Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis

Bravo Automated Liquid Handling Platform with Enzymatic and ULS Labeling

Protocol

Version 1.1, November 2010

Agilent Technologies

Notices

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CAUTION

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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 Agilent's recommended operational procedures to analyze DNA copy number variations using Agilent 60-mer oligonucleotide microarrays for array-based comparative genomic hybridization (aCGH) analysis. This protocol is specifically developed and optimized to 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 DNA 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 and Feature Extraction

This chapter describes the steps to hybridize, wash and scan Agilent CGH microarrays and to extract data using the Agilent Feature Extraction Software.

6 Troubleshooting

This chapter contains the 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 protocol.

What's New in Version 1.1?

- Processing of 1x1M, 2x400K, 4x180K and 8x60K SurePrint G3 CGH+SNP microarrays for enzymatic labeling is supported.
- Guidelines for gDNA Quantitation and Quality Analysis are expanded.
- Proteinase K step in DNA Isolation is lowered to 56°C.

Content

Contents

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, 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

- **ULS-Cy3, ULS-Cy5, Cyanine 3-dUTP and Cyanine 5-dUTP 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.**
- **2X 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 Agilent's 2X Hi-RPM Hybridization Buffer.**
- **Agilent 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 entire 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.

Catalog CGH Microarray Kits

- Five 1-inch x 3-inch glass slides for 1x and 2x Microarray Kits
- Three 1-inch x 3-inch glass slides for 4x and 8x Microarray Kits
- Disk that contains microarray design files in various file formats

Design files can be downloaded from https://earray.chem.agilent.com/earray.

See Table 1 and Table 2 for available designs. For more information on CGH designs, go to http://www.genomics.agilent.com. Under **Products**, click **By Application > Comparative Genomic Hybridization (CGH)**.

Table 1 Available Catalog SurePrint HD CGH Microarray Kits

Part Number	Description
G4411B	Human Genome CGH Microarray Kit 1x244K
G4412A	Human Genome CGH Microarray Kit 2x105K
G4417A	Human CNV Association Microarray Kit 2x105K
G4413A	Human Genome CGH Microarray Kit 4x44K
G4415A	Mouse Genome CGH Microarray Kit 1x244K
G4416A	Mouse Genome CGH Microarray Kit 2x105K
G4435A	Rat Genome CGH Microarray Kit 1x244K
G4436A	Rat Genome CGH Microarray Kit 2x105K

Table 2 Available Catalog SurePrint G3 CGH Microarray Kits

Part Number	Description
G4447A	SurePrint G3 Human CGH Microarray Kit 1x1M
G4448A	SurePrint G3 Human CGH Microarray Kit 2x400K

Table 2 Available Catalog SurePrint G3 CGH Microarray Kits (continued)

Part Number	Description
G4449A	SurePrint G3 Human CGH Microarray Kit 4x180K
G4450A	SurePrint G3 Human CGH Microarray Kit 8x60K
G4506A	SurePrint G3 Human High-Resolution Discovery Microarray 1x1M
G4507A	SurePrint G3 Human CNV Microarray Kit 2x400K
G4838A	SurePrint G3 Mouse CGH Microarray Kit 1x1M
G4839A	SurePrint G3 Mouse CGH Microarray Kit 4x180K
G4840A	SurePrint G3 Rat CGH Microarray Kit 1x1M
G4841A	SurePrint G3 Rat CGH Microarray Kit 4x180K
G4842A*	SurePrint G3 Human CGH+SNP Microarray Kit 2x400K
G4890A*	SurePrint G3 Human CGH+SNP Microarray Kit 4x180K

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

Unrestricted SurePrint HD and G3 CGH Microarray Kits

- One, two, four or eight microarray(s) printed on each 1-inch x 3-inch glass slide
- Number of microarrays varies per kit and per order
- Disk that contains microarray design files in various file formats

See Table 3 and Table 4 for available designs.

