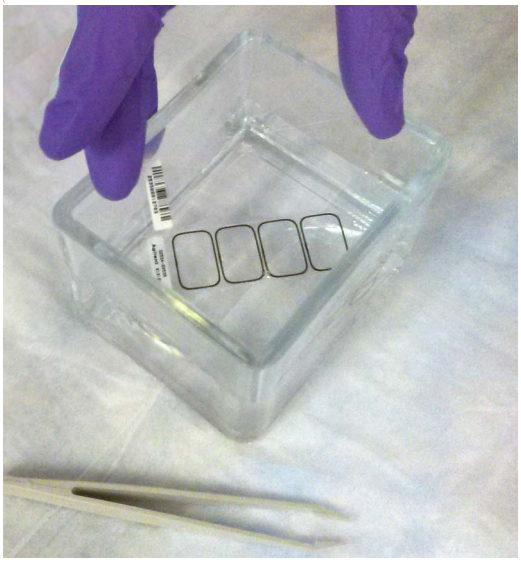


Figure 18. Removal of the microarray slide from the staining dish

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 42 lists the wash conditions for the Wash Procedure B with **Stabilization and Drying Solution**.

Table 42 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 73.

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.

CAUTION Fingerprints cause errors in the fluorescence detection. Touch only the edges of the slide and always use gloves when handling slides.

For SureScan microarray scanner

1. Before you insert the slide into a slide holder, inspect the slide holder for any dust or fingerprints. If found, remove the dust or fingerprints with compressed air or a soft, dust-free cloth. If the slide holder is scratched, worn, or damaged, has a lid that does not close tightly, or has a hinge that does not move freely, discard the slide holder and select a different one.
2. Place the slide holder on a flat surface, with the clear cover facing up, and the tab on the right. This helps to ensure that you have the slide aligned properly when you insert it into the slide holder.
3. Gently push in and pull up on the tabbed end of the clear plastic cover to open the slide holder.

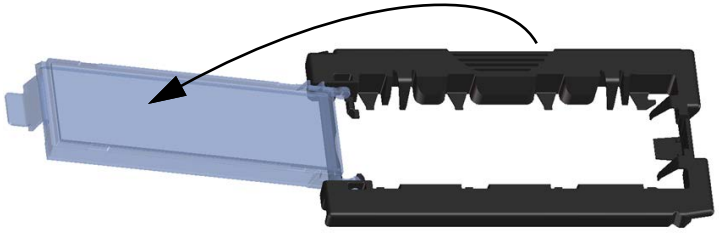


Figure 19. Opening the slide holder

4. Insert the slide into the holder.
   a. Hold the slide at the barcode end and position the slide over the open slide holder. Make sure that the active microarray surface faces up with the barcode on the left, as shown in **Figure 20**.
   b. Carefully place the end of the slide without the barcode label onto the slide ledge. See **Figure 20**.

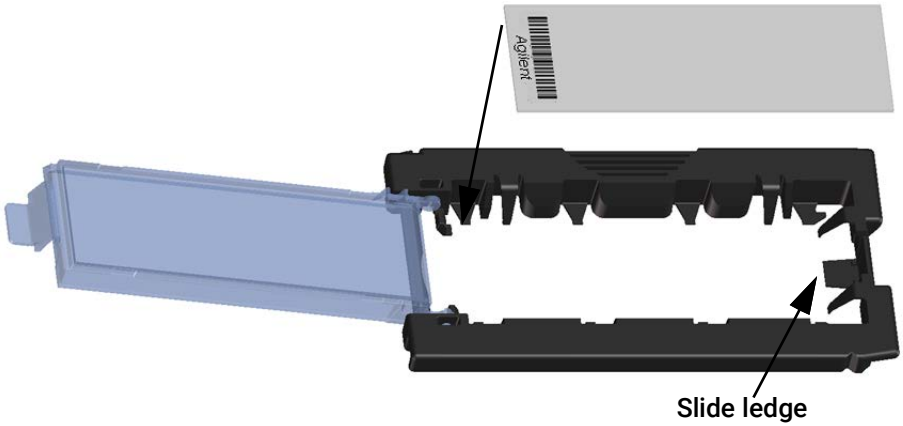


Figure 20. Inserting slide into the slide holder

c Gently lower the slide into the slide holder. See **Figure 21**.

d Close the plastic slide cover, pushing on the tab end until you hear it "click." This moves the slide into position in the holder.

