Oligonucleotide Array-Based CGH for Genomic DNA Analysis

Bravo Automated Liquid Handling Platform with Enzymatic and ULS Labeling

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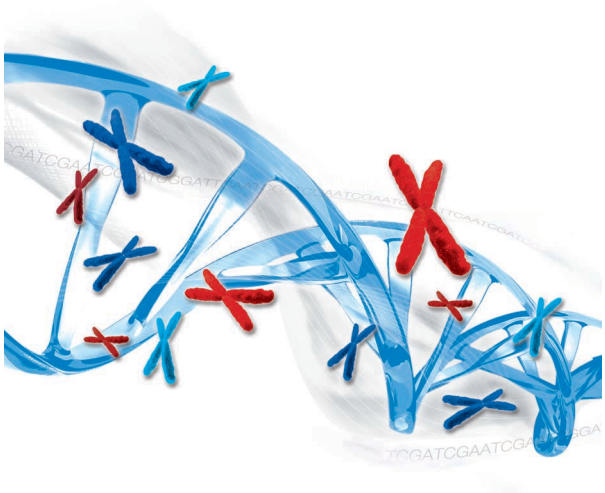
Version 3.0, April 2025



Notices

Manual Part Number

G4410-90040 Revision C0
April 2025

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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 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 use the Bravo Automated Liquid Handling Platform for the labeling (ULS and enzymatic), purification and pre-hybridization steps.

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, frozen tissues, or FFPE samples prior to labeling.

3 Sample Preparation for Bravo Platform

This chapter describes how to prepare the samples to be used on the Bravo Automated Liquid Handling Platform.

4 Sample Labeling on the Bravo Platform

This chapter describes the fragmentation, labeling (ULS and enzymatic), clean-up and preparation of labeled gDNA for hybridization done on the Bravo platform.

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. It also tells you how to get the Agilent Reference Genotype Files (Male and Female) for use in Agilent CytoGenomics and Agilent Genomic Workbench.

6 Troubleshooting

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

7 Reference

This chapter contains reference information related to the labeling kits and the protocol.

What's new in 3.0

- 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 128.
- Expanded instructions and new images in the **Microarray Processing** procedures to help avoid common problems and optimize hybridization of your sample to the microarray.
- Updated web addresses for Agilent materials.
- Updated **Safety Notes**.
- Removed microarray scanning instructions for the Agilent B scanner.
- Update to number of slides that can be processed with the Oligo aCGH/ChIP-on-chip Hybridization Kit. See **Table 20** on page 22.

What's new in 2.3

- Added reference to compatibility matrix for non-Agilent scanners.
- Updated product labeling statement.

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, frozen tissues or FFPE samples.
- If the DNA isolation procedure described in this document cannot be followed, make sure that the DNA is free of RNA and protein contamination. For ULS labeling, also make sure the DNA is in one of the following buffers compatible with ULS labeling:
  - TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5 or pH 8)
  - 10 mM LiCl
  - 10 to 100 mM Na acetate
  - 10 mM NaCl
- 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.
- Agilent-CGHblock 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

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 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†

* 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)
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

Table 3 Catalog CGH Microarray Kits - Human (continued)

Part Number	Description
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

Table 4 Catalog CNV Microarray Kits - Human

Part Number	Description
G4506A	SurePrint G3 Human CNV Microarray Kit 1×1M (5 slides)
G4824A-023642	SurePrint G3 Human CNV Microarray Slide 1×1M
G4507A	SurePrint G3 Human CNV Microarray Kit 2×400K (5 slides)
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
G4417A	Human CNV Association Microarray Kit 2×105K (5 slides)
G4425B-022837	Human CNV Association Microarray Slide 2×105K

Table 5 Catalog CGH Microarrays- Mouse

Part Number	Description
G4838A	SurePrint G3 Mouse CGH Microarray Kit 1×1M (5 slides)
G4824A-027414	SurePrint G3 Mouse CGH Microarray Slide 1×1M
G4839A	SurePrint G3 Mouse CGH Microarray Kit 4×180K (3 slides)
G4826A-027411	SurePrint G3 Mouse CGH Microarray Kit 4×180K
G4415A	Mouse Genome CGH Microarray Kit 1×244K (5 slides)
G4423B-014695	Mouse Genome CGH Microarray Slide 1×244K
G4416A	Mouse Genome CGH Microarray Kit 2×105K (5 slides)
G4425B-014699	Mouse Genome CGH Microarray Slide 2×105K

Table 6 Catalog CGH Microarrays - Rat

Part Number	Description
G4840A	SurePrint G3 Rat CGH Microarray Kit 1×1M (5 slides)
G4824A-027065	SurePrint G3 Rat CGH Microarray Slide 1×1M
G4841A	SurePrint G3 Rat CGH Microarray Kit 4×180K (3 slides)
G4826A-027064	SurePrint G3 Rat CGH Microarray Slide 4×180K
G4435A	Rat Genome CGH Microarray Kit 1×244K (5 slides)
G4423B-015223	Rat Genome CGH Microarray Slide 1×244K
G4436A	Rat Genome CGH Microarray Kit 2×105K (5 slides)
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
For Enzymatic Labeling: Bravo Automated Liquid Handling Platform with 96 Channel Disposable LT Pipetting Head, Riser and 2 CPAC Ultraflat heated/cooled Deck Pads	Agilent p/n G5409A Option 002
For ULS labeling: Bravo Automated Liquid Handling Platform with 96 Channel Disposable LT Pipetting Head	Agilent p/n G5409A Option 003
Robotic Pipetting Tips (250 µL)	Agilent p/n 19477-002
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 65°C for ULS and 67°C for Enzymatic Labeling	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
PlateLoc Thermal Microplate Sealer *and* Peelable Aluminum Seal *or* Heat Sealer Peel-it-lite Foil (removable)	Agilent p/n G5402A *and* Agilent p/n 24210-001 Eppendorf p/n 591000010 *and* Eppendorf p/n 0030127854
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

Table 11 Required equipment (continued)

Description	Vendor and part number
Thermal cycler with heated lid	Eppendorf p/n 950000015 or equivalent
Full skirted 96-well PCR plate (compatible with Bravo platform and thermal cycler)	Eppendorf p/n 951020401 or equivalent
Microcentrifuge	Eppendorf p/n 5430 or equivalent
Centrifuge (for 96-well plate)	Eppendorf p/n 5810 or equivalent
384-Well Deep Well Microplates	Greiner Bio-One p/n 781270
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
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
Deep reservoir, 12 column partitions, V bottom, natural polypropylene	V&P Scientific p/n VP 572DC
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 set to 37°C, 56°C, and 90°C (for DNA extraction)	
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.

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

Table 12 Required equipment for hybridization preparation for 1-pack microarrays.

Description	Vendor and part number
Deep-well plate	ABgene p/n AB-0859
Eppendorf ThermoStat *or* BioShake iQ Thermomixer	Eppendorf p/n 022670204 Q.Instruments p/n 1808-0506
Eppendorf Deep-Well Plate Block	Eppendorf p/n 022670565
Adapter for Deep-Well Plates	Q.Instruments p/n 1808-1141

Table 13 Required equipment for hybridization preparation for 2-pack microarrays.

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

Table 14 Required equipment for 4-pack and 8-pack microarrays.

Description	Vendor and part number
Vacuum Concentrator	Thermo Scientific Savant SpeedVac p/n DNA130-115 or equivalent

Table 15 Optional equipment for DNA extraction from tissue or FFPE samples.

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

Required Reagents

Table 16 Required reagents for gDNA isolation

Description	Vendor and part number
Phosphate Buffered Saline pH 7.4 (PBS)	Amresco p/n E504-500ML
E-Gel General Purpose Agarose Gels, 1.2%	Thermo Fisher Scientific p/n G551801
SYBR Gold Nucleic Acid Gel Stain	Thermo Fisher Scientific p/n S11494
SYBR photographic filter	Thermo Fisher Scientific p/n S7569
TrackIt 1 Kb DNA Ladder	Thermo Fisher Scientific p/n 10488085
DNase/RNase-free distilled water	Thermo Fisher Scientific p/n 10977-015
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
Sodium thiocyanate (NaSCN)†	Sigma-Aldrich 467871-50G
Ethanol (95% to 100% molecular biology grade)	Sigma-Aldrich p/n E7023-6×500ML
Tween 20†	Sigma-Aldrich p/n P9416-50ML

* Optional.

† Optional components if isolating DNA from FFPE samples.

Table 17 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
AutoScreen A, 96-well plates	GE Healthcare Life Sciences p/n 25-9005-98
1×TE (pH 8.0), Molecular grade	Thermo Fisher Scientific p/n AM9849

* Kit content is listed in **"Reagent Kit Components"** on page 126.

† Included in the **SurePrint G3 CGH Bundle**.

Table 18 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
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

* Kit content is listed in **"Reagent Kit Components"** on page 126.

Table 19 Required reagents for ULS sample prep and labeling*

Description	Vendor and part number
Genomic DNA High-Throughput ULS Labeling Kit	Agilent p/n 5190-0450
Genomic DNA 96-well Purification Module	Agilent p/n 5190-0451
Oligo aCGH Labeling Kit	Agilent p/n 5190-0420
DNase/RNase-free distilled water	Thermo Fisher Scientific p/n 10977015
For possible use as a reference sample: • Human Genomic DNA *or* • Mouse Genomic DNA *or* • Rat Genomic DNA	 • Promega p/n G1521 (female) or p/n G1471 (male) • Jackson Labs p/n 000664 (female and male) • Harlan Sprague Dawley (custom)

* ULS is not supported for CGH+SNP.

Table 20 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 24 slides) or p/n 5188-5380 (minimum 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 18440-016 Applied Genetics p/n RHB
DNase/RNase-free distilled water	Thermo Fisher Scientific p/n 10977015
Milli-Q ultrapure water	Millipore
Acetonitrile*	Sigma-Aldrich p/n 271004-1L

* Optional components recommended if wash procedure B is selected.

Required Bravo Platform Protocols

- The Agilent Oligo aCGH Bravo Platform protocols are on the CD that came with your Bravo platform or may be obtained by contacting your local Agilent product specialist.

Required Hardware and Software

- Refer to the *Bravo Platform User Guide* and the *VWorks Automation Control User Guide* for minimum and recommended computer requirements. Go to **www.agilent.com**.
- 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

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**.

This chapter describes the Agilent recommended procedure for isolating gDNA from blood, cells, frozen or FFPE tissues using the **DNeasy Blood & Tissue Kit**. Minor differences exist in the DNA extraction procedure between the ULS and Enzymatic processing method that you will choose in Chapter 4.

FFPE samples are not supported when you process SurePrint G3 CGH+SNP microarrays.

For DNA isolated from FFPE tissues, ULS processing is the only option. Follow the instructions in **"FFPE Tissues"** on page 33.

NOTE

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

"DNA Isolation" on page 23

"Sample Preparation for Bravo Platform" on page 37

"Microarray Processing" on page 89

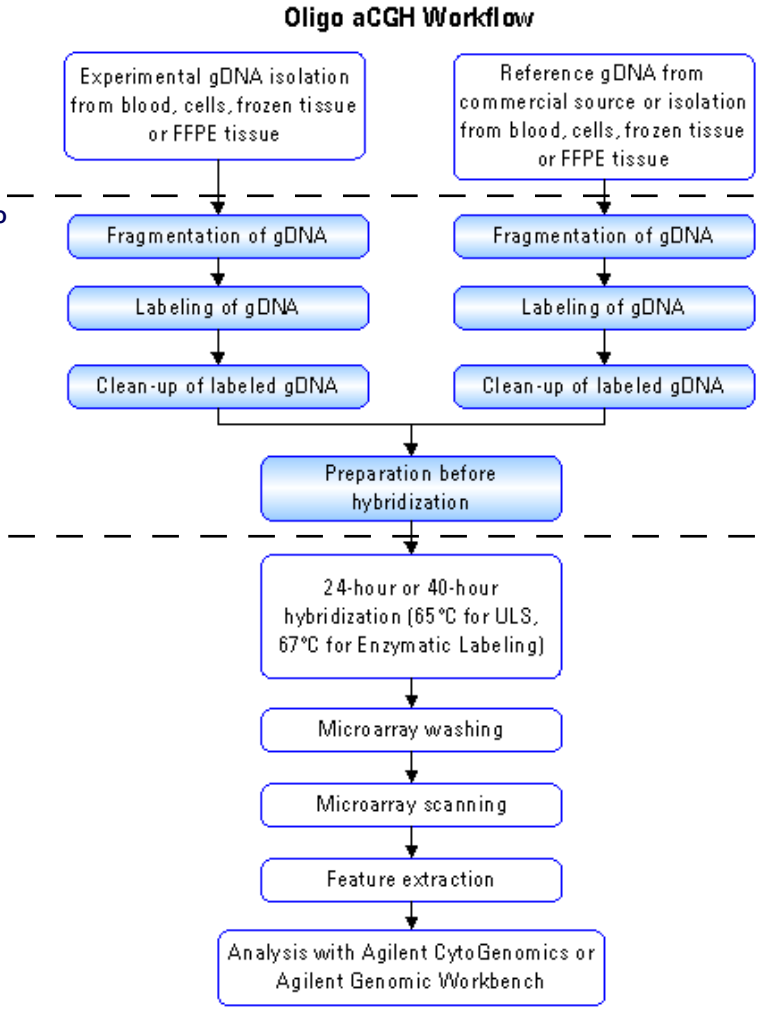


Figure 1. Workflow diagram for sample preparation and microarray processing. Steps that are in blue use the Bravo Automated Liquid Handling Platform.

Blood, Cells or Frozen Tissues

This section describes the recommended procedure to isolate gDNA from blood, cells, or frozen tissues using the **DNeasy Blood & Tissue Kit**.

Table 21 Required gDNA amount from blood, cells or frozen tissues

Microarray format	ULS gDNA input amount requirement (ng)*	Enzymatic gDNA input amount requirement (ng) for CGH*	Enzymatic gDNA input amount requirement (ng) for CGH+SNP*
1-pack	1500	500	1000
2-pack	1000	500	1000
4-pack	500	500	1000
8-pack	250	200	500

* You can use more gDNA, but the gDNA needs to be at a higher concentration. See **Table 24**, **Table 25** and **Table 26** on page 39 for required concentrations.

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 For Enzymatic labeling: 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 For ULS labeling: Prepare a fresh 80% **Ethanol** solution by adding 40 mL 100% **Ethanol** to 10 mL of **DNase/RNase-free distilled water**.

CAUTION

For ULS labeling: Do *not* use Buffer AW2 supplied with the DNeasy Blood & Tissue Kit for the subsequent step because salt from Buffer AW2 will interfere with the subsequent labeling reaction. This is especially important if you need to do a concentration step before labeling.

15 Add the amount below 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.

- For ULS labeling: 500 µL 80% **Ethanol**
- For Enzymatic labeling: 500 µL **Buffer AW2**

16 Put the **DNeasy Mini Spin Column** in a clean **1.5-mL RNase-free Microfuge Tube**, and pipette the amount below directly onto the center of the DNeasy column membrane.

- For ULS labeling: 200 µL of **DNase/RNase-free distilled water**
- For Enzymatic labeling: 200 µL of **Buffer AE**

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

18 Repeat elution with **DNase/RNase-free distilled water** (for ULS labeling) or **Buffer AE** (for Enzymatic labeling) once as described in **step 16** and **step 17**. Combine the duplicate samples in one microcentrifuge tube for a final volume of 400 µL.

NOTE

For ULS labeling: If long term storage is needed, store DNA that was eluted in water at -20°C. Make small aliquots before you freeze the DNA so as to avoid repeated freeze-thaw cycles.

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. gDNA isolated from FFPE samples typically exhibits varying degrees of degradation depending on the age of the tissue and the paraffin embedding protocol used. See **"FFPE Tissues"** on page 33 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 2000 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 22** together on a vortex mixer for 2 to 3 seconds.

Table 22 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 **DNase/RNase-free distilled water** (for ULS labeling) or **Buffer AE** (for Enzymatic labeling) 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.

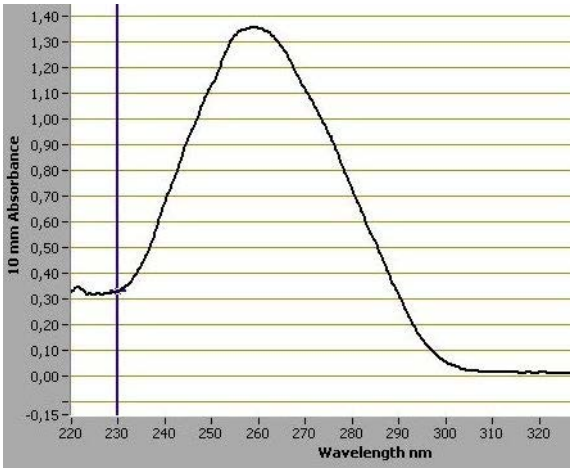


Figure 2. Typical spectrum of pure DNA

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 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**.