CAUTION An improperly inserted slide can damage the scanner.

e Gently push in and pull up on the tabbed end of the clear plastic cover to open it again and verify that the slide is correctly positioned.

f Close the plastic slide cover, gently pushing on the tab end until you hear it "click". See **Figure 22**. Make sure that the slide holder is completely closed.

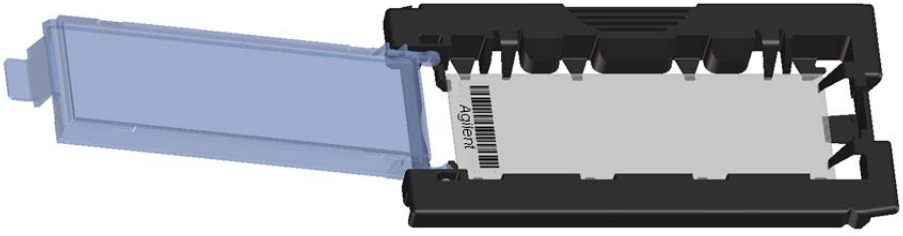


Figure 21. Slide inserted in slide holder – cover open

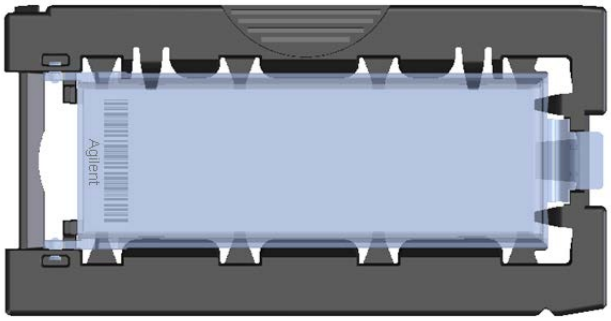


Figure 22. Slide inserted in slide holder – cover closed

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 23**. Refer to the *Agilent Ozone-Barrier Slide Cover User Guide* (publication G2505-90550), included with the slide cover, for more information.

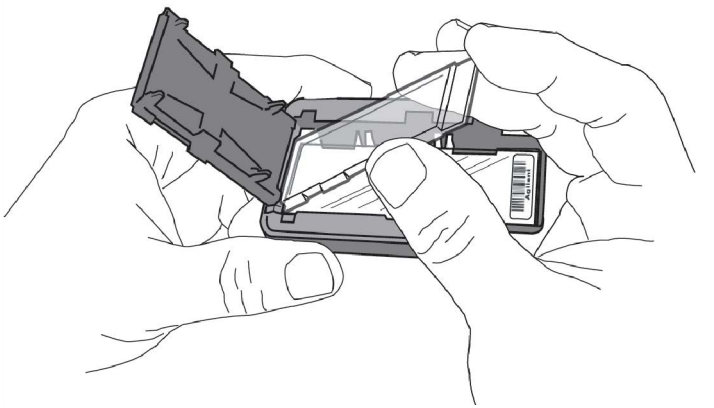


Figure 23. Inserting the ozone-barrier slide cover

- 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" (publication G1662-90043) for scanner compatibility and settings.

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. Refer to **Figure 24** and **Figure 25**.

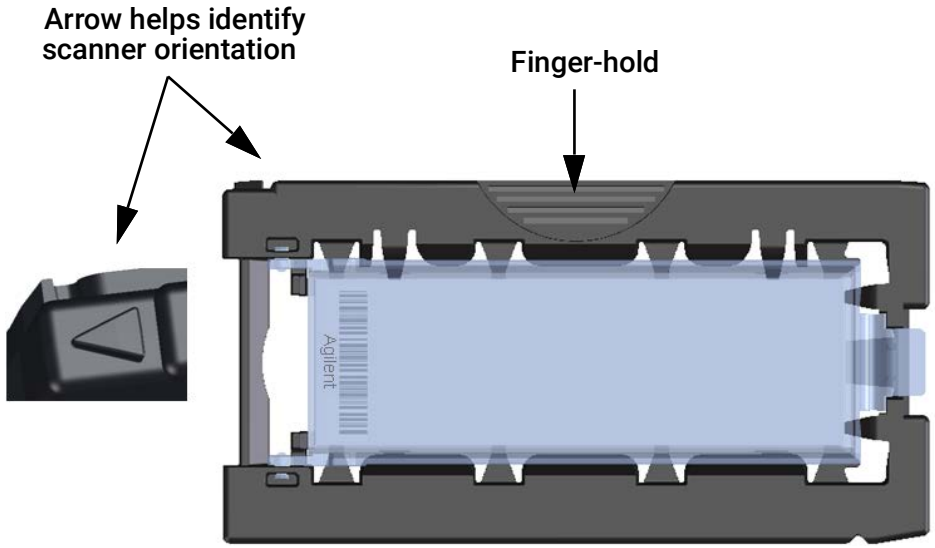


Figure 24. Slide holder helps you to insert slides correctly

Figure 25. Inserting slide holder into cassette

2 Select **Protocol AgilentG3_CGH** for G3 microarrays. Select **Protocol AgilentHD_CGH** for HD microarrays.

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** for G3 microarrays. Select **Profile AgilentHD_CGH** for HD microarrays.

4 Verify scan settings. See **Table 43**.

Table 43 C Scanner Scan Settings

	For HD Microarray Formats	For G3 Microarray Formats
Dye channel	R+G (*red and green*)	R+G (*red and green*)
Scan region	Agilent HD (61 x 21.6 mm)	Agilent HD (61 x 21.6 mm)
Scan resolution	5 µm	3 µm
Tiff file dynamic range	16 bit	16 bit

Table 43 C Scanner Scan Settings (continued)

	For HD Microarray Formats	For G3 Microarray Formats
Red PMT gain	100%	100%
Green PMT gain	100%	100%
XDR	<No 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.

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.

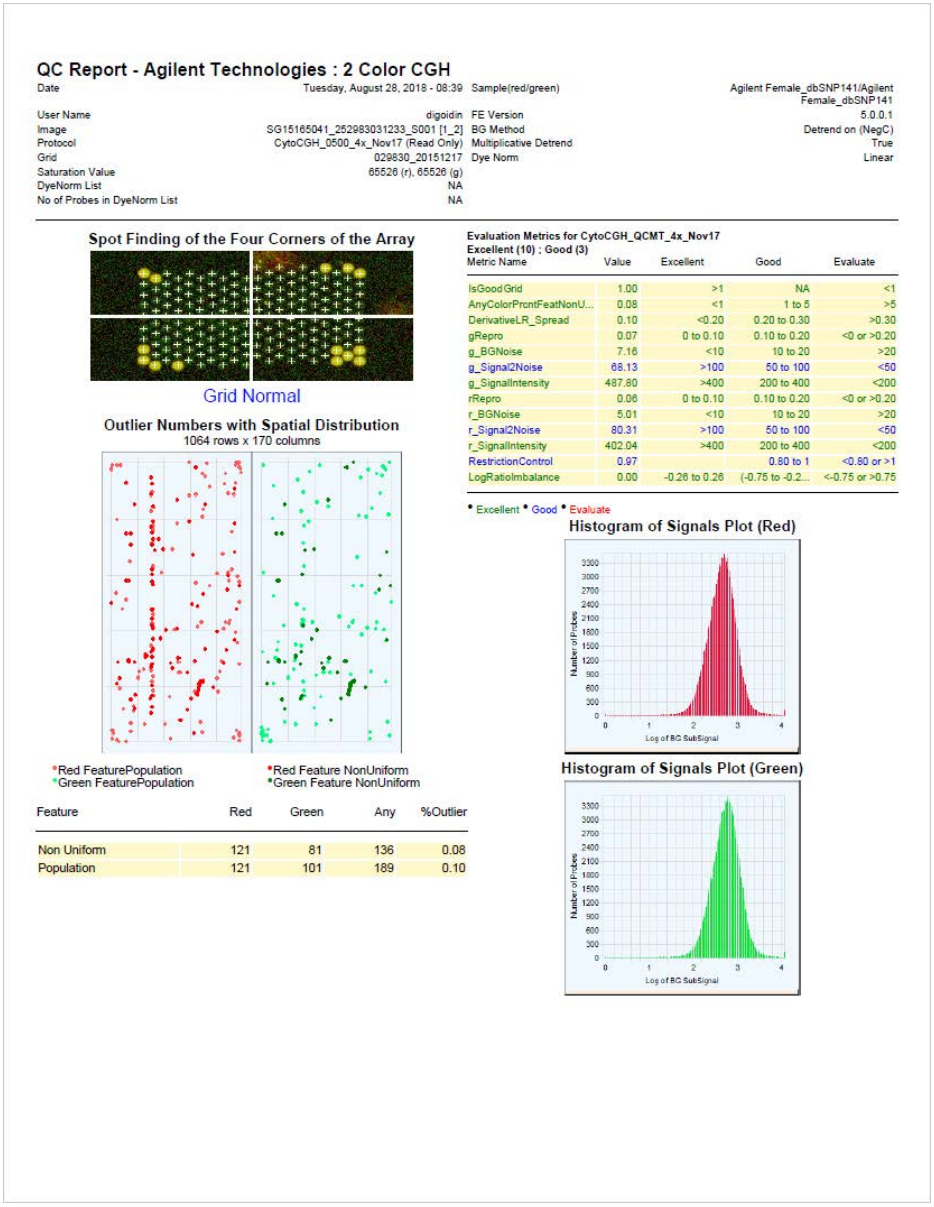
Microarray QC Metrics for high DNA quality samples