FFPE Tissues

This section describes the recommended procedure to isolate gDNA from formalin-fixed paraffin-embedded (FFPE) samples and is based on the method described by van Beers et al. (Br J Cancer. 2006 Jan 30; 94(2):333-7) using the **DNeasy Blood & Tissue Kit**. Determine the number of FFPE sections needed for your experiment based on the estimates summarized in **Table 23**. One 20 micron FFPE section containing 1 cm^2 of tissue is estimated to generate a minimal yield of 500 ng of gDNA.

Table 23 Estimated number of 20 micron FFPE sections needed per microarray

Microarray format	gDNA input amount requirement (ng)	Estimated number of 20 micron FFPE sections
1-pack	2000	4 to 5
2-pack	1000	3
4-pack	500	2
8-pack	250	1

Step 1. Paraffin Removal

1. Equilibrate a heat block or water bath to 90°C and a thermomixer to 37°C.
2. Put up to 5 20-micron FFPE sections into a **1.5-mL RNase-free Microfuge Tube**.
3. Prepare 10% **Tween 20**, by adding 100 µL **Tween 20** to 900 µL of **DNase/RNase-free distilled water**. The solution can be prepared in advance and stored up to 6 months at room temperature.
4. Add 480 µL **Phosphate Buffered Saline pH 7.4 (PBS)** and 20 µL 10% **Tween 20** to the FFPE sections in the **1.5-mL RNase-free Microfuge Tube**.
5. Transfer the sample tube to a circulating water bath or heat block at 90°C. Incubate at 90°C for 10 minutes.
6. Spin immediately for 15 minutes at 10,000 × g in a microcentrifuge.
7. Put the sample tube on ice for 2 minutes.

8 Remove the resulting wax disc with a pipette tip or tweezers. Remove and discard the supernatant without disturbing the pellet.

9 Add 1 mL of 100% **Ethanol** to the pellet and vortex briefly.

10 Spin for 5 minutes at 10,000 × g in a microcentrifuge.

11 Remove **Ethanol** without disturbing the pellet and let the sample tube sit at room temperature with the lid open until residual ethanol has completely evaporated.

12 Prepare a 1M NaSCN solution by adding 10 g of **Sodium thiocyanate (NaSCN)** to 123 mL of **DNase/RNase-free distilled water**. The solution can be prepared in advance and stored up to 1 month at room temperature.

13 Add 400 µL 1M **Sodium thiocyanate (NaSCN)** to the dry pellet and briefly mix on a vortex mixer.

14 Transfer the sample tube to a thermomixer at 37°C. Incubate overnight at 37°C while shaking at 450 rpm.

Step 2. Proteinase K Treatment

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

1 Equilibrate a thermomixer to 56°C.

2 Transfer the sample tube to a microcentrifuge. Spin for 20 minutes at 10,000 × g.

3 Remove and discard the supernatant without disturbing the pellet.

4 Add 400 µL **Phosphate Buffered Saline pH 7.4 (PBS)** to the pellet and vortex briefly.

5 Spin again for 20 minutes at 10,000 × g in a microcentrifuge.

6 Remove and discard the supernatant without disturbing the pellet.

7 Add 360 µL of **Buffer ATL**.

8 Add 40 µL **Proteinase K**, mix well on a vortex mixer, and incubate overnight in a thermomixer at 56°C shaking at 450 rpm.

9 Transfer the sample tube to a microcentrifuge. Spin for 30 seconds at 6,000 × g to drive the contents off the walls and lid.

10 Add 40 µL **Proteinase K**, mix well on a vortex mixer, and incubate in a thermomixer for approximately 6 to 8 hours at 56°C shaking at 450 rpm.

11 At the end of the day, transfer the sample tube to a microcentrifuge and spin for 30 seconds at 6,000 × g to drive the contents off the walls and lid.

12 Add 40 µL **Proteinase K**, mix well on a vortex mixer and incubate overnight in a thermomixer at 56°C shaking at 450 rpm.

Step 3. gDNA Extraction

1 Equilibrate a heat block or water bath to 56°C.

2 Let samples 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.

3 Add 8 µL of **RNase A (100 mg/mL)**, mix on a vortex mixer, and incubate for 2 minutes at room temperature. Transfer the sample tube to a microcentrifuge and spin for 30 seconds at 6,000 × g to drive the contents off the walls and lid.

4 Add 400 µL **Buffer AL**, mix thoroughly on a vortex mixer, and incubate in a circulating water bath or heat block at 56°C for 10 minutes. Transfer the sample tube to a microcentrifuge and spin for 30 seconds at 6,000 × g to drive the contents off the walls and lid.

5 Add 440 µL 100% **Ethanol**, and mix thoroughly on a vortex mixer. Transfer the sample tube to a microcentrifuge and spin for 30 seconds at 6,000 × g to drive the contents off the walls and lid.

6 Put two **DNeasy Mini Spin Columns** in two clean **2 mL Collection Tubes**. Split the entire sample mixture onto two **DNeasy Mini Spin Columns** (i.e., 660 µL each).

NOTE

Use two **DNeasy Mini Spin Columns** per sample to prevent clogging.

7 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 fresh **2 mL Collection Tube**.

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

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

10 Prepare a fresh 80% ethanol solution by adding 40 mL 100% **Ethanol** to 10 mL of **DNase/RNase-free distilled water**.

CAUTION

Do not use Buffer AW2 supplied with the DNeasy Blood & Tissue Kit for the subsequent step because salt from Buffer AW2 will interfere with the subsequent labeling reaction. This is especially important if you need to do a concentration step before labeling.

11 Add 500 µL of 80% **Ethanol** onto each column, and spin in a microcentrifuge for 3 minutes at 20,000 × g to dry the column membrane. Discard the flow-through and collection tube.

12 Put the **DNeasy Mini Spin Column** in a clean **1.5-mL RNase-free Microfuge Tube**, and add 50 µL of **DNase/RNase-free distilled water** directly to the center of each spin column.

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

14 Combine the purified DNA from the same sample in one microcentrifuge tube for a final total volume of 100 µL.

Measure gDNA concentration and purity, and analyze on an agarose gel as described in **"Step 2. gDNA Quantitation and Quality Analysis"** on page 29.

NOTE

If long term storage is needed, store DNA that was eluted in water at -20°C. Make small aliquots before you freeze the DNA so as to avoid repeated freeze-thaw cycles.

3 Sample Preparation for Bravo Platform

This chapter describes how to prepare the samples to be used on the Bravo Automated Liquid Handling Platform.

There is no need to re-determine the concentration of the **Human Reference DNA (Male and Female)**, its concentration is 200 ng/µL as measured by both spectrophotometer and fluorometer.

CAUTION

Use equal amounts of gDNA for both the experimental and reference samples. The required gDNA input amount and concentration depends on the microarray format, labeling method and DNA source used (see Table 21 and Table 23).

Follow the DNA isolation procedure described in Chapter 2, "DNA Isolation". Failure to clean samples thoroughly will result in unsatisfactory microarray results.

If the DNA isolation procedure described in this document cannot be followed, make sure that the DNA is free of RNA and protein contamination. For ULS labeling, also make sure the DNA is in one of the following buffers compatible with ULS labeling:

- **TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5 or pH 8)**
- **10 mM LiCl**
- **10 to 100 mM Na acetate**
- **10 mM NaCl**

If needed, repurify already isolated DNA. Start from step 6 on page 27 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 > 350 ng/µL, dilute 1:2 in **DNase/RNase-free distilled water** (if proceeding with ULS labeling), **Buffer AE** or **1×TE (pH 8.0)** (if following the enzymatic labeling) and quantitate again to make sure dilution is accurate.

Step 1. gDNA Dilution

- Dilute the gDNA with nuclease-free water to the concentration listed in **Table 24** through **Table 26**.

Make sure that the volume is higher than the minimum volume requirement for the Bravo platform (µL).

If the gDNA concentration is less than those listed in **Table 24** through **Table 26**, concentrate the sample using a concentrator (such as Speed Vac) to dryness and resuspend in **DNase/RNase-free distilled water** to the final concentration in those tables.

CAUTION **Do not excessively dry the gDNA as the pellets will become difficult to resuspend.**

Table 24 gDNA concentration and input amount required per microarray for ULS labeling

Microarray format	Concentration requirement (ng/µL)*	Minimum volume requirement for Bravo platform (µL)	Volume that Bravo platform will use
1-pack (non-FFPE samples)	100	17	15
1-pack (FFPE samples)	133.5	17	15
2-pack	100	12	10
4-pack	100	7	5
8-pack	50	7	5

* If you want to use more gDNA than what is listed in **Table 21** on page 26, use gDNA that is at a higher concentration than is listed in this table.

Table 25 For CGH: gDNA concentration and input amount required per microarray for Enzymatic labeling

Microarray format	Concentration requirement (ng/µL)*	Minimum volume requirement for Bravo platform (µL)	Volume that Bravo platform will use
1-, 2-, and 4-pack	100	7	5
8-pack	50	6	4

* If you want to use more gDNA than what is listed in **Table 21** on page 26, use gDNA that is at a higher concentration than is listed in this table.

Table 26 For CGH+SNP: gDNA concentration and input amount required per microarray for Enzymatic labeling

Microarray format	Concentration requirement (ng/µL)*	Minimum volume requirement for Bravo platform (µL)	Volume that Bravo platform will use
1-, 2-, and 4-pack	50	22	20
8-pack	71.4	9	7

* If you want to use more gDNA than what is listed in **Table 21** on page 26, use gDNA that is at a higher concentration than is listed in this table.

Step 2. gDNA Placement in a 96-Well Plate

1. In a **Full skirted 96-well PCR plate**, put the experimental gDNA samples in columns 1 through 6 of the 96-well plate as indicated in **Figure 3**.
2. Put the reference gDNA in columns 7 through 12 as indicated in **Figure 3**.

   Make sure that the experimental and reference gDNA samples meet the minimum volume requirements indicated in **Table 24** on page 38, or **Table 25** or **Table 26** on page 39.
3. Make sure that no air bubbles are stuck at the bottom of the well. If needed, spin the plate in a centrifuge for 30 seconds at 6,000 × g to drive the contents off the walls.

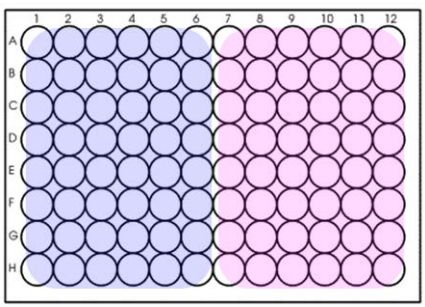


Figure 3. 96-well plate layout of gDNA before labeling. Columns 1 through 6 contain 48 experimental gDNA samples (blue). Columns 7 through 12 contain 48 reference gDNA samples (pink).

You can process between 1 and 48 experimental samples, but to minimize reagent waste, choose 8, 16, 24, 32, 40 or 48 samples. If you process fewer than 48 samples, add gDNA to the wells column-wise. Fill columns 1 and 7 before you continue to columns 2 and 8.

CAUTION

The Agilent Oligo aCGH Bravo platform protocols are based on the plate configuration in Figure 3. Therefore, the first 6 columns are always experimental samples and the last 6 columns are always references. The reference sample for A1 is in A7, the reference sample for B1 is in B7, and so on. The pattern for reference DNAs must match the sample pattern. If this configuration is not followed, the samples will not process correctly.

4 Sample Labeling on the Bravo Platform

This chapter describes the fragmentation, labeling, clean-up and preparation of labeled gDNA for hybridization done on the Bravo platform.

The Agilent Oligo aCGH Bravo Platform protocols are on the CD that came with your Bravo platform or may be obtained by contacting your local Agilent product specialist. Nine protocols are available, three each for ULS processing, Enzymatic processing for CGH, and Enzymatic processing for CGH+SNP.

For ULS processing

- FragmentationLabelingProtocol_ULS (contains both the Fragmentation and Labeling protocol)
- PurificationProtocol_ULS
- HybridizationPrepProtocol_ULS

For Enzymatic CGH processing

- FragmentationLabelingProtocol_Enz (contains both the Fragmentation and Labeling protocol)
- PurificationProtocol_Enz
- HybridizationPrepProtocol_Enz

For Enzymatic CGH+SNP processing

- CGH+SNP_RestrictionDigestionLabelingProtocol_Enz (contains both the Restriction Digestion and Labeling protocol)
- CGH+SNP_PurificationProtocol_Enz
- CGH+SNP_HybridizationPrepProtocol_Enz

For DNA isolated from blood, cells or frozen tissue choose either ULS or Enzymatic processing methods. For DNA isolated from FFPE tissues choose the ULS processing method.

ULS Labeling

The **Genomic DNA High-Throughput ULS Labeling Kit** uses a non-enzymatic procedure to differentially label gDNA samples with fluorescent dyes. The kit contains two-color labeling reaction reagents sufficient for:

- 16 1-pack arrays (blood, cells, tissue samples)
- 12 1-pack arrays (FFPE samples) *or*
- 24 2-pack arrays *or*
- 48 4-pack arrays *or*
- 96 8-pack arrays

You also need to order the **Genomic DNA 96-well Purification Module** to purify the labeled DNA.

Enzymatic Labeling for CGH or CGH+SNP

If you choose the Enzymatic processing method for the CGH or CGH+SNP microarrays, you need a **Bravo Automated Liquid Handling Platform with 96 Channel Disposable LT Pipetting Head, Riser and 2 CPAC Ultraflat heated/cooled Deck Pads**.

The processing of CGH+SNP microarrays requires the restriction digestion of the gDNA with **Alu I** and **Rsa I** (included in the **SureTag Complete DNA Labeling Kit** and the **SureTag DNA Labeling Kit**) prior to labeling, to enable the genotyping of SNPs located in the enzymes' recognition sites for the detection of copy-neutral LOH/UPD.

The **SureTag Complete DNA Labeling Kit** and **SureTag DNA Labeling Kit** use random primers and the Exo (-) Klenow fragment to differentially label gDNA samples with fluorescent-labeled nucleotides. The kit contains sufficient two-color labeling reaction reagents for:

- 25 1-pack, 2-pack and 4-pack arrays *or*
- 50 8-pack arrays

You also need to order the **AutoScreen A, 96-well plate** to purify the labeled DNA.

For Agilent's Oligo aCGH application (including CGH+SNP microarrays), the experimental sample is labeled with one dye (Cy5), while the reference sample is labeled with the other dye (Cy3). The Agilent Oligo aCGH protocols available for the Bravo platform will label the experimental samples and reference samples with their respective dye.

About the Bravo Platform

The Bravo platform is a versatile liquid handler with a nine plate-location platform deck, suitable for handling 96-well, 384-well, and 1536-well plates. The Bravo platform is controlled by the VWorks Automation Control software. Fitted with a choice of seven interchangeable fixed-tip or disposable-tip pipette heads, it accurately dispenses fluids from 0.1 µL to 250 µL.

Use **Figure 4** to familiarize yourself with the location numbering convention on the Bravo platform deck.

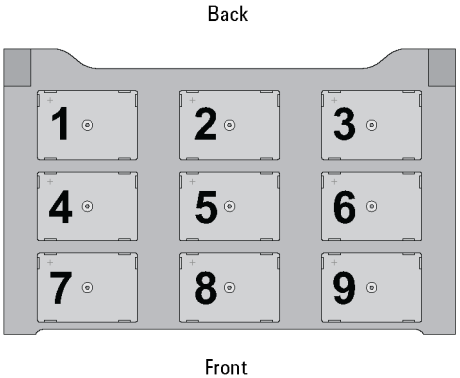


Figure 4. Bravo platform deck

CAUTION **Before you begin, make sure that you have read and understand operating, maintenance and safety instructions for using your Bravo platform. Refer to the *Bravo Platform User Guide* (publication G5409-90004) and the *VWorks Software User Guide* (publication G5415-90002).**

Setting up the VWorks Software

To communicate with and to control the robot and integrated devices, the VWorks software uses a device file. You need to create a device file and link the protocols to that device file. See the VWorks User Guide for instructions.