These metrics are only appropriate for high-quality DNA 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 the microarray format (1-pack, 2-pack, 4-pack or 8-pack), biological sample source, quality of starting gDNA, 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.

To achieve a high SNP call rate and accuracy when processing SurePrint G3 CGH+SNP microarrays, make sure the DLRSD is <0.2.

Table 44 QC metric thresholds for Enzymatic labeling

Metric	Excellent	Good	Evaluate
BGNoise	< 10	10 to 20	> 20
Signal Intensity	> 150	50 to 150	< 50
Signal to Noise	> 100	30 to 100	< 30
Reproducibility	< 0.05	0.05 to 0.2	> 0.2
DLRSD	< 0.2	0.2 to 0.3	> 0.3



QC Report - Agilent Technologies : 2 Color CGH

Date	Tuesday, August 28, 2018 - 08:39	Sample(red/green)	Agilent Female_dbSNP141/Agilent Female_dbSNP141
User Name	digoidin	FE Version	5.0.0.1
Image	SG15165041_252983031233_S001 [1_2]	BG Method	Detrend on (NegC)
Protocol	CytoCGH_0500_4x_Nov17 (Read Only)	Multiplicative Detrend	True
Grid	029830_20151217	Dye Norm	Linear
Saturation Value	65526 (r), 65526 (g)		
DyeNorm List	NA		
No of Probes in DyeNorm List	NA		

Spot Finding of the Four Corners of the Array

Grid Normal

Outlier Numbers with Spatial Distribution
1064 rows x 170 columns

*Red FeaturePopulation
*Green FeaturePopulation
*Red Feature NonUniform
*Green Feature NonUniform

Feature	Red	Green	Any	%Outlier
Non Uniform	121	81	136	0.08
Population	121	101	189	0.10

Evaluation Metrics for CytoCGH_QCMT_4x_Nov17
Excellent (10) ; Good (3)

Metric Name	Value	Excellent	Good	Evaluate
IsGoodGrid	1.00	>1	NA	<1
AnyColorPrcntFeatNonU...	0.08	<1	1 to 5	>5
DerivativeLR_Spread	0.10	<0.20	0.20 to 0.30	>0.30
gRepro	0.07	0 to 0.10	0.10 to 0.20	<0 or >0.20
g_BGNoise	7.16	<10	10 to 20	>20
g_Signal2Noise	68.13	>100	50 to 100	<50
g_SignalIntensity	487.80	>400	200 to 400	<200
rRepro	0.06	0 to 0.10	0.10 to 0.20	<0 or >0.20
r_BGNoise	5.01	<10	10 to 20	>20
r_Signal2Noise	80.31	>100	50 to 100	<50
r_SignalIntensity	402.04	>400	200 to 400	<200
RestrictionControl	0.97		0.80 to 1	<0.80 or >1
LogRatioImbalance	0.00	-0.26 to 0.26	(-0.75 to -0.2...	<-0.75 or >0.75

• Excellent • Good • Evaluate

Histogram of Signals Plot (Red)

Histogram of Signals Plot (Green)