Step 1. Log in to the VWorks software

1. Double-click the VWorks icon on the Windows desktop to start the software.
2. In the VWorks window, click **Log in** on the toolbar. The User Authentication dialog box opens.
3. Type your VWorks user name and password, and click **OK**. (If no user account is set up, contact the administrator.)

Step 2. Open and start a protocol

1. Click **File > Open**. The Open dialog box opens.
2. Locate and select the protocol (.pro) file that you want to open, and click **Open**.
3. Click **Simulation is on** in the VWorks toolbar to turn off the simulation mode.

   The button changes to “Simulation is off”.
4. Click **Start** on the toolbar.

   The Run Configuration Wizard dialog box opens.

5 For each prompt, respond as follows. Click **Next** between each response:

Run protocol this many times	1
Determine when the protocol will start	As soon as possible
Current state of the tip boxes	*Select tip box location and click the icon on the right. Make sure that the state of the tip boxes matches that in the prompt. For example, tip box at location 1 is empty and tip box at location 2 appears full in the prompt.*
Add notes about the protocol	*Optional.*

6 Click **Finish**.

The Confirm Labware Placement dialog box opens.

7 Confirm that the labware on the Bravo platform deck matches the diagram in the software and click **Continue**.

The working plate location appears empty in the VWorks program.

8 In the Array format dialog box, type the number that corresponds to the microarray format, and then click **Continue**.

1×1M or 1×244K microarrays	1
2×400K or 2×105K microarrays	2
4×180K or 4×44K microarrays	4
8×60K or 8×15K microarrays	8

The Select columns dialog box opens.

9 Type the number of experimental samples you will process in the run. *Do not count the number of reference samples*.

You can process between 1 and 48 samples. To optimize reagent use, select 8, 16, 24, 32, 40 or 48 samples.

ULS Labeling

Follow these steps if you are using ULS Labeling. Otherwise, continue to **"Enzymatic Labeling for CGH"** on page 62 or **"Enzymatic Labeling for CGH+SNP"** on page 74.

Step 1. ULS Labeling

In this step, the Bravo platform transfers the correct amount of gDNA from the 96-well plate that was prepared in **Chapter 3**, "Sample Preparation for Bravo Platform", to the working plate. You fragment the gDNA in a thermal cycler, the Bravo platform adds the Labeling Master Mix to the fragmented gDNA, then the reactions occur in a thermal cycler.

NOTE

Do not fragment gDNA isolated from FFPE tissues. Adjust the fragmentation time of the intact reference gDNA so that the average molecular weight is the same as that of the gDNA isolated from FFPE tissues. This recommendation is based on the method described by Craig JM *et al*. "DNA Fragmentation Simulation Method (FSM) and Fragment Size Matching Improve aCGH Performance of FFPE Tissues," PLoS One 7(6): e38881.

Bravo Platform Protocol

FragmentationLabelingProtocol_ULS

Initial Deck Layout

Location	Content
1 and 7	Empty tip box
2 and 8	Full tip box
6	384 deep well plate: • Cy5 Labeling Master Mix in quadrant 1 • Cy3 Labeling Master Mix in quadrant 3
4	Empty **Full skirted 96-well PCR plate**
5	Sample plate containing the gDNA (prepared in Chapter 3)

1 Based on your microarray format and sample type, prepare one Cy3 and one Cy5 Labeling Master Mix by mixing the respective components in **Table 27** through **Table 31**.

Table 27 Multiplier to calculate the volume of Labeling Master Mix (for 1-pack microarrays using non-FFPE samples)*

Components	Per reaction (µL)	Total volume needed	Volume to add per well of 384 deep well plate
DNase/RNase-free distilled water	1.5	(1.5 µL × N) + 9.6 µL	
ULS-Cy3 Reagent or **ULS-Cy5 Reagent**	1.5	(1.5 µL × N) + 9.6 µL	
10× Labeling Solution	2	(2 µL × N) + 12.8 µL	
Final volume of Labeling Master Mix	**5**	**(5 µL × N) + 32 µL**	**((5 µL × N) + 24 µL) / 8**

* N is the number of experimental samples (8, 16, 24, 32, 40 or 48). Do not count reference samples.

Table 28 Multiplier to calculate the volume of Labeling Master Mix (for 1-pack microarrays using FFPE samples)*

Components	Per reaction (µL)	Total volume needed	Volume to add per well of 384 deep well plate
DNase/RNase-free distilled water	1	(1 µL × N) + 6.4 µL	
ULS-Cy3 Reagent or **ULS-Cy5 Reagent**	2	(2 µL × N) + 12.8 µL	
10× Labeling Solution	2	(2 µL × N) + 12.8 µL	
Final volume of Labeling Master Mix	**5**	**(5 µL × N) + 32 µL**	**((5 µL × N) + 24 µL) / 8**

* N is the number of experimental samples (8, 16, 24, 32, 40 or 48). Do not count reference samples.

Table 29 Multiplier to calculate the volume of Labeling Master Mix (for 2-pack microarrays using non-FFPE and FFPE samples)*

Components	Per reaction (µL)	Total volume needed	Volume to add per well of 384 deep well plate
DNase/RNase-free distilled water	7	(7 µL × N) + 22.4 µL	
ULS-Cy3 Reagent or ULS-Cy5 Reagent	1	(1 µL × N) + 3.2 µL	
10× Labeling Solution	2	(2 µL × N) + 6.4 µL	
Final volume of Labeling Master Mix	**10**	**(10 µL × N) + 32 µL**	**((10 µL × N) + 24 µL) / 8**

* N is the number of experimental samples (8, 16, 24, 32, 40 or 48). Do not count reference samples.

Table 30 Multiplier to calculate the volume of Labeling Master Mix (for 4-pack microarrays using non-FFPE and FFPE samples)*

Components	Per reaction (µL)	Total volume needed	Volume to add per well of 384 deep well plate
DNase/RNase-free distilled water	3.5	(3.5 µL × N) + 22.4 µL	
ULS-Cy3 Reagent or ULS-Cy5 Reagent	0.5	(0.5 µL × N) + 3.2 µL	
10× Labeling Solution	1	(1 µL × N) + 6.4 µL	
Final volume of Labeling Master Mix	**5**	**(5 µL × N) + 32 µL**	**((5 µL × N) + 24 µL) / 8**

* N is the number of experimental samples (8, 16, 24, 32, 40 or 48). Do not count reference samples.

Table 31 Multiplier to calculate the volume of Labeling Master Mix (for 8-pack microarrays using non-FFPE and FFPE samples)*

Components	Per reaction (µL)	Total volume needed	Volume to add per well of 384 deep well plate
DNase/RNase-free distilled water	3.75	(3.75 µL × N) + 24 µL	
ULS-Cy3 Reagent or ULS-Cy5 Reagent	0.25	(0.25 µL × N) + 1.6 µL	
10× Labeling Solution	1	(1 µL × N) + 6.4 µL	
Final volume of Labeling Master Mix	**5**	**(5 µL × N) + 32 µL**	**((5 µL × N) + 24 µL) / 8**

* N is the number of experimental samples (8, 16, 24, 32, 40 or 48). Do not count reference samples.

2 In a 384 deep well plate:

- Refer to **Table 27** through **Table 31** for volumes.
- Add the Cy5 Labeling Master Mix to 8 wells in quadrant 1 (location A1, C1, E1, G1, I1, K1, M1, and O1).
- Add the Cy3 Labeling Master Mix to 8 wells in quadrant 3 (location B1, D1, F1, H1, J1, L1, N1, and P1).

See **Figure 5**.

Make sure that no air bubbles are stuck at the bottom of the well. If needed, spin the plate in a centrifuge for 30 seconds at 6,000 × g to drive the contents off the walls.

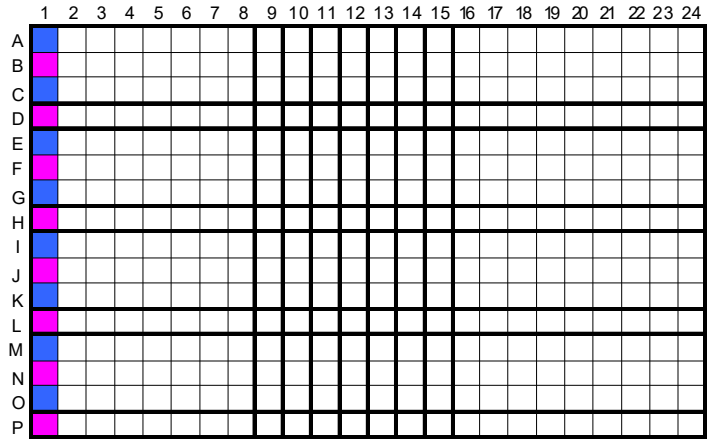


Figure 5. 384 deep well plate for ULS labeling. Well plate contains Cy5 Labeling Master Mix (blue) in quadrant 1 and Cy3 Labeling Master Mix (pink) in quadrant 3.

3 Put the 384 deep well plate at location 6 on the Bravo platform deck.

4 Start the FragmentationLabelingProtocol_ULS protocol run.

The Bravo platform transfers the correct amount of gDNA from the 96-well plate that was prepared in **Chapter 3**, "Sample Preparation for Bravo Platform" to the working plate.

5 When the Bravo platform pauses at the Protocol User Message task:

a Remove the gDNA sample plate from location 5. If needed, store the leftover gDNA at 4°C for short term storage or at -20°C for long term storage.

b Remove the working plate from location 4.

c Seal the plate and spin in a centrifuge for 30 seconds at 6,000 × g to drive the contents off the walls and lid.

d Transfer the working plate to a thermal cycler.

e Program the thermal cycler according to **Table 32** and run the program.

Table 32 DNA fragmentation using a thermal cycler

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

6 Spin the plate in a centrifuge for 30 seconds at 6,000 × g to drive the contents off the walls and lid.

7 Remove the seal and transfer the working plate back to location 4 on the Bravo platform deck.

8 Click **Continue** in the VWorks software.

The Bravo platform adds the Labeling Master Mix to the fragmented gDNA.

9 When the protocol run is finished:

a Remove the working plate from location 4.

b Seal the plate and spin in a centrifuge for 30 seconds at 6,000 × g to drive the contents off the walls and lid.

c Transfer the working plate to a thermal cycler.

d Program the thermal cycler according to **Table 33** and run the program.

Table 33 DNA labeling using a thermal cycler

Step	Temperature	Time
Step 1	85°C	30 minutes
Step 2	4°C	Hold

10 Spin the plate in a centrifuge for 30 seconds at 6,000 × g to drive the contents off the walls and lid.

11 Keep the plate at 4°C in the dark until ready to do **"Step 2. Purification after ULS labeling"** on page 53.

Step 2. Purification after ULS labeling

In this step, the Bravo platform prepares the **Genomic DNA 96-well Purification Module**, transfers the labeled DNA to the purification plate, then you collect the labeled, purified DNA by centrifugation.

Bravo Platform Protocol Used

PurificationProtocol_ULS

Initial Deck Layout

Location	Content
1	Full tip box
4	Agilent reservoir containing **DNase/RNase-free distilled water**
7	When prompted: 96-well plate that contains the labeled gDNA
8	After **step 4**: Pre-spun purification plate/wash plate sandwich

Use the same centrifuge speed and length for all three spinning steps (**step 3**, **step 6** and **step 10**). If you spin only one plate, make sure that you counterbalance the plate.

1. Carefully remove the top and bottom seals of the purification plate. Once the bottom seal is removed, keep the 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.
2. Put the purification plate in a re-usable deep well wash plate (supplied).
3. Pre-spin the purification plate/wash plate sandwich in a centrifuge for 3 minutes at 3,000 × g.
4. Discard the flow-through from the wash plate, and put the purification plate back on the same wash plate. Transfer the purification plate/wash plate sandwich to location 8 on the Bravo platform.
5. Start the PurificationProtocol_ULS protocol run.

   The Bravo platform adds water to the purification plate/wash plate sandwich.
6. When the Bravo platform pauses at the Protocol User Message task, remove the purification plate/wash plate sandwich from location 8 and spin again in a centrifuge for 3 minutes at 3000 × g.
7. Discard the flow-through.

8 Transfer the purification plate to a sample collection plate (supplied) and transfer the purification plate/sample collection plate sandwich to location 8 on the Bravo platform. Put the 96-well plate containing the labeled gDNA on location 7.

9 Click **Continue** in the VWorks software.

For 4-pack and 8-pack microarrays only, the Bravo platform adds water to the labeled DNA. The Bravo platform transfers the labeled DNA to the purification plate/sample collection plate sandwich.

10 When the Bravo protocol run is finished, remove the purification plate/sample collection plate sandwich from location 8 and spin in a centrifuge for 3 minutes at 3,000 × g to collect the purified labeled gDNA. For 1-pack and 2-pack microarrays the volume per sample will be approximately 19 µL. For 4-pack and 8-pack microarrays the volume per sample will be approximately 12.5 µL.

11 Take 1.5 µL of each sample to determine the yield and degree of labeling. See **"To determine yield, degree of labeling or specific activity"** on page 54.

12 For 8-pack microarrays only, use a vacuum concentrator to concentrate the labeled samples to 4.5 µL. If needed, you can concentrate the labeled samples to dryness and resuspend in 4.5 µL water.

CAUTION **Do not excessively dry the samples as the pellets will become difficult to resuspend.**

13 Keep the plate at 4°C in the dark until ready for **"Step 3. Preparation of ULS Labeled gDNA for Hybridization"** on page 56.

To determine yield, degree of labeling or specific activity

Use the 8-channel NanoDrop 8000 or single channel NanoDrop 2000 UV-VIS Spectrophotometer to measure the yield, degree of labeling or specific activity.

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

2 Use 1.5 µL of 1× labeling solution (dilute 10× labeling solution 1:10) to blank the instrument.

3 Mix the samples.

You mix the samples before you measure to get a more accurate quantitation.

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

5 Calculate the Degree of Labeling or Specific Activity of the labeled gDNA:

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

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

*pmol dyes per µg gDNA

Note that the Specific Activity is Degree of Labeling divided by 0.034.

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

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

As a general guideline, an optimal Cy5 degree of labeling lies between 0.75% and 2.5% and an optimal Cy3 degree of labeling lies between 1.75% and 3.5%, with a Cy3 minus Cy5 range between 1% and 2%. Because the ULS-labeling does not copy or amplify the input DNA, the yield after the labeling should be the same as the input amount of DNA.

Step 3. Preparation of ULS Labeled gDNA for Hybridization

In this step, the Bravo platform combines the Cy3 and Cy5 samples and adds the Hybridization Master Mix to the labeled purified DNA samples. You then incubate the sample in a thermal cycler, and the Bravo platform adds the **Agilent-CGHblock**.

Bravo Platform Protocol Used

HybridizationPrepProtocol_ULS

Initial Deck Layout

Location	1-pack microarray	2-pack microarray	4-pack and 8-pack microarray
1	Empty tip box	Empty tip box	Empty tip boxT
2	Full tip box	Full tip box	Full tip box
4	V&P Scientific Reservoir: • column 1: Hybridization Master Mix • column 2: **Agilent-CGHblock**	V&P Scientific Reservoir: • column 1: Hybridization Master Mix • column 2: **Agilent-CGHblock**	V&P Scientific Reservoir: • column 1: Hybridization Master Mix • column 2: **Agilent-CGHblock**
5		**Tall Chimney PCR plate** placed in **Full skirted 96-well PCR plate**)	T
7	**Deep-well plate**		
8	When you are prompted: Working plate containing the labeled, purified DNA.	When you are prompted: Working plate containing the labeled, purified DNA.	Working plate containing the labeled, purified DNA.

1 Prepare the 100× aCGH Blocking Agent:

   a Add 135 µL of **DNase/RNase-free distilled water** to the vial containing lyophilized **10× aCGH Blocking Agent** (included in the **Oligo aCGH/ChIP-on-chip Hybridization Kit**).

   b Mix briefly on a vortex mixer and leave at room temperature for 60 minutes to reconstitute the **10× aCGH Blocking Agent** before use or storage.

   c On the vial of lyophilized **10× aCGH Blocking Agent**, cross out "10×" and write "100×". For ULS labeled samples, you will make and use 100× of the blocking agent.

   The 100× aCGH Blocking Agent can be prepared in advance and stored at -20°C.

2 Prepare the Hybridization Master Mix by mixing the components in the tables below according to the microarray format used.

Table 34 Multiplier to calculate the volume of Hybridization Master Mix for 1-pack microarrays, non-FFPE and FFPE samples*

Components	Per reaction (µL)	Total volume needed
DNase/RNase-free distilled water	39.8	(39.8 µL × N) + 157 µL
Cot-1 DNA (1.0 mg/mL)†	50	(50 µL × N) + 197.2 µL
100× aCGH Blocking Agent‡	5.2	(5.2 µL × N) + 20.5 µL
2× HI-RPM Hybridization Buffer‡	260	(260 µL × N) + 1025.4 µL
Final Volume of Hybridization Master Mix	**355**	**(355 µL × N) + 1400 µL**

* N is the number of experimental samples (8, 16, 24, 32, 40 or 48). Do not count reference samples.

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

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

Table 35 Multiplier to calculate the volume of Hybridization Master Mix for 2-pack microarrays, non-FFPE and FFPE samples*

Components	Per reaction	Total volume needed
DNase/RNase-free distilled water	2.4	(2.4 µL × N) + 21 µL
Cot-1 DNA (1.0 mg/mL)†	25	(25 µL × N) + 218.8 µL
100× aCGH Blocking Agent‡	2.6	(2.6 µL × N) + 22.8 µL
2× HI-RPM Hybridization Buffer‡	130	(130 µL × N) + 1137.5 µL
Final Volume of Hybridization Master Mix	**160**	**(160 µL × N) + 1400 µL**

* N is the number of experimental samples (8, 16, 24, 32, 40 or 48). Do not count reference samples.

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

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

Table 36 Multiplier to calculate the volume of Hybridization Master Mix for 4-pack microarrays, non-FFPE and FFPE samples*

Components	Per reaction (µL)	Total volume needed
Cot-1 DNA (1.0 mg/mL)†	5	(5 µL × N) + 114.7 µL
100× aCGH Blocking Agent‡	1	(1 µL × N) + 23.0 µL
2× HI-RPM Hybridization Buffer‡	55	(55 µL × N) + 1262.3 µL
Final Volume of Hybridization Master Mix	**61**	**(61 µL × N) + 1400 µL**

* N is the number of experimental samples (8, 16, 24, 32, 40 or 48). Do not count reference samples.

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

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

Table 37 Multiplier to calculate the volume of Hybridization Master Mix for 8-pack microarrays, non-FFPE and FFPE samples*

Components	Per reaction (µL)	Total volume needed
Cot-1 DNA (1.0 mg/mL)†	2	(2 µL × N) + 112 µL
100× aCGH Blocking Agent‡	0.5	(0.5 µL × N) + 28 µL
2× HI-RPM Hybridization Buffer‡	22.5	(22.5 µL × N) + 1260 µL
Final Volume of Hybridization Master Mix	**25**	**(25 µL × N) + 1400 µL**

* N is the number of experimental samples (8, 16, 24, 32, 40 or 48). Do not count reference samples.

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

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

3 Add hybridization mix to column 1 of the reservoir in location 4. Be careful not to generate bubbles.

4 Use **Table 38** to calculate how much **Agilent-CGHblock** (included in the **Genomic DNA High-Throughput ULS Labeling Kit**) to use.

Table 38 Multiplier to calculate volume of **Agilent-CGHblock***

Format	Total volume needed
1-pack	(130 µL × N) + 1400 µL
2-pack	(65 µL × N) + 1400 µL
4-pack	(27 µL × N) + 1400 µL
8-pack	(11 µL × N) + 1400 µL

* N is the number of experimental samples (8, 16, 24, 32, 40 or 48). Do not count reference samples.

5 Bring the **Agilent-CGHblock** to room temperature.

6 Add the **Agilent-CGHblock** to the 2nd column of the reservoir in location 4.

The addition of **Agilent-CGHblock** to the hybridization is needed to eliminate background noise on the microarray. The **Agilent-CGHblock** contains components that cannot be heated to 95°C.

7 Start the HybridizationPrepProtocol_ULS protocol run.

For 1-pack and 2-pack microarrays, the Bravo platform transfers the hybridization master mix to the empty deep-well or tall-chimney plates. Continue at **step 8**.

For 4-pack and 8-pack microarrays, the Bravo platform combines the Cy5 and Cy3 labeled, purified samples and adds the hybridization master mix. Continue at **step 9**.

8 For 1-pack and 2-pack microarrays:

a When the Bravo platform pauses at the Protocol User Message task, put the working plate that contains the labeled, purified DNA at location 8.

b Click **Continue** in the VWorks software.

The Bravo platform adds the Cy3 and Cy5 labeled, purified gDNA to the hybridization master mix.

NOTE

When the number of samples is greater than 40, the Bravo platform runs out of tips before the end of the protocol run.

If you see the error message **Error in automatic tip operation**, put more tips into the Bravo platform. Go to **Tools > Tip State Editor**, and update the status of the tip boxes.

9 When the Bravo platform pauses at the Protocol User Message task:

a Remove, seal and transfer the deep-well plate from location 7, tall-chimney plate from location 5, or the PCR plate from location 8 to a thermal cycler.

b Program the thermal cycler according to **Table 39** and run the program.

For the 1-pack microarray deep-well plates, use an incubator that is compatible with deep-well plates, such as the Eppendorf ThermoStat (Eppendorf p/n 022670204 with deep-well plate block p/n 022670565) or BioShake iQ (Q.Instruments p/n 1808-0506).

Table 39 DNA preparation before hybridization

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

10 Spin the plate in a centrifuge for 30 seconds at 6,000 × g to drive the contents off the walls and lid.

11 Remove the seal and transfer the deep-well plate back to location 7, tall-chimney plate back to location 5, or the PCR plate back to location 8 on the Bravo platform deck.

12 Click **Continue** in the VWorks software.

The Bravo platform adds the **Agilent-CGHblock** to the samples.

The samples are ready for the Hybridization step. Continue at **Chapter 5**, "Microarray Processing."

Enzymatic Labeling for CGH

Follow the procedure in this section if you are using Enzymatic labeling for CGH with the **SureTag Complete DNA Labeling Kit** or **SureTag DNA Labeling Kit**.

Step 1. Enzymatic Labeling

In this step, the Bravo platform transfers the correct amount of gDNA from the 96-well plate that was prepared in **Chapter 3**, "Sample Preparation for Bravo Platform" to the working plate. You denature and fragment the gDNA in the presence of random primers in a thermal cycler, the Bravo platform adds the Labeling Master Mix to the fragmented gDNA, and the labeling reactions are done in a thermal cycler.

Bravo Platform Protocol

FragmentationLabelingProtocol_Enz

Initial Deck Layout

Location	Content
1 and 7	Empty tip box
2 and 8	Full tip box
5	Sample plate containing the gDNA (prepared in Chapter 3)
4 (cooled)	Empty **Full skirted 96-well PCR plate**
6 (cooled)	384 deep well plate: • Random primers in quadrant 1 • Cy5 Labeling Master Mix in quadrant 2 • Cy3 Labeling Master Mix in quadrant 4

1 Turn on the Bravo Platform CPAC Ultraflat heated/cooled Deck Pads on Positions 4 and 6, set to 4°C.

2 Use **Table 40** to calculate how much **Random Primer** to use.

Table 40 Multiplier to calculate volume of Random Primer*

Format	Total volume needed	Volume to add per well of 384 deep well plate
1-pack, 2-pack or 4-pack	(5 µL × N × 2) + 32 µL	((5 µL × N × 2) + 24 µL)/8
8-pack	(2.5 µL × N × 2) + 32 µL	((2.5 µL × N × 2) + 24 µL)/8

* N is the number of experimental samples (8, 16, 24, 32, 40 or 48). Do not count reference samples.

3 Refer to **Table 40** for volumes. Add **Random Primer** to 8 wells in quadrant 1 (location A1, C1, E1, G1, I1, K1, M1, and O1). See **Figure 6**.

Make sure that no air bubbles are stuck at the bottom of the well. If needed, spin the plate in a centrifuge for 30 seconds at 6,000 × g to drive the contents off the walls.

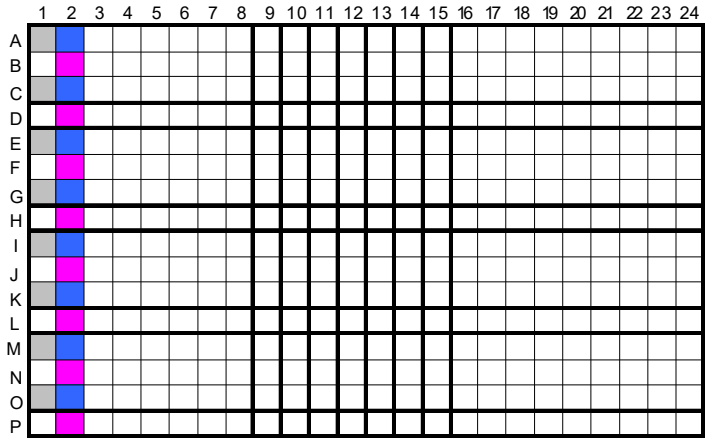


Figure 6. 384 deep well plate for Enzymatic labeling containing **Random Primer** (gray) in quadrant 1, Cy5 Labeling Master Mix (blue) in quadrant 2 and Cy3 Labeling Master Mix (pink) in quadrant 4

4 Prepare one Cy3 and one Cy5 Labeling Master Mix by mixing the components in **Table 41** and **Table 42**, based on your microarray format.

5 In a 384 deep well plate:

- Refer to **Table 41** and **Table 42** for volumes.
- Add the Cy5 Labeling Master Mix to 8 wells in quadrant 2 (location A2, C2, E2, G2, I2, K2, M2, and O2).
- Add the Cy3 Labeling Master Mix to 8 wells in quadrant 4 (location B2, D2, F2, H2, J2, L2, N2, and P2).

See **Figure 6**.

6 Spin the plate in a centrifuge for 30 seconds at 6,000 × g to drive the contents off the walls.

Make sure that no air bubbles are stuck at the bottom of the well.

7 Put the 384 deep well plate at location 6 on the Bravo platform deck.

Table 41 Multiplier to calculate the volume of Labeling Master Mix (for 1-, 2-, or 4-pack microarrays)*

Component	Per reaction (µL)	Total volumes needed	Volume to add per well of 384 deep well plate
Nuclease-Free Water	1	(1 µL × N) + 2.1 µL	
5× Reaction Buffer	5	(5 µL × N) + 10.7 µL	
10× dNTPs†	5	(5 µL × N) + 10.7 µL	
Cyanine 3-dUTP or Cyanine 5-dUTP	3	(3 µL × N) + 6.4 µL	
Exo (-) Klenow	1	(1 µL × N) + 2.1 µL	
Final volume of Labeling Master Mix	**15**	**(15 µL × N) + 32 µL**	**((15 µL × N) + 24 µL)/8**

* N is the number of experimental samples (8, 16, 24, 32, 40 or 48). Do not count reference samples.

† used as a 5× dNTP mix

Table 42 Multiplier to calculate the volume of Labeling Master Mix (for 8-pack microarrays)

Component	Per reaction (µL)	Total volumes needed	Volume to add per well of 384 deep well plate
Nuclease-Free Water	9	(9 µL × N) + 15.6 µL	
5× Reaction Buffer	5	(5 µL × N) + 8.6 µL	
10× dNTPs	2.5	(2.5 µL × N) + 4.3 µL	
Cyanine 3-dUTP or Cyanine 5-dUTP	1.5	(1.5 µL × N) + 2.6 µL	
Exo (-) Klenow	0.5	(0.5 µL × N) + 0.9 µL	
Final volume of Labeling Master Mix	**18.5**	**(18.5 µL × N) + 32 µL**	**((18.5 µL × N) + 24 µL)/8**

8 Start the FragmentationLabelingProtocol_Enz protocol run.

The Bravo platform adds **Random Primer** to the empty working plate. The Bravo platform transfers the correct amount of gDNA from the 96-well plate that was prepared in **Chapter 3**, "Sample Preparation for Bravo Platform" to the working plate.

9 When the Bravo platform pauses at the Protocol User Message task:

- **a** Remove the gDNA sample plate from location 5. If needed, store the leftover gDNA at 4°C for short term storage or at -20°C for long term storage.
- **b** Remove the working plate from location 4.
- **c** Seal the plate and spin in a centrifuge for 30 seconds at 6,000 × g to drive the contents off the walls and lid.
- **d** Transfer the working plate to a thermal cycler.
- **e** Program the thermal cycler according to **Table 43** and run the program.

Table 43 DNA fragmentation using a thermal cycler

Step	Temperature	Time
Step 1	98°C	10 minutes
Step 2	4°C	Hold

10 Spin the plate in a centrifuge for 30 seconds at 6,000 × g to drive the contents off the walls and lid.

11 Remove the seal and transfer the working plate back to location 4 on the Bravo platform deck.

12 Click **Continue** in the VWorks software.

The Bravo platform adds the Labeling Master Mix to the fragmented gDNA.

NOTE

When the number of samples is greater than 40, the Bravo platform runs out of tips before the end of the protocol run.

If you see the error message **Error in automatic tip operation**, put more tips into the Bravo platform. Go to **Tools > Tip State Editor**, and update the status of the tip boxes.

13 When the protocol run is finished:

- **a** Remove the working plate from location 4.
- **b** Seal the plate and spin in a centrifuge for 30 seconds at 6,000 × g to drive the contents off the walls and lid.
- **c** Transfer the working plate to a thermal cycler.
- **d** Program the thermal cycler according to **Table 44** and run the program.

Table 44 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

14 Turn off the Bravo Platform CPAC Ultraflat heated/cooled Deck Pads on Positions 4 and 6.

15 Spin the plate in a centrifuge for 30 seconds at 6,000 × g to drive the contents off the walls and lid.

16 Keep the plate at 4°C in the dark until ready to do **"Step 2. Purification after Enzymatic Labeling"** on page 67.

Step 2. Purification after Enzymatic Labeling

In this step, the Bravo platform prepares the AutoScreen-96A Well plates, transfers the labeled DNA to the purification plate, then you collect the labeled purified DNA by centrifugation.

Bravo Platform Protocol Used

PurificationProtocol_Enz

Initial Deck Layout

Location	Content
1	Full tip box
4	Agilent reservoir containing **DNase/RNase-free distilled water**
7	When prompted: 96-well plate containing the labeled gDNA
8	After **step 6**: Pre-spun purification plate/wash plate sandwich

Use the same centrifuge speed and length for all three spinning steps (**step 5**, **step 8** and **step 12**). If you spin only one plate, make sure that you counterbalance.

1. Get a **Full skirted 96-well PCR plate** to use as a wash plate, and label it as "Wash Plate".

   The wash plate can be reused.
2. Get another 96-well PCR plate to use as a collection plate, and label it as "Collection Plate".
3. Carefully remove the top and bottom seals of the purification plate. Once the bottom seal is removed, keep the 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.
4. Put the purification plate in a reusable wash plate.
5. Pre-spin the purification plate/wash plate sandwich in a centrifuge for 5 minutes at 910 × g.
6. Discard the flow-through from the wash plate and put the purification plate back on the same wash plate. Transfer the purification plate/wash plate sandwich to location 8 on the Bravo platform.
7. Start the PurificationProtocol_Enz protocol run.

   The Bravo platform adds water to the purification plate/wash plate sandwich.

8 When the Bravo platform pauses at the Protocol User Message task, remove the purification plate/wash plate sandwich from location 8 and spin again in a centrifuge for 5 minutes at 910 × g.

9 Discard the flow-through.

10 Transfer the purification plate to a sample collection plate and transfer the purification plate/sample collection plate sandwich to location 8 on the Bravo platform. Put the 96-well plate containing the labeled gDNA on location 7.

11 Click **Continue** in the VWorks software.

The Bravo platform transfers the labeled DNA to the purification plate/sample collection plate sandwich.

12 When the protocol run is finished, remove the purification plate/sample collection plate sandwich from location 8 and 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 ~19 µL.

13 Take 1.5 µL of each sample to determine the yield and degree of labeling or specific activity. See **"To determine yield, degree of labeling or specific activity"** on page 68.

14 For 8-pack microarrays only, use a vacuum concentrator to concentrate the labeled samples to 8 µL. If needed, you can concentrate the labeled samples to dryness and resuspend in 8 µL **1×TE (pH 8.0)**.

CAUTION **Do not excessively dry the samples, or the pellets will become difficult to resuspend.**

15 Keep the plate at 4°C in the dark until ready for **"Step 3. Preparation of Enzymatic Labeled gDNA for Hybridization"** on page 70.

To determine yield, degree of labeling or specific activity

Use the 8-channel NanoDrop 8000 or single channel NanoDrop 2000 UV-VIS Spectrophotometer to measure yield, degree of labeling or specific activity.