Figure 26. Feature Extraction QC Report, page 1

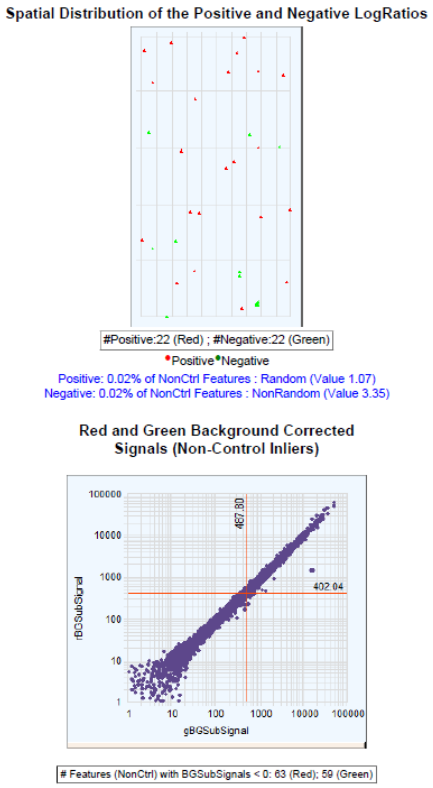


Figure 27. Feature Extraction QC Report, page 2

Step 3. Set up Analysis Workflow in CytoGenomics

- Set up Analysis Workflow in the CytoGenomics program to analyze your data.

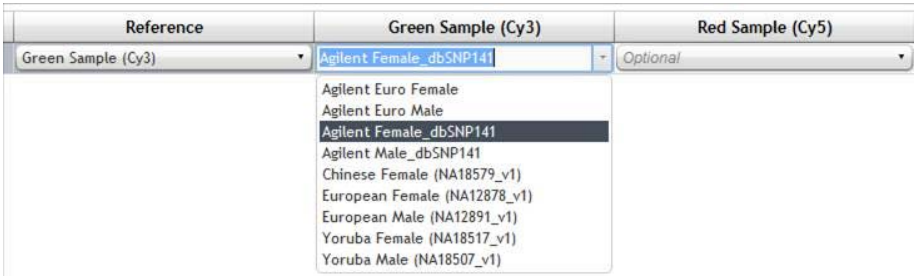
  You must use **Agilent Female_dbSNP141** or **Agilent Male_dbSNP141** as the **Reference** for:

  - **GenetiSure Cancer Research CGH+SNP, 2×400K** microarrays
  - **GenetiSure Postnatal Research CGH+SNP, 2×400K†** microarrays
  - All custom microarrays designed after December 15, 2015

  Use only CytoGenomics 3.0.6 or higher. Older versions of CytoGenomics and Agilent Genomic Workbench do not support the GenetiSure microarrays or the newer SNP content.

  For all other designs, you can also use the older **Agilent Euro Female** or **Agilent Euro Male** files as the Reference.

  The dbSNP141 files contain all of the active SNP probes of the Euro files, plus 50,000 new probes.



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 you have a low OD260/230 or OD260/280 value

A low OD260/230 value can indicate contaminants, such as residual salt or organic solvents. A low OD260/280 value indicates residual protein. Either condition can result in low specific activity (pmol dye/µg DNA) or Degree of Labeling. See **"To determine yield, and degree of labeling or specific activity"** on page 55.

Repurify the DNA using the Qiagen DNeasy protocol. See **"DNA Isolation"** on page 25. This procedure includes a proteinase K treatment.

If you must do a phenol/chloroform DNA extraction, do not get too close to the interface.

Phenol has an absorbance maximum of 270–275 nm, which is close to that of DNA. Phenol contamination mimics both higher purity and higher yields, because of an upward shift in the OD260 value.

Make sure to calibrate the spectrophotometer with the appropriate buffer.

If you have poor sample quality due to residual RNA

The input amount of DNA for the experimental sample must be the same as for the reference sample. RNA absorbs at the same wavelength as DNA. Therefore, RNA-contaminated sample can result in a DNA overestimation.

Repurify the DNA using the Qiagen DNeasy protocol. See **"DNA Isolation"** on page 25. This procedure includes a RNase A treatment.

If you get poor sample quality due to degradation

For non-FFPE samples: On a 1 to 1.5% agarose gel, intact gDNA should appear as a compact, high-molecular weight band with no lower molecular weight smears. Degraded DNA results in biased labeling.