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

2 Use 1.5 µL of TE to blank the instrument.

3 Mix the samples.

You mix the samples before you measure to get a more accurate quantitation.

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

5 Calculate the Degree of Labeling or Specific Activity of the labeled gDNA:

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

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

*pmol dyes per µg gDNA

Note that the Specific Activity is Degree of Labeling divided by 0.034.

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

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

Refer to **Table 45** for expected yield of labeled gDNA and specific activity after labeling and clean-up, when starting with high quality gDNA.

Table 45 Expected Yield and Specific Activity after Labeling and Clean-up with the SureTag Complete DNA Labeling Kit or SureTag DNA Labeling Kit

Input gDNA (µg)	Yield (µg)	Specific Activity of Cyanine-3 Labeled Sample (pmol/µg)	Specific Activity of Cyanine-5 Labeled Sample (pmol/µg)
0.5	11 to 14	22 to 29	18 to 25
0.2*	5 to 7	18 to 27	16 to 24

* Half labeling reaction (half the amount of random primers, dye, enzyme and dNTPs)

Check that the Cy3 and Cy5 yield after labeling are the same. If not, refer to **"Troubleshooting"** on page 119.

Step 3. Preparation of Enzymatic Labeled gDNA for Hybridization

In this step, the Bravo platform combines the Cy3 and Cy5 samples, adds the Hybridization Master Mix to the labeled purified DNA samples, and you incubate the sample in a thermal cycler.

Bravo Platform Protocol Used

HybridizationPrepProtocol_Enz

Initial Deck Layout

Location	1-pack microarray	2-pack microarray	4-pack and 8-pack microarray
1	Empty tip box	Empty tip box	Empty tip box
2	Full tip box	Full tip box	Full tip box
4	V&P Scientific Reservoir: • column 1: Hybridization Master Mix • If number of samples > 32, column 2: Hybridization Master Mix	V&P Scientific Reservoir: • column 1: Hybridization Master Mix	V&P Scientific Reservoir: • column 1: Hybridization Master Mix
5		**Tall Chimney PCR plate** placed in a **Full skirted 96-well PCR plate**	
7	**Deep-well plate**		
8	When you are prompted: Working plate containing the labeled, purified DNA.	When you are prompted: Working plate containing the labeled, purified DNA.	Working plate containing the labeled, purified DNA.

1 Prepare the **10× aCGH Blocking Agent**:

   a Add 1350 µL of **DNase/RNase-free distilled water** to the vial containing lyophilized **10× aCGH Blocking Agent** (included in the **Oligo aCGH/ChIP-on-chip Hybridization Kit**).

   b Mix briefly on a vortex mixer and leave at room temperature for 60 minutes to reconstitute the Blocking Agent before use or storage.

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

2 Prepare the Hybridization Master Mix by mixing the components in the tables below according to the microarray format used.

Table 46 Multiplier to calculate the volume of Hybridization Master Mix for 1-pack microarrays*

Component	Per reaction (µL)	Total volume needed in column 1 if N <= 32	Additional volume needed in column 2 if N > 32
1×TE (pH 8.0)	123	(123 µL × N) + 355.1 µL	(123 µL × (N-32)) + 355.1 µL
Cot-1 DNA (1.0 mg/mL)†	50	(50 µL × N) + 144.3 µL	(50 µL × (N-32)) + 144.3 µL
10× aCGH Blocking Agent‡	52	(52 µL × N) + 150.1 µL	(52 µL × (N-32)) + 150.1 µL
2× HI-RPM Hybridization Buffer‡	260	(260 µL × N) + 750.5 µL	(260 µL × (N-32)) + 750.5 µL
Final Volume of Hybridization Master Mix	**485**	**(485 µL × N) + 1400 µL**	**(485 µL × (N-32)) + 1400 µL**

* N is the number of experimental samples (8, 16, 24, 32, 40 or 48). Do not count reference samples.

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

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

Table 47 Multiplier to calculate the volume of Hybridization Master Mix for 2-pack microarrays

Component	Per reaction (µL)	Total volume needed
1×TE (pH 8.0)	44	(44 µL × N) + 273.8 µL
Cot-1 DNA (1.0 mg/mL)*	25	(25 µL × N) + 155.6 µL
10× aCGH Blocking Agent†	26	(26 µL × N) + 161.8 µL
2× HI-RPM Hybridization Buffer†	130	(130 µL × N) + 808.9 µL
Final Volume of Hybridization Master Mix	**225**	**(225 µL × N) + 1400 µL**

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

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

Table 48 Multiplier to calculate the volume of Hybridization Master Mix for 4-pack microarrays

Component	Per reaction (µL)	Total volume needed
1×TE (pH 8.0)	4	(4 µL × N) + 74.7µL
Cot-1 DNA (1.0 mg/mL)*	5	(5 µL × N) + 93.3 µL
10× aCGH Blocking Agent†	11	(11 µL × N) + 205.3 µL
2× HI-RPM Hybridization Buffer†	55	(55 µL × N) + 1026.7 µL
Final Volume of Hybridization Master Mix	**75**	**(75 µL × N) + 1400 µL**

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

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

Table 49 Multiplier to calculate the volume of Hybridization Master Mix for 8-pack microarrays

Component	Per reaction (µL)	Total volume needed
Cot-1 DNA (1.0 mg/mL)*	2	(2 µL × N) + 96.6 µL
10× aCGH Blocking Agent†	4.5	(4.5 µL × N) + 217.2 µL
2× HI-RPM Hybridization Buffer†	22.5	(22.5 µL × N) + 1086.2 µL
Final Volume of Hybridization Master Mix	**29**	**(29 µL × N) + 1400 µL**

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

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

3 Add hybridization master mix to the reservoir in location 4. Be careful not to generate bubbles.
   - For 1-pack and ≤ 32 samples, 2-pack, 4-pack and 8-pack: add to column 1.
   - For 1-pack and > 32 samples: add to column 1 and 2.

4 Start the HybridizationPrepProtocol_Enz protocol run.

   For 1-pack and 2-pack microarrays, the Bravo platform transfers the hybridization master mix to the empty deep-well or tall-chimney plates. Continue at **step 5**.

   For 4-pack and 8-pack microarrays, the Bravo platform combines the Cy5 and Cy3 labeled, purified samples and adds the hybridization master mix. Continue at **step 6**.

5 For 1-pack and 2-pack microarrays:
   a When the Bravo platform pauses at the Protocol User Message task, put the working plate that contains the labeled, purified DNA at location 8.

b Click **Continue** in the VWorks software.

The Bravo platform adds the Cy3 and Cy5 labeled, purified gDNA to the hybridization master mix.

NOTE

When the number of samples is greater than 40, the Bravo platform runs out of tips before the end of the protocol run.

If you see the error message **Error in automatic tip operation**, put more tips into the Bravo platform. Go to **Tools > Tip State Editor**, and update the status of the tip boxes.

6 When the protocol run is finished, remove, seal, spin, and transfer the deep-well plate from location 7, tall-chimney plate from location 5, or the PCR plate from location 8 to a thermal cycler. Program the thermal cycler according to **Table 50** and run the program.

For the 1-pack microarray deep-well plates, use an incubator that is compatible with deep-well plates, such as the Eppendorf ThermoStat (Eppendorf p/n 022670204 with deep-well plate block p/n 022670565) or BioShake iQ (Q.Instruments p/n 1808-0506).

Table 50 DNA preparation before hybridization

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

7 Spin the plate in a centrifuge for 30 seconds at 6,000 × g to drive the contents off the walls and lid.

The samples are ready for the Hybridization step.

Enzymatic Labeling for CGH+SNP

Follow the procedure in this section if you are using Enzymatic labeling for CGH+SNP with the **SureTag Complete DNA Labeling Kit** or **SureTag DNA Labeling Kit**.

Step 1. Enzymatic Labeling

In this step, the Bravo platform combines the correct amount of gDNA from the 96-well plate that was prepared in **Chapter 3**, "Sample Preparation for Bravo Platform" with the Restriction Digestion Master Mix in the working plate. The digested gDNA is denatured in the presence of random primers in a thermal cycler, the Bravo platform adds the Labeling Master Mix to the digested gDNA, and the labeling reactions are done in a thermal cycler.

Bravo Platform Protocol

CGH+SNP_RestrictionDigestionLabelingProtocol_Enz

Initial Deck Layout

Location	Content
1 and 7	Empty tip box
2 and 8	Full tip box
5	Sample plate containing the gDNA (prepared in Chapter 3)
4 (cooled)	Empty **Full skirted 96-well PCR plate**
6 (cooled)	384 deep well plate: • Restriction Digestion Master Mix in quadrant 3 • Only when prompted: Random Primers and Labeling Master Mix Random Primers in quadrant 1 Cy5 Labeling Master Mix in quadrant 2 Cy3 Labeling Master Mix in quadrant 4

1 Turn on the Bravo Platform CPAC Ultraflat heated/cooled Deck Pads on Positions 4 and 6, set to 4°C.

2 Prepare the Restriction Digestion Master Mix by mixing the components in **Table 51** through **Table 52**, based on your microarray format.

Table 51 Multiplier to calculate the volume of Restriction Digestion Master Mix (for 1-, 2-, and 4-pack microarrays)

Components	Per reaction (µL)	Total Volumes Needed*	Volume to add per well of 384 deep well plate
Nuclease-Free Water†	2.2	(2.2 µL × N × 2) + 11.7	
10× Restriction Enzyme Buffer†	2.6	(2.6 µL × N × 2) + 13.9	
BSA†	0.2	(0.2 µL × N × 2) + 1.1	
Alu I†	0.5	(0.5 µL × N × 2) + 2.7	
Rsa I†	0.5	(0.5 µL × N × 2) + 2.7	
Final volume of Restriction Digestion Master Mix	**6**	**(6 µL × N × 2) + 32**	**((6 µL × N × 2) + 24 µL)/8**

* N is the number of experimental samples (8, 16, 24, 32, 40 or 48). Do not count reference samples.

† Included in the **SureTag Complete DNA Labeling Kit** and **SureTag DNA Labeling Kit**

Table 52 Multiplier to calculate the volume of Restriction Digestion Master Mix (for 8-pack microarrays)

Components	Per reaction (µL)	Total Volumes Needed*	Volume to add per well of 384 deep well plate
Nuclease-Free Water†	4.1	(4.1 µL × N × 2) + 21.9	
10× Restriction Enzyme Buffer†	1.3	(1.3 µL × N × 2) + 6.9	
BSA†	0.1	(0.1 µL × N × 2) + 0.5	
Alu I†	0.25	(0.25 µL × N × 2) + 1.3	
Rsa I†	0.25	(0.25 µL × N × 2) + 1.3	
Final volume of Restriction Digestion Master Mix	**6**	**(6 µL × N × 2) + 32**	**((6 µL × N × 2) + 24 µL)/8**

* N is the number of experimental samples (8, 16, 24, 32, 40 or 48). Do not count reference samples.

† Included in the **SureTag Complete DNA Labeling Kit** and **SureTag DNA Labeling Kit**

3 In a 384 deep well plate, add Restriction Digestion Master Mix to 8 wells in quadrant 3 (location B1, D1, F1, H1, J1, L1, N1, and P1). See **Figure 7**.

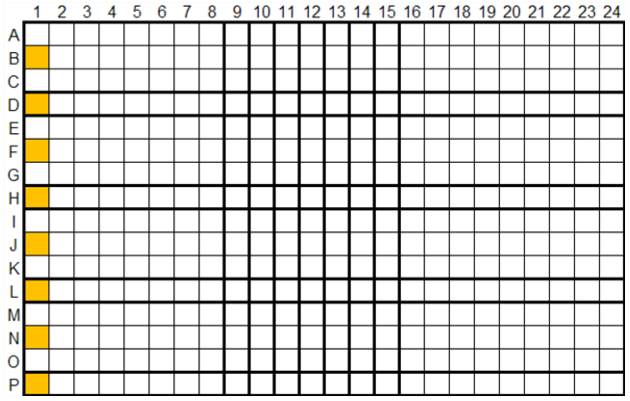


Figure 7. 384 deep well plate for CGH+SNP Enzymatic Labeling containing Restriction Digestion Master Mix in quadrant 3

4. Spin the plate in a centrifuge for 30 seconds at 6,000 × g to drive the contents off the walls and to remove any bubbles that are stuck at the bottom of the well.
5. Put the 384 deep well plate at location 6 on the Bravo platform deck.
6. Start the **CGH+SNP_RestrictionDigestionLabelingProtocol_Enz** protocol run.

   The Bravo platform adds the Restriction Digestion Master Mix to the empty working plate. The Bravo platform transfers the correct amount of gDNA from the 96-well plate that was prepared in **Chapter 3**, "Sample Preparation for Bravo Platform" to the working plate.
7. When the Bravo platform pauses at the Protocol User Message task:
   a. Remove the gDNA sample plate from location 5. If needed, store the leftover gDNA at 4°C for short term storage, or at -20°C for long term storage.
   b. Remove the working plate from location 4.
   c. Seal the plate and spin in a centrifuge for 30 seconds at 6,000 × g to drive the contents off the walls and lid.
   d. Transfer the working plate to a thermal cycler.
   e. Program the thermal cycler according to **Table 53** and run the program.

Table 53 DNA restriction digestion using a thermal cycler

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

8 When the incubation is finished, remove the plate from the thermal cycler and spin the plate in a centrifuge for 30 seconds at 6,000 × g to drive the contents off the walls and lids.

Keep the plate at 4°C until ready to continue to the addition of **Random Primer** and Labeling Master Mix.

9 Use **Table 54** to calculate how much **Random Primer** to use.

Table 54 Multiplier to calculate volume of Random Primer*

Format	Total volume needed	Volume to add per well of 384 deep well plate
1-, 2-, and 4-pack	(5 µL × N × 2) + 32 µL	((5 µL × N × 2) + 24 µL)/8
8-pack	(2.5 µL × N × 2) + 32 µL	((2.5 µL × N × 2) + 24 µL)/8

* N is the number of experimental samples (8, 16, 24, 32, 40 or 48). Do not count reference samples.

10 Refer to **Table 54** for volumes. Add the **Random Primer** to 8 wells in quadrant 1 (location A1, C1, E1, G1, I1, K1, M1, and O1). See **Figure 8**.

Make sure that no air bubbles are stuck at the bottom of the well. If needed, spin the plate in a centrifuge for 30 seconds at 6,000 × g to drive the contents off the walls.

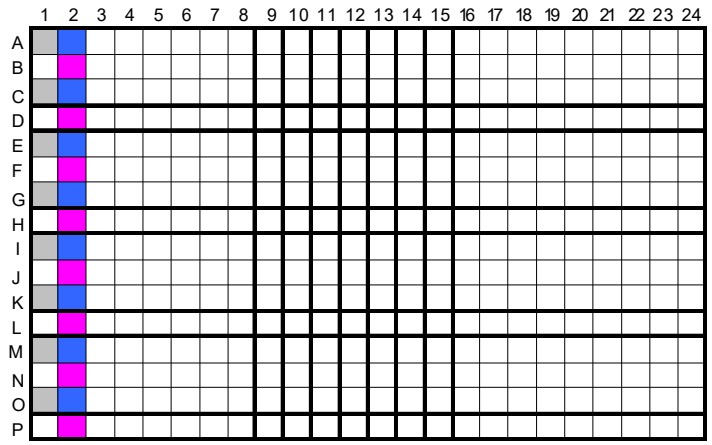


Figure 8. 384 deep well plate for Enzymatic labeling containing **Random Primer** (gray) in quadrant 1, Cy5 Labeling Master Mix (blue) in quadrant 2 and Cy3 Labeling Master Mix (pink) in quadrant 4

11 Prepare one Cy3 and one Cy5 Labeling Master Mix by mixing the components in **Table 55** and **Table 56**, based on your microarray format.

12 In the 384 deep well plate:

- Refer to **Table 55** and **Table 56** for volumes.
- Add the Cy5 Labeling Master Mix to 8 wells in quadrant 2 (location A2, C2, E2, G2, I2, K2, M2, and O2).
- Add the Cy3 Labeling Master Mix to 8 wells in quadrant 4 (location B2, D2, F2, H2, J2, L2, N2, and P2).

See **Figure 8**.

13 Spin the plate in a centrifuge for 30 seconds at 6,000 × g to drive the contents off the walls.

Make sure that no air bubbles are stuck at the bottom of the well.

14 Put the 384 deep well plate at location 6 on the Bravo platform deck.

Table 55 Multiplier to calculate the volume of Labeling Master Mix (for 1-, 2-, and 4-pack microarrays)*

Component	Per reaction (µL)	Total volumes needed	Volume to add per well of 384 deep well plate
5× Reaction Buffer	10	(10 µL × N) + 16.8 µL	
10× dNTPs†	5	(5 µL × N) + 8.4 µL	
Cyanine 3-dUTP or **Cyanine 5-dUTP**	3	(3 µL × N) + 5.1 µL	
Exo (-) Klenow	1	(1 µL × N) + 1.7 µL	
Final volume of Labeling Master Mix	**19**	**(19 µL × N) + 32 µL**	**((19 µL × N) + 24 µL)/8**

* N is the number of experimental samples (8, 16, 24, 32, 40 or 48). Do not count reference samples.

† used as a 5× dNTP mix

Table 56 Multiplier to calculate the volume of Labeling Master Mix (for 8-pack microarrays)

Component	Per reaction (µL)	Total volumes needed	Volume to add per well of 384 deep well plate
5× Reaction Buffer	5	(5 µL × N) + 16.8 µL	
10× dNTPs	2.5	(2.5 µL × N) + 8.4 µL	
Cyanine 3-dUTP or **Cyanine 5-dUTP**	1.5	(1.5 µL × N) + 5.1 µL	
Exo (-) Klenow	0.5	(0.5 µL × N) + 1.7 µL	
Final volume of Labeling Master Mix	**9.5**	**(9.5 µL × N) + 32 µL**	**((9.5 µL × N) + 24 µL)/8**

15 Remove the seal and transfer the working plate back to location 4 on the Bravo platform deck.

16 Press the **Go** button to continue the **CGH+SNP_RestrictionDigestionLabelingProtocol_Enz** protocol run.

The Bravo platform adds the **Random Primer** to the working plate that contains the digested gDNA.

17 When the Bravo platform pauses at the Protocol User Message task:

a Remove the working plate from location 4.

b Seal the plate and spin in a centrifuge for 30 seconds at 6,000 × g to drive the contents off the walls and lid.

c Transfer the working plate to a thermal cycler.

d Program the thermal cycler according to **Table 57** and run the program.

Table 57 DNA denaturation using a thermal cycler

Step	Temperature	Time
Step 1	98°C	3 minutes
Step 2	4°C	Hold

18 Spin the plate in a centrifuge for 30 seconds at 6,000 × g to drive the contents off the walls and lid.

19 Remove the seal and transfer the working plate back to location 4 on the Bravo platform deck.

20 Click **Continue** in the VWorks software.

The Bravo platform adds the Labeling Master Mix to the digested gDNA.

NOTE

When the number of samples is 24 or greater, the Bravo platform runs out of tips before the end of the protocol run.

If you see the error message **Error in automatic tip operation**, put more tips into the Bravo platform. Go to **Tools > Tip State Editor**, and update the status of the tip boxes.

21 When the protocol run is finished:

a Remove the working plate from location 4.

b Seal the plate and spin in a centrifuge for 30 seconds at 6,000 × g to drive the contents off the walls and lid.

c Transfer the working plate to a thermal cycler.

d Program the thermal cycler according to **Table 58** and run the program.

Table 58 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

22 Turn off the Bravo Platform CPAC Ultraflat heated/cooled Deck Pads on Positions 4 and 6.

23 Spin the plate in a centrifuge for 30 seconds at 6,000 × g to drive the contents off the walls and lid.

24 Keep the plate at 4°C in the dark until ready to do **"Step 2. Purification after Enzymatic Labeling"** on page 81.

Step 2. Purification after Enzymatic Labeling

In this step, the Bravo platform prepares the AutoScreen-96A Well plates, transfers the labeled DNA to the purification plate, then you collect the labeled purified DNA by centrifugation.

Bravo Platform Protocol Used

CGH+SNP_PurificationProtocol_Enz

Initial Deck Layout

Location	Content
1	Full tip box
4	Agilent reservoir containing **DNase/RNase-free distilled water**
7	When prompted: 96-well plate containing the labeled gDNA
8	After **step 6**: Pre-spun purification plate/wash plate sandwich

Use the same centrifuge speed and length for all spinning steps (**step 5**, **step 8**, **step 12** and **step 14**). If you spin only one plate, make sure that you counterbalance.

1 Get a **Full skirted 96-well PCR plate** to use as a wash plate, and label it as "Wash Plate".

The wash plate can be reused.

2 Get another 96-well PCR plate to use as a collection plate, and label it as "Collection Plate".

3 Carefully remove the top and bottom seals of the purification plate. Once the bottom seal is removed, keep the 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.

4 Put the purification plate in a reusable wash plate.

5 Pre-spin the purification plate/wash plate sandwich in a centrifuge for 5 minutes at 910 × g.

6 Discard the flow-through from the wash plate and put the purification plate back on the same wash plate. Transfer the purification plate/wash plate sandwich to location 8 on the Bravo platform.

7 Start the CGH+SNP_PurificationProtocol_Enz protocol run. You are prompted to choose the microarray format.

The Bravo platform adds water to the purification plate/wash plate sandwich.

8 When the Bravo platform pauses at the Protocol User Message task, remove the purification plate/wash plate sandwich from location 8 and spin again in a centrifuge for 5 minutes at 910 × g.

9 Discard the flow-through.

10 Transfer the purification plate to a sample collection plate and transfer the purification plate/sample collection plate sandwich to location 8 on the Bravo platform. Put the 96-well plate containing the labeled gDNA on location 7.

11 Click **Continue** in the VWorks software.

The Bravo platform transfers 25 µL of the labeled DNA to the purification plate/sample collection plate sandwich.

12 Remove the purification plate/sample collection plate sandwich from location 8 and 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 ~19 µL.

For 1-, 2-, and 4-pack microarray formats, return the purification plate/sample collection plate sandwich to location 8.

CAUTION **Make sure the plate is positioned in the deck in the same orientation as in the step before. Otherwise, the Cy5-labeled and Cy3-labeled samples will get mixed.**

13 Click **Continue** in the VWorks software.

The Bravo platform transfers the remaining 25 µL of labeled DNA to the same purification plate/sample collection plate sandwich.

14 When the protocol run is finished, remove the purification plate/sample collection plate sandwich from location 8 and spin in a centrifuge for 5 minutes at 910 × g to collect the purified labeled gDNA in the sample collection plate.

The recovered volume per sample will be approximately 19 µL. The total recovered volume per sample will be approximately 38 µL.

15 Take 1.5 µL of each sample to determine the yield and degree of labeling or specific activity. See **"To determine yield, degree of labeling or specific activity"** on page 83.

16 For 4-pack and 8-pack microarrays, use a vacuum concentrator to concentrate the labeled samples:

- For 8-pack microarrays, concentrate to 8 µL. If needed, concentrate the labeled samples to dryness and resuspend in 8 µL of **1×TE (pH 8.0)**.
- For 4-pack microarrays, concentrate to 24.5 µL. If needed, concentrate the labeled samples to dryness and resuspend in 24.5 µL of **1×TE (pH 8.0)**.

  As an alternative, transfer 24.5 µL of each experimental and reference labeled DNA to a new plate that will be used for Hybridization preparation. In this case, not all labeled DNA will be hybridized, and consequently the signals will be lower.

CAUTION **Do not excessively dry the samples, or the pellets will become difficult to resuspend.**

17 Keep the plate at 4°C in the dark until ready for **"Step 3. Preparation of Enzymatic Labeled gDNA for Hybridization"** on page 85.

To determine yield, degree of labeling or specific activity

Use the 8-channel NanoDrop 8000 or single channel NanoDrop 2000 UV-VIS Spectrophotometer to measure yield, degree of labeling or specific activity.

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

2 Use 1.5 µL of TE to blank the instrument.

3 Mix the samples.

  You mix the samples before you measure to get a more accurate quantitation.

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

5 Calculate the Degree of Labeling or Specific Activity of the labeled gDNA:

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

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

*pmol dyes per µg gDNA

Note that the Specific Activity is Degree of Labeling divided by 0.034.

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

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

Refer to **Table 59** for expected yield of labeled gDNA and specific activity after labeling and clean-up, when starting with high quality gDNA.

Table 59 Expected Yield and Specific Activity after Labeling and Clean-up with the SureTag Complete DNA Labeling Kit or SureTag DNA Labeling Kit

Input gDNA (µg)	Yield (µg)	Specific Activity of Cyanine-3 Labeled Sample (pmol/µg)	Specific Activity of Cyanine-5 Labeled Sample (pmol/µg)
0.5 to 1	12 to 16	19 to 30	15 to 25

Check that the Cy3 and Cy5 yield after labeling are the same. If not, refer to **"Troubleshooting"** on page 119.

Step 3. Preparation of Enzymatic Labeled gDNA for Hybridization

In this step, the Bravo platform combines the Cy3 and Cy5 samples, adds the Hybridization Master Mix to the labeled purified DNA samples, and you incubate the sample in a thermal cycler.

Bravo Platform Protocol Used

CGH+SNP_HybridizationPrepProtocol_Enz

Initial Deck Layout

Location	1-pack microarray	2-pack microarray	4-pack and 8-pack microarray
1	Empty tip box	Empty tip box	Empty tip box
2	Full tip box	Full tip box	Full tip box
4	V&P Scientific Reservoir: • column 1: Hybridization Master Mix • If number of samples > 32, column 2: Hybridization Master Mix	V&P Scientific Reservoir: • column 1: Hybridization Master Mix	V&P Scientific Reservoir: • column 1: Hybridization Master Mix
5		**Tall Chimney PCR plate** placed in a **Full skirted 96-well PCR plate**	
7	**Deep-well plate**		
8	When you are prompted: Working plate containing the labeled, purified DNA.	When you are prompted: Working plate containing the labeled, purified DNA.	Working plate containing the labeled, purified DNA.

1 Prepare the 100× aCGH Blocking Agent:

   a Add 135 µL of **DNase/RNase-free distilled water** to the vial containing lyophilized **10× aCGH Blocking Agent** (included in the **Oligo aCGH/ChIP-on-chip Hybridization Kit**).

   b Mix briefly on a vortex mixer and leave at room temperature for 60 minutes to reconstitute the sample before use or storage.

c On the vial of lyophilized blocking agent, cross out "10×" and write "100×". For labeled samples that will hybridize to CGH+SNP arrays, you make and use 100× of the blocking agent.

The 100× aCGH Blocking Agent can be prepared in advance and stored at -20°C.

2 Prepare the Hybridization Master Mix by mixing the components in the tables below according to the microarray format used.

Table 60 Multiplier to calculate the volume of Hybridization Master Mix for 1-pack microarrays*

Component	Per reaction (µL)	Total volume needed in column 1 if N <= 32	Additional volume needed in column 2 if N > 32
1×TE (pH 8.0)	131.8	(131.8 µL × N) + 412.8 µL	(131.8 µL × (N-32)) + 412.8 µL
Human Cot-1 DNA	50	(50 µL × N) + 156.6 µL	(50 µL × (N-32)) + 156.6 µL
100× aCGH Blocking Agent†	5.2	(5.2 µL × N) + 16.3 µL	(5.2 µL × (N-32)) + 16.3 µL
2× HI-RPM Hybridization Buffer†	260	(260 µL × N) + 814.3 µL	(260 µL × (N-32)) + 814.3 µL
Final Volume of Hybridization Master Mix	**447**	**(447 µL × N) + 1400 µL**	**(447 µL × (N-32)) + 1400 µL**

* N is the number of experimental samples (8, 16, 24, 32, 40 or 48). Do not count reference samples.

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

Table 61 Multiplier to calculate the volume of Hybridization Master Mix for 2-pack microarrays*

Component	Per reaction (µL)	Total volume needed
1×TE (pH 8.0)	29.4	(29.4 µL × N) + 220.1 µL
Human Cot-1 DNA	25	(25 µL × N) + 187.2 µL
100× aCGH Blocking Agent†	2.6	(2.6 µL × N) + 19.5 µL
2× HI-RPM Hybridization Buffer†	130	(130 µL × N) + 973.3 µL
Final Volume of Hybridization Master Mix	**187**	**(187 µL × N) + 1400 µL**

* N is the number of experimental samples (8, 16, 24, 32, 40 or 48). Do not count reference samples.

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

Table 62 Multiplier to calculate the volume of Hybridization Master Mix for 4-pack microarrays*

Component	Per reaction (µL)	Total volume needed
Human Cot-1 DNA	5	(5 µL × N) + 114.6 µL
100× aCGH Blocking Agent†	1.1	(1.1 µL × N) + 25.2 µL
2× HI-RPM Hybridization Buffer†	55	(55 µL × N) + 1260.2 µL
Final Volume of Hybridization Master Mix	**61.1**	**(61.1 µL × N) + 1400 µL**

* N is the number of experimental samples (8, 16, 24, 32, 40 or 48). Do not count reference samples.

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

Table 63 Multiplier to calculate the volume of Hybridization Master Mix for 8-pack microarrays*

Component	Per reaction (µL)	Total volume needed
1×TE (pH 8.0)	4.05	(4.05 µL × N) + 195.5 µL
Human Cot-1 DNA	2	(2 µL × N) + 96.6 µL
100× aCGH Blocking Agent†	0.45	(0.45 µL × N) + 21.7 µL
2× HI-RPM Hybridization Buffer†	22.5	(22.5 µL × N) + 1086.2 µL
Final Volume of Hybridization Master Mix	**29**	**(29 µL × N) + 1400 µL**

* N is the number of experimental samples (8, 16, 24, 32, 40 or 48). Do not count reference samples.

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

3 Add hybridization mix to the reservoir in location 4. Be careful not to generate bubbles.
   - For 1-pack and ≤ 32 samples, 2-pack, 4-pack and 8-pack: add to column 1.
   - For 1-pack and > 32 samples: add to column 1 and 2.

4 Start the **CGH+SNP_HybridizationPrepProtocol_Enz** protocol run.

   For 1-pack and 2-pack microarrays, the Bravo platform transfers the hybridization master mix to the empty deep-well or tall-chimney plates. Continue at **step 5**.

   For 4-pack and 8-pack microarrays, the Bravo platform combines the Cy5 and Cy3 labeled, purified samples and adds the hybridization master mix. Continue at **step 6**.

5 For 1-pack and 2-pack microarrays:

   a When the Bravo platform pauses at the Protocol User Message task, put the working plate that contains the labeled, purified DNA at location 8.

   b Click **Continue** in the VWorks software.

   The Bravo platform adds the Cy3 and Cy5 labeled, purified gDNA to the hybridization master mix.

NOTE

When the number of samples is greater than 40, the Bravo platform runs out of tips before the end of the protocol run.

If you see the error message **Error in automatic tip operation**, put more tips into the Bravo platform. Go to **Tools > Tip State Editor**, and update the status of the tip boxes.

6 When the protocol run is finished, remove, seal, spin, and transfer the deep-well plate from location 7, tall-chimney plate from location 5, or the PCR plate from location 8 to a thermal cycler. Program the thermal cycler according to **Table 64** and run the program.

   For the 1-pack microarray deep-well plates, use an incubator that is compatible with deep-well plates, such as the Eppendorf ThermoStat (Eppendorf p/n 022670204 with deep-well plate block p/n 022670565) or BioShake iQ (Q.Instruments p/n 1808-0506).

Table 64 DNA preparation before hybridization

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

7 Spin the plate in a centrifuge for 30 seconds at 6,000 × g to drive the contents off the walls and lid.

   The samples are ready for the Hybridization step.

5 Microarray Processing

Microarray processing consists of hybridization, washing, and scanning.

Microarray Hybridization

Familiarize yourself with the assembly and disassembly instructions for use with the Agilent microarray hybridization chamber and gasket slides. Please refer to the Agilent Microarray Hybridization Chamber User Guide (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 128 and **"Microarray Handling Tips"** on page 130.

Step 1. Prepare the hybridization assembly

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.

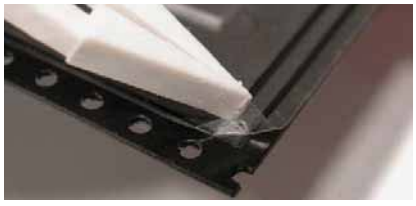


Figure 9. Removal of clear plastic covering

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.