Check DNA on a 1 to 1.5% agarose gel. If DNA that was isolated from cells, blood or frozen tissue is degraded, then repurify the DNA using the Qiagen DNeasy protocol. See **"DNA Isolation"** on page 25.

If you replace the restriction digestion step by a longer incubation at 98°C, make sure that the DNA is not over-fragmented prior to labeling. Possible causes are incorrect temperature or length of incubation at 98°C, or evaporation (use a thermal cycler with heated lid). Make sure most of the heat fragmented products are between 1000 and 3000 bases in length.

For processing FFPE samples, refer to the *Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis (ULS Labeling for Blood, Cells, Tissues or FFPE) Protocol* (publication G4410-90020). FFPE samples are not supported when you process SurePrint G3 CGH+SNP microarrays.

If the estimated concentration is too high or low

The input amount of DNA for the experimental sample must be the same as for the reference sample. Precipitated DNA or DNA that is at a very high concentration cannot be quantitated accurately.

Contaminants such as organic solvents and RNA also absorb at 260 nm, which results in an inaccurate DNA quantitation.

Make sure that the gDNA is completely in solution by pipetting up and down. If needed, incubate at 37°C for 30 minutes. If the gDNA concentration is > 350 ng/µL, dilute 1:2 in **Buffer AE** or **1×TE (pH 8.0)**. Quantitate again to make sure quantitation is accurate.

Different DNA isolation methods can create different quantitation artifacts, the risk of assay noise is higher when the experimental and reference DNA samples are isolated from different sources. If you used a spectrophotometer (such as the Nanodrop) for the initial measurement, also use a double-stranded DNA-based fluorometer (such as the Qubit) to verify.

If needed, repurify the DNA using the Qiagen DNeasy protocol. See **"DNA Isolation"** on page 25.

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 or use a plate **Heat Sealer** to avoid evaporation.

Make sure that the pipettors are not out of calibration.

Make sure that the gDNA, 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 gDNA 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 gDNA"** on page 51 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 67°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 or B, 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 77.

If you have high BGNoise values

High BGNoise can cause lower signal-to-noise values (see **Table 44** 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* (publication 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 44** 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.

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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.

DNeasy Blood & Tissue Kit

DNeasy Mini Spin Column
2 mL Collection Tube
Buffer ATL
Buffer AL
Buffer AW1
Buffer AW2
Buffer AE
Proteinase K

Qubit dsDNA BR Assay Kit

Qubit dsDNA BR reagent
Qubit dsDNA BR buffer
Qubit dsDNA BR standard #1
Qubit dsDNA BR standard #2

SureTag Complete DNA Labeling Kit and SureTag DNA Labeling Kit

Human Reference DNA (Male and 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 Primers

* Included in the **SureTag Complete DNA Labeling Kit** only.

GenomePlex Complete Whole Genome Amplification Kit

1× Library Preparation Buffer
10× Fragmentation Buffer
Control Human Genomic DNA
Library Preparation Enzyme
10× Amplification Master Mix
Library Stabilization Solution
WGA DNA Polymerase
Nuclease-Free Water

GenElute PCR Clean-Up Kit

Column Preparation Solution
Binding Solution
Wash Solution Concentrate
Elution Solution
GenElute plasmid mini spin column
2 mL Collection Tube

Oligo aCGH/ChIP-on-chip Hybridization Kit*

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

Oligo aCGH/ChIP-on-chip Wash Buffer Kit

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

"Secure Fit" Slide Box Opening Instructions

Agilent now ships all microarray slides in a newly designed "secure fit" slide box. The instructions below describe how to remove the slide box from the shipping pouch, how to open the slide box, and how to properly remove a microarray slide.

1 Use scissors to cut below the seal and remove box from its foil pouch.

After breaking foil on microarray pouch, store microarray slides in the slide box at room temperature (in the dark) under a vacuum desiccator or nitrogen purge box.

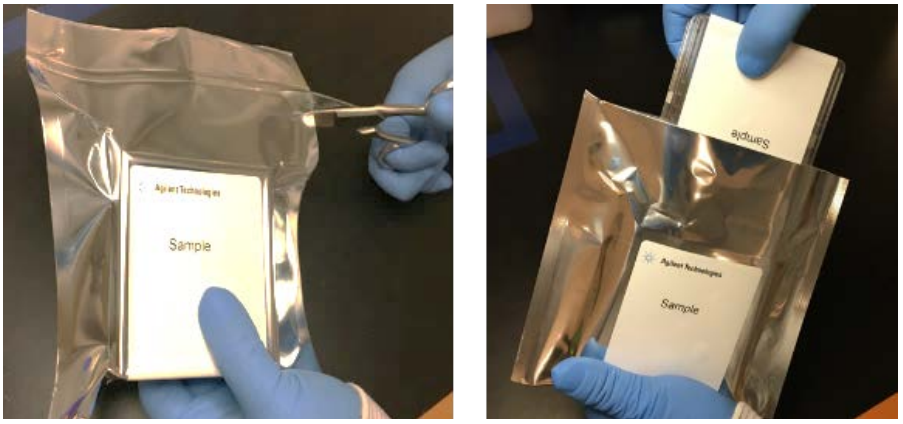

Figure 28. Opening foil pouch (left) and removing the slide box (right)

2 Place the slide box on a flat surface. While stabilizing the box from the top with one hand, use a sharp edge to cut the sealing tape on both sides of the box before opening.

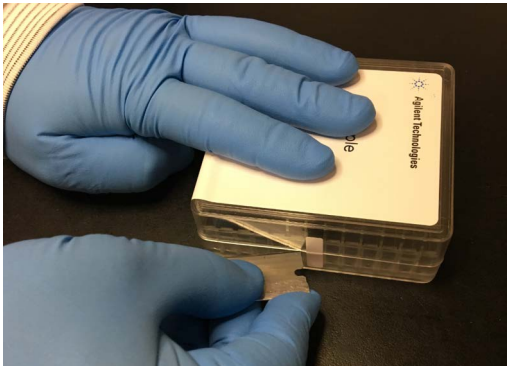

Figure 29. Cutting the sealing tape

3 With one hand, firmly hold the base of the box on the sides with the indentations (or dimples) for added grip.

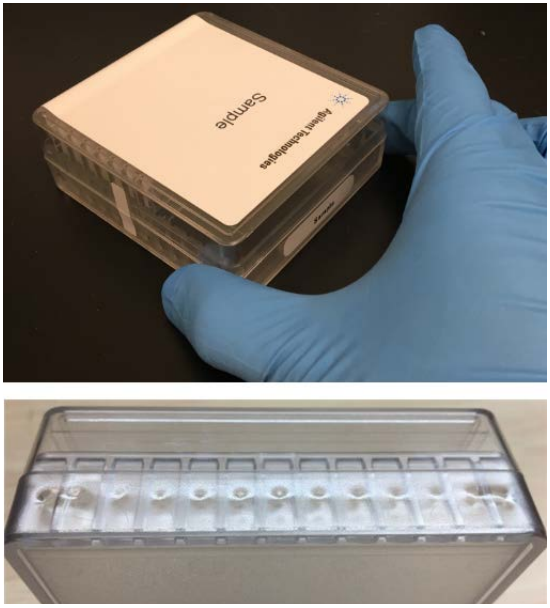

Figure 30. Gripping the base at the indentations (top) and close-up of the indentations (bottom)

4 Using your free hand, grasp the lid and gently lift it away from the base as if it is hinged. Set the lid aside.

Depending on your preference, you can reverse the hand positions so that the left hand holds the base while the right hand grasps the lid.