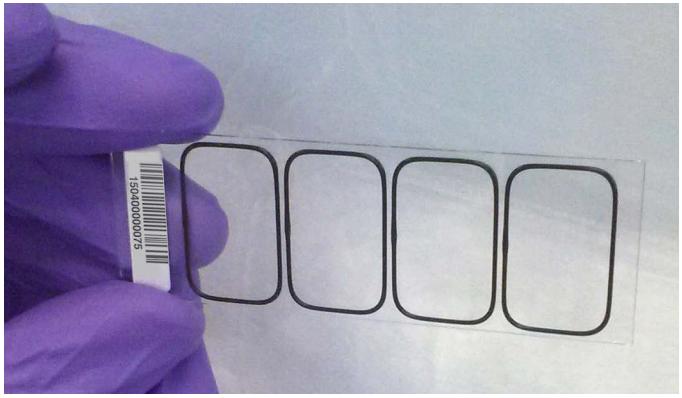


Figure 10. Gasket slide, gasket side

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 11**) with the barcode label resting over the base's rectangular barcode guide.

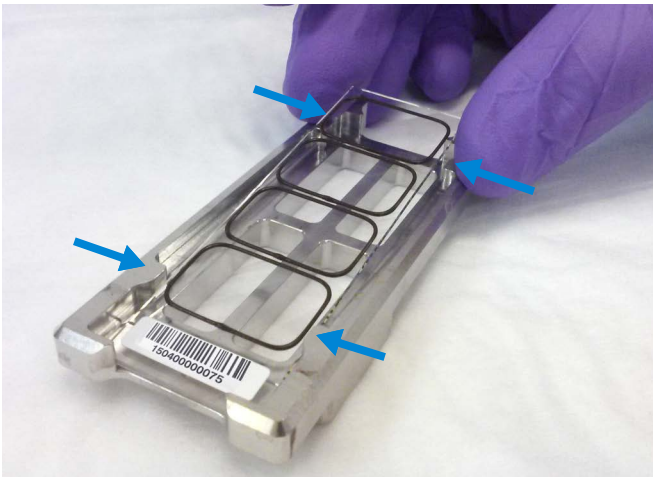


Figure 11. Chamber base, guide posts denoted with arrows

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 12. 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 13. 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 14** for proper technique on holding the microarray slide with both hands.

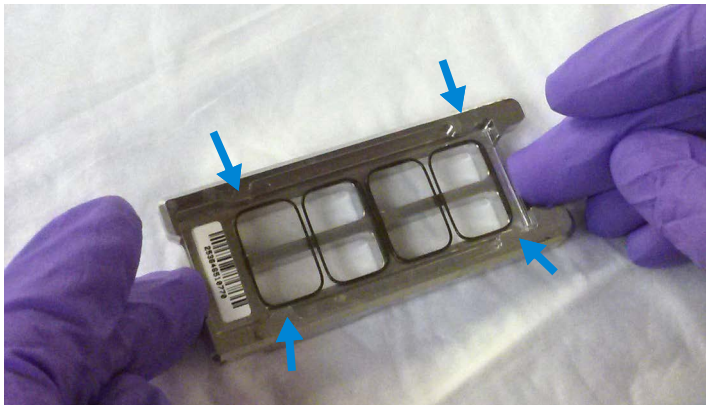


Figure 14. Chamber base with gasket and microarray slide applied, guide posts denoted with arrows

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.

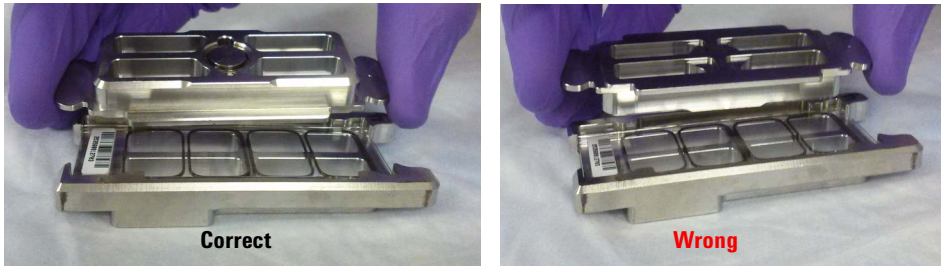


Figure 15. Chamber cover in correct (left) and incorrect (right) orientations

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.

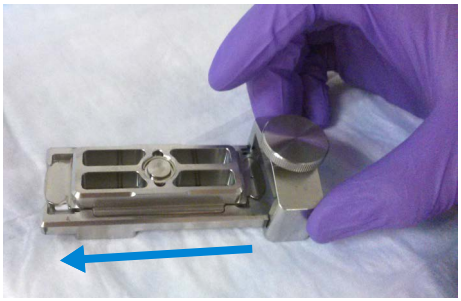


Figure 16. Slipping the clamp onto the chamber base

3 Firmly tighten the thumbscrew fully.

The slides will not be harmed by hand-tightening.

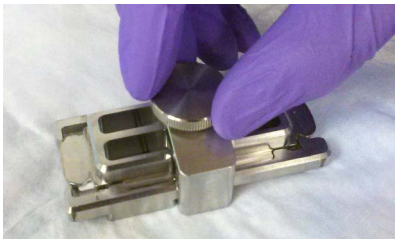


Figure 17. Tightening of the thumbscrew on the clamp

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 18**).

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

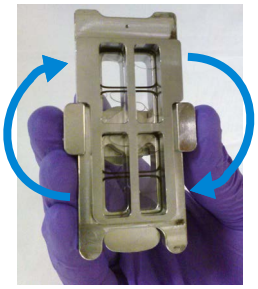


Figure 18. Rotation of the final assembled chamber

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.

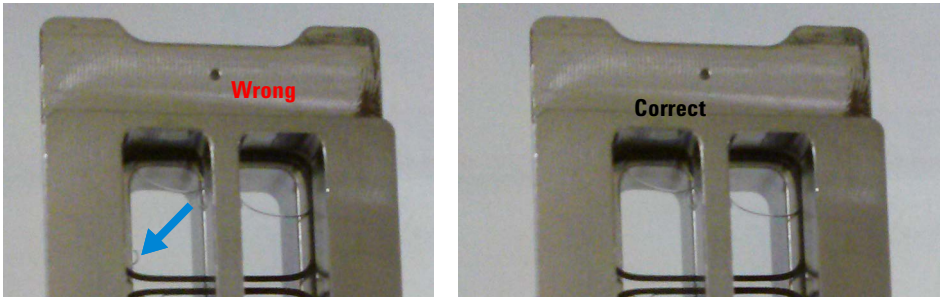


Figure 19. The slide on the left shows a stray, stationary bubble (denoted with arrow), which must be removed before hybridization. The slide on the right shows only large mixing bubbles, which move freely around the chamber when rotated. Bubbles are acceptable, as long as they move freely when you rotate 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 20. 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 65°C for ULS and 67°C for Enzymatic Labeling for:

- 24 hours for blood, cell and tissue samples (4-pack and 8-pack microarrays)
- 40 hours for blood, cell and tissue samples (1-pack and 2-pack microarrays)
- 40 hours for FFPE samples (1-pack, 2-pack, 4-pack and 8-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 99.

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. These products are available through filter suppliers.

Before you begin, determine which wash procedure to use:

Table 65 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 101	No
> 5 ppb < 10 ppb	**"Wash Procedure A (without Stabilization and Drying Solution)"** on page 101	Yes
> 10 ppb	**"Wash Procedure B (with Stabilization and Drying Solution)"** on page 105	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 105.

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

Table 66 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.

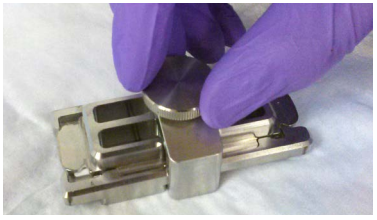


Figure 21. Loosening of the thumbscrew

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

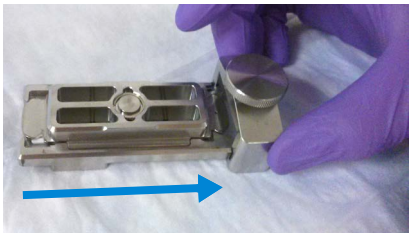


Figure 22. Removal of the clamp

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!*

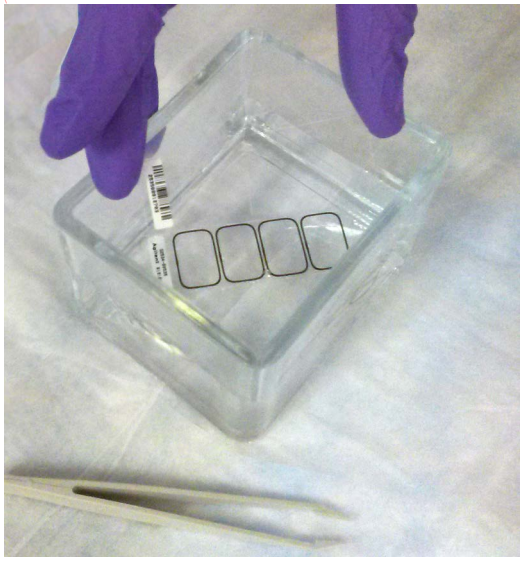


Figure 23. Removal of the microarray slide from the staining dish

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

Table 67 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 101.

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

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

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

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

NOTE

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

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

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

Step 6. Put slides in a slide holder

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

For SureScan microarray scanner

1. 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.

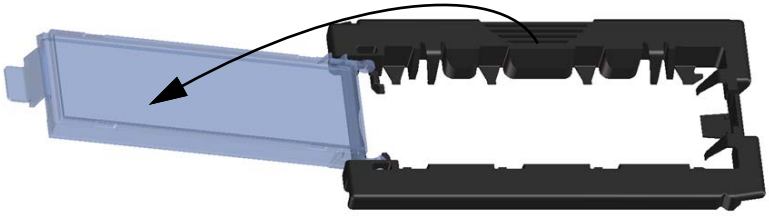


Figure 24. Opening the slide holder

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 25**.
   - **b** Carefully place the end of the slide without the barcode label onto the slide ledge. See **Figure 25**.

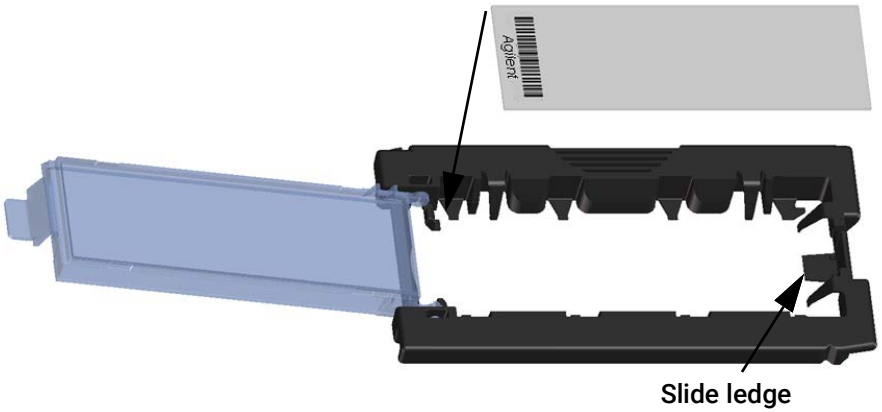


Figure 25. Inserting slide into the slide holder

c Gently lower the slide into the slide holder. See **Figure 26**.

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 27**. Make sure that the slide holder is completely closed.

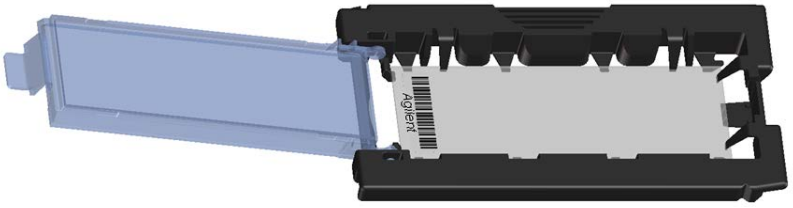


Figure 26. Slide inserted in slide holder – cover open

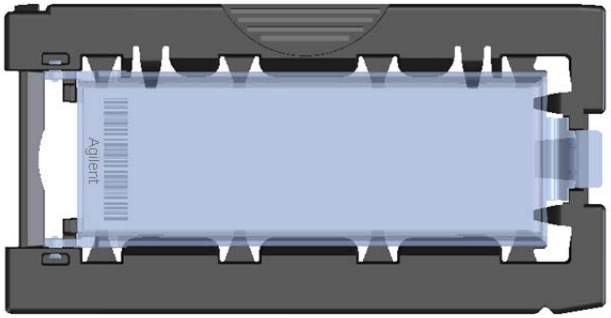


Figure 27. Slide inserted in slide holder – cover closed

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

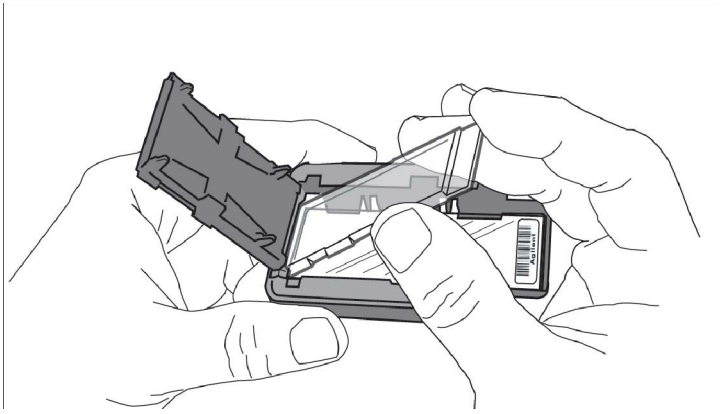


Figure 28. Inserting the ozone-barrier slide cover

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

Microarray Scanning and Analysis

Step 1. Scan the microarray slides

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

Agilent provides support for Agilent microarrays scanned on select non-Agilent scanners. Please see "Feature Extraction Compatibility Matrix for Non Agilent scanners" for scanner compatibility and settings (**www.agilent.com/cs/library/usermanuals/Public/G1662-90043_ScannerCompatibilityMatrix.pdf**).

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

Agilent SureScan Microarray Scanner

1 Put assembled slide holders into the scanner cassette. Refer to **Figure 29** and **Figure 30**.

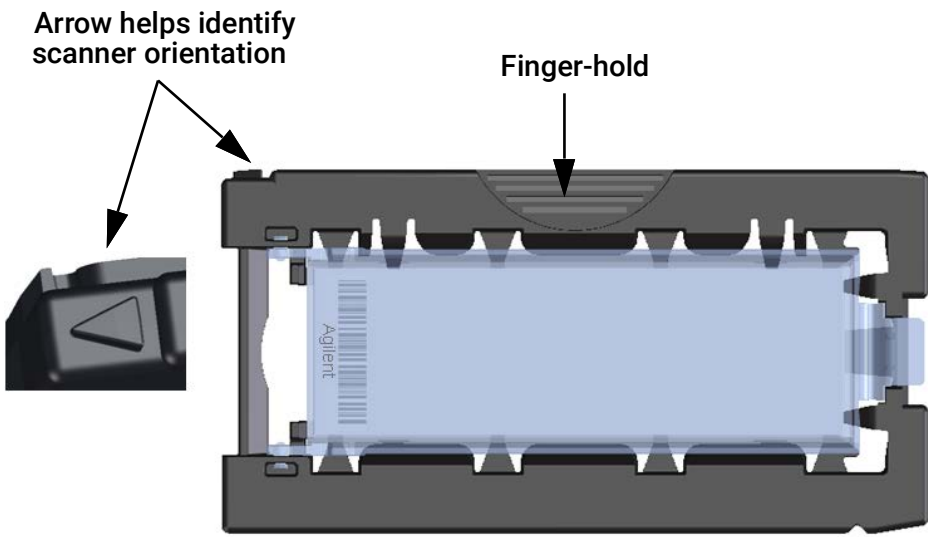


Figure 29. Slide holder helps you to insert slides correctly

Figure 30. 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 68**.

Table 68 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
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

These metrics are only appropriate for samples analyzed with Agilent CGH microarrays by following the standard operational procedures provided in this user guide. These metrics are reported in the Feature Extraction QC report generated by Feature Extraction (standalone or as included in the Agilent CytoGenomics software). They can be used to assess the relative data quality from a set of microarrays in an experiment. In some cases, they can indicate potential processing errors that have occurred or suggest that the data from particular microarrays might be compromised. Many factors can influence the range of these metrics, including 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.

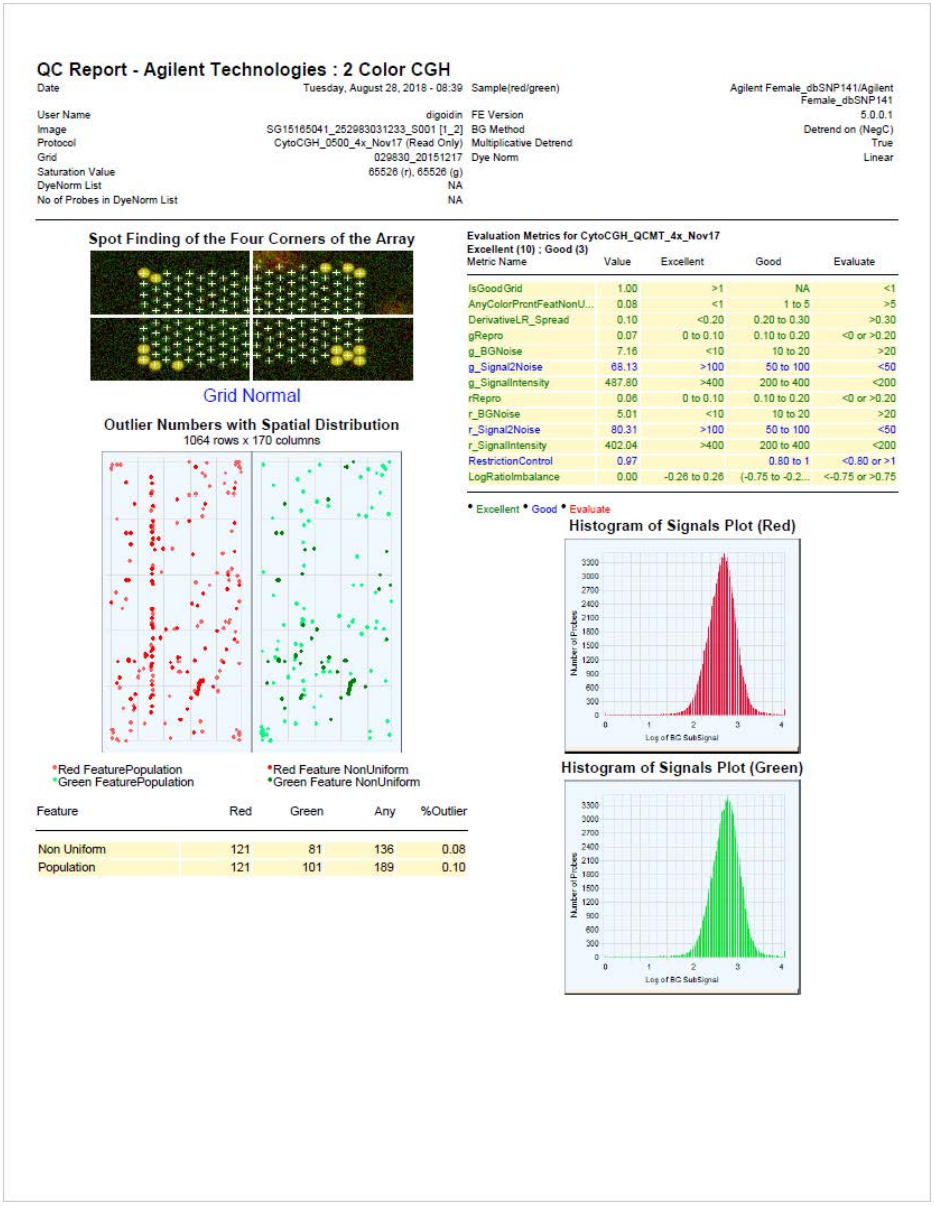
To achieve a high SNP call rate and accuracy when processing SurePrint G3 CGH+SNP microarrays, make sure the DLRSD is <0.2.

Table 69 QC metric thresholds for ULS labeling

	Blood and Cell Samples	Tissue Samples	FFPE Samples
BGNoise	<15	<15	<15
Signal Intensity	>90	>90	>90
Signal to Noise	>20	>20	>10
Reproducibility	<0.2	<0.2	<0.2
DLRSD	<0.2	<0.3	<0.4

Table 70 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 31. Feature Extraction QC Report, page 1

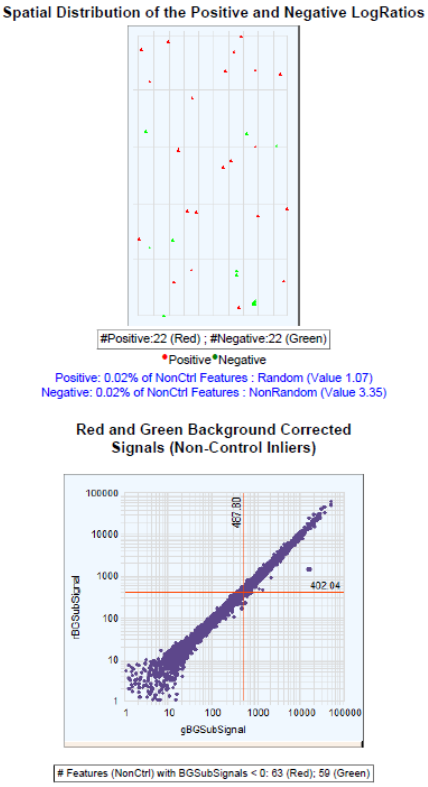


Figure 32. Feature Extraction QC Report, page 2

Step 3. Download Agilent Reference Genotype File

To analyze your data when you have used the Human Reference DNA (Male and Female) and CGH+SNP microarrays with Agilent CytoGenomics 2.0 or Genomic Workbench 6.5 or higher, you need to get the Agilent Reference Genotype File (Male and Female) from Agilent.

The Reference Genotype File is included with Agilent CytoGenomics 2.7.

1 Go to **www.genomics.agilent.com/article.jsp?pageId=3299**.

2 Click the **Download** link for **Agilent Reference Genotype Files (Male and Female)**.

3 Click **Save** to save the files.

To load the Agilent Reference Genotype Files (Male and Female), refer to the user guide for Agilent CytoGenomics or Agilent Genomic Workbench.

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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.

For troubleshooting information for the Bravo platform, refer to the *Bravo Platform User Guide* (publication G5409-90004) and the *VWorks Software User Guide* (publication G5415-90002).

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, degree of labeling or specific activity"** on page 54, **page 68** or **page 83**.

Repurify the DNA using the Qiagen DNeasy protocol. See **"DNA Isolation"** on page 23. 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.

The ULS labeling is very sensitive to salt contamination. Use 80% EtOH instead of AW2 in the DNA extraction step and elute in water.

The ULS Labeling also labels proteins and RNA that compete for dye. Make sure that the DNA is free of proteins and RNA. See **"DNA Isolation"** on page 23.

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 23. This procedure includes a RNase A treatment.

The ULS labeling also labels RNA that will hybridize to the probe.

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 23.

Make sure that the DNA is not over-fragmented prior to labeling. Possible causes are incorrect temperature or length of incubation at 95°C for ULS or 98°C for Enzymatic Labeling, 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.

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 water (or any of the recommended buffers) for ULS labeling, or **Buffer AE** or **1×TE (pH 8.0)** for Enzymatic labeling. 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 23.

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 after ULS labeling"** on page 53 or **"Step 2. Purification after Enzymatic Labeling"** on page 67 or **page 81** to remove unreacted dye. Many other columns result in the loss of shorter fragments.

If you have post-labeling signal loss

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

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

Check that the oven temperature is 65°C for ULS and 67°C for Enzymatic Labeling. If needed, recalibrate the hybridization oven. Follow the steps in *Agilent G2545A Hybridization Calibration Procedure* (publication 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 107.

If you have high BGNoise values

High BGNoise can cause lower signal-to-noise values (see **Table 70** or **Table 71** 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 70** or **Table 71** 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.

7 Reference

This chapter contains reference information that pertains to this protocol.

Refer also to the following Agilent publications:

- Bravo Automated Liquid Handling Platform User Guide (publication G5409-90004)
- VWorks Automation Control Setup Guide (publication G5415-90003)
- VWorks Automation Control User Guide (publication G5415-90002)
- VWorks Software Quick Start (publication G5415-90004)

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 Primer

Genomic DNA High-Throughput ULS Labeling Kit

Agilent-CGHblock
10× Labeling Solution
ULS-Cy5 Reagent
ULS-Cy3 Reagent

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

† Not used in this protocol.

Genomic DNA 96-well Purification Module

96-well Agilent-KREApure purification plate
96-well collection plate
96-well wash plate

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.

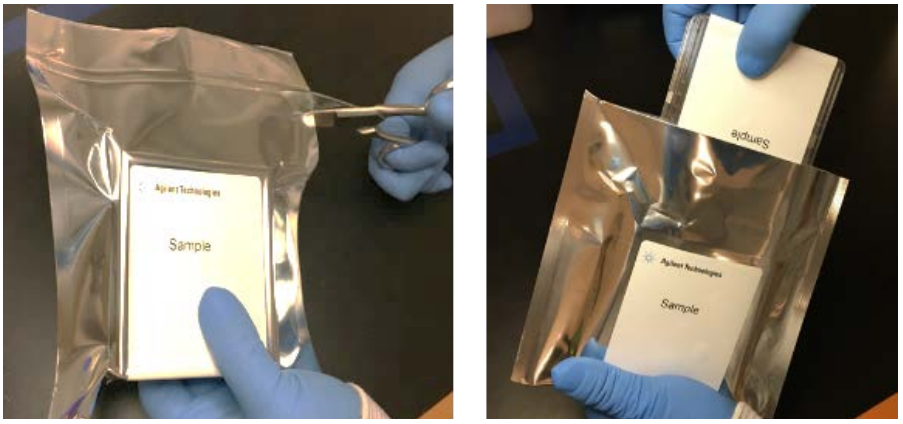

Figure 33. Opening foil pouch (left) and removing the slide box (right)

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.

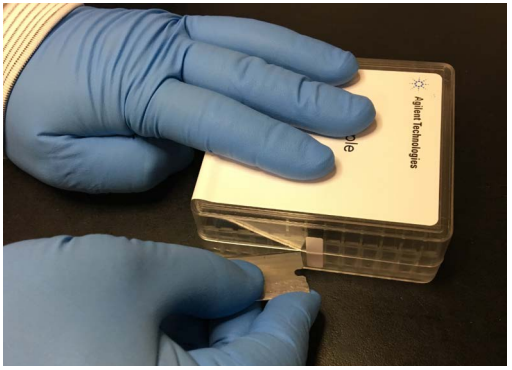

Figure 34. Cutting the sealing tape

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

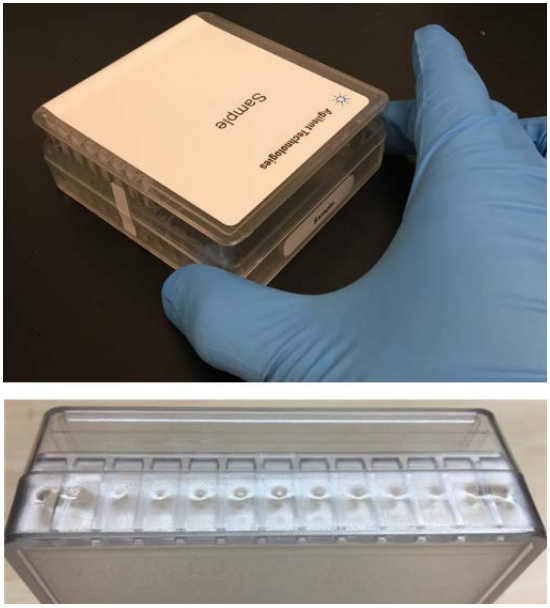

Figure 35. Gripping the base at the indentations (top) and close-up of the indentations (bottom)

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.

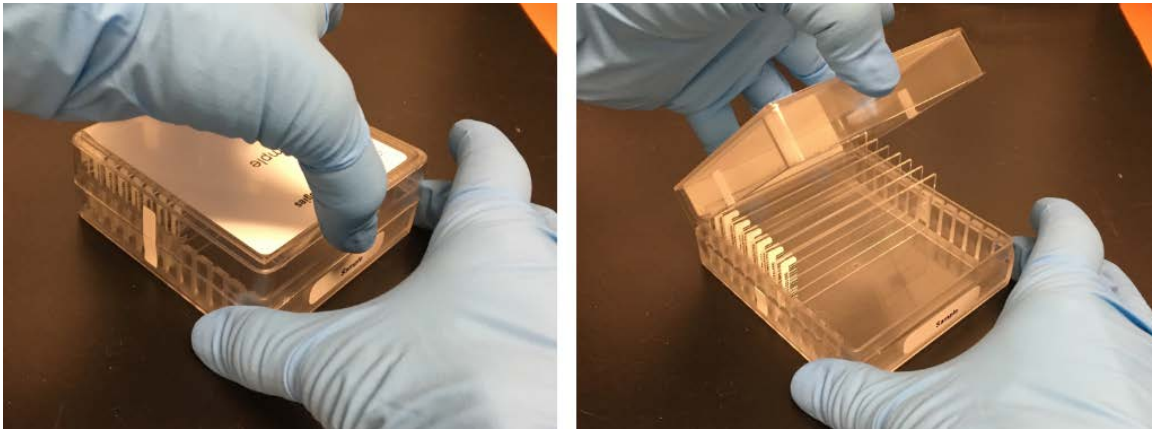

Figure 36. Grasping the lid (left) and lifting the lid from the base (right)

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 (p/n 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

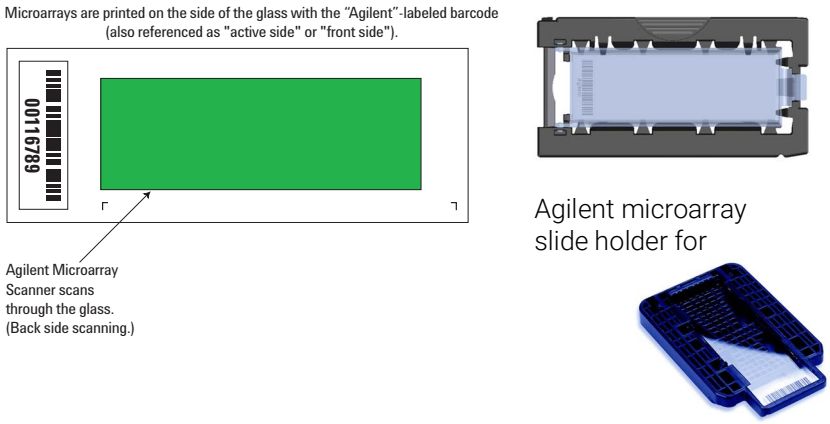

Figure 37. Agilent microarray slide and slide holder

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 37 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 38**).

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

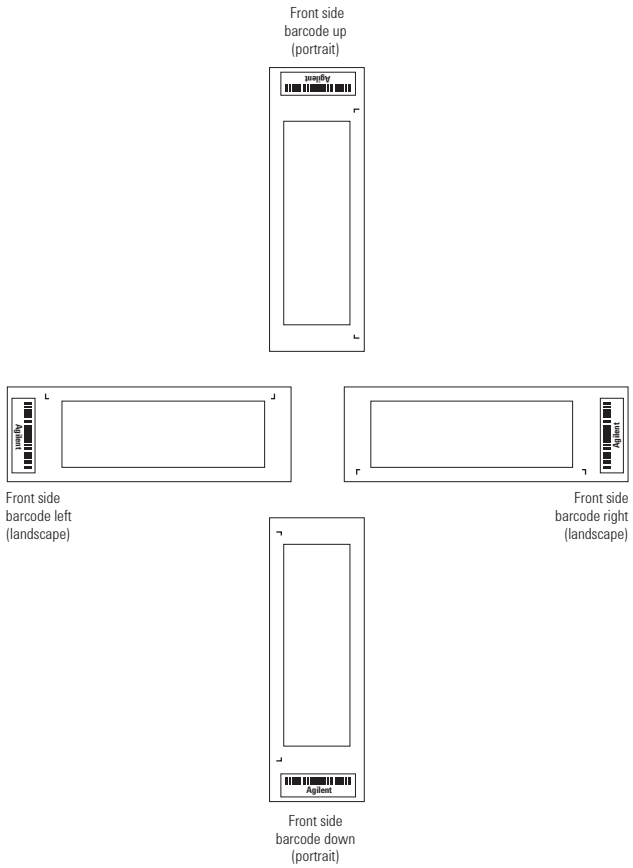

Figure 38. Microarray slide orientation

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 39. 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 40. 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 41. 8-pack microarray slide

In This Book

This guide contains information to run the Oligonucleotide Array-Based CGH for Genomic DNA Analysis - Bravo Automated Liquid Handling Platform with Enzymatic and ULS Labeling protocol.

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April 2025
Version 3.0

G4410-90040 Revision C0

Agilent