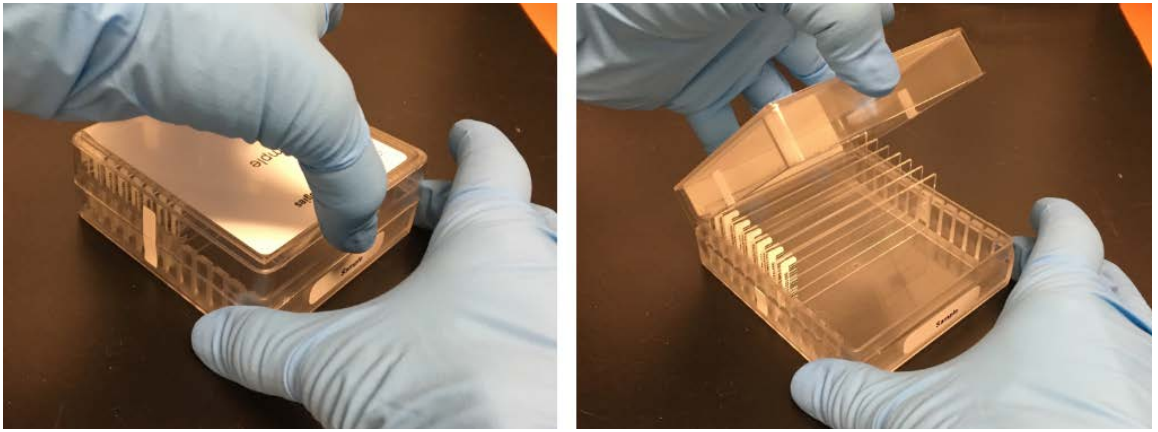

Figure 31. Grasping the lid (left) and lifting the lid from the base (right)

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

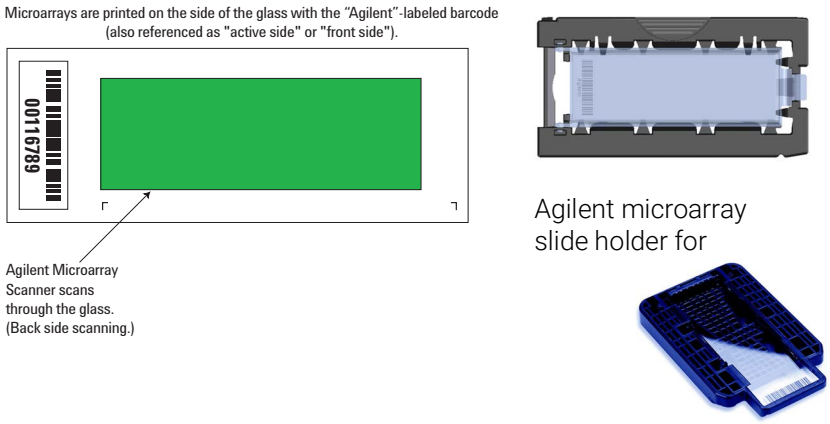

Figure 32. Agilent microarray slide and slide holder

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 or Scanner B). 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 32 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 33**).

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

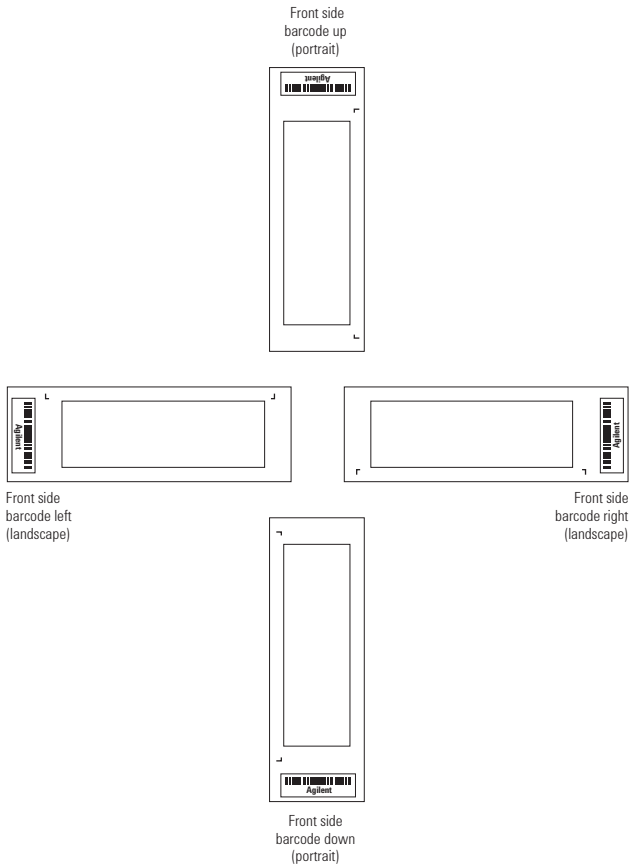

Figure 33. Microarray slide orientation

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**
BARCODE	**Sample:**	**Sample:**

Barcode Number _____

Figure 34. 2-pack microarray slides

Arrays

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

Barcode Number _____

Figure 35. 4-pack microarray slides

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 36. 8-pack microarray slide

In This Book

This guide contains information to run the Oligonucleotide Array-Based CGH for Genomic DNA Analysis – Enzymatic Labeling for Blood, Cells, or Tissues (with a High Throughput option) protocol.

www.agilent.com

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