Table 3 Unrestricted High-Definition CGH Microarrays

Part Number	Description
G4423B, AMADID 014693	Unrestricted HD-CGH Microarray, 1x244K, Human (same design as G4411B)
G4423B, AMADID 016266	Unrestricted HD-CGH Microarray, 1x244K supplemental, Human
G4423B, AMADID 014695	Unrestricted HD-CGH Microarray, 1x244K, Mouse (same design as G4415A)
G4423B, AMADID 015223	Unrestricted HD-CGH Microarray, 1x244K, Rat (same design as G4435A)
G4423B, AMADID 019553	Unrestricted HD-CGH Microarray, 1x244K, Chicken
G4425B, AMADID 014698	Unrestricted HD-CGH Microarray, 2x105K, Human (same design as G4412A)
G4425B, AMADID 022837	Unrestricted HD-CGH Microarray, 2x105K, Human (same design as G4417A)
G4425B, AMADID 014699	Unrestricted HD-CGH Microarray, 2x105K, Mouse (same design as G4416A)
G4425B, AMADID 015235	Unrestricted HD-CGH Microarray, 2x105K, Rat (same design as G4436A)
G4426B, AMADID 014950	Unrestricted HD-CGH Microarray, 4x44K, Human (same design as G4413A)
G4426B, AMADID 015028	Unrestricted HD-CGH Microarray, 4x44K, Mouse

Table 4 Unrestricted SurePrint G3 CGH Microarrays

Part Number	Description
G4824A, AMADID 021529	Unrestricted SurePrint G3 CGH Microarray, 1x1M, Human (same design as G4447A)
G4824A, AMADID 023642	Unrestricted SurePrint G3 CGH Microarray, 1x1M, Human (same design as G4506A)
G4824A, AMADID 027414	Unrestricted SurePrint G3 CGH Microarray, 1x1M, Mouse (same design as G4838A)
G4824A, AMADID 027065	Unrestricted SurePrint G3 CGH Microarray, 1x1M, Rat (same design as G4840A)
G4825A, AMADID 021850	Unrestricted SurePrint G3 CGH Microarray, 2x400K, Human (same design as G4448A)
G4825A, AMADID 021365	Unrestricted SurePrint G3 CGH Microarray, 2x400K, Human (same design as G4507A)
G4826A, AMADID 022060	Unrestricted SurePrint G3 CGH Microarray, 4x180K, Human (same design as G4449A)
G4826A, AMADID 027411	Unrestricted SurePrint G3 CGH Microarray, 4x180K, Mouse (same design as G4839A)
G4826A, AMADID 027064	Unrestricted SurePrint G3 CGH Microarray, 4x180K, Rat (same design as G4841A)
G4826A, AMADID 024422	Unrestricted SurePrint G3 CGH Microarray, 4x180K, Chimpanzee
G4826A, AMADID 024419	Unrestricted SurePrint G3 CGH Microarray, 4x180K, Rhesus Macaque
G4826A, AMADID 025242	Unrestricted SurePrint G3 CGH Microarray, 4x180K, Bovine
G4826A, AMADID 025522	Unrestricted SurePrint G3 CGH Microarray, 4x180K, Canine
G4826A, AMADID 025843	Unrestricted SurePrint G3 CGH Microarray, 4x180K, Rice
G4827A, AMADID 021924	Unrestricted SurePrint G3 CGH Microarray, 8x60K, Human (same design as G4450A)

Custom SurePrint HD and G3 Microarray Kits

- 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 Table 5 and Table 6 for available formats.

Table 5 Custom High-Definition CGH Microarrays

Part Number	Description
G4423A	Custom HD-CGH Microarray, 1x244K
G4425A	Custom HD-CGH Microarray, 2x105K
G4426A	Custom HD-CGH Microarray, 4x44K
G4427A	Custom HD-CGH Microarray, 8x15K

Table 6 Custom SurePrint G3 CGH Microarrays

Part Number	Description
G4123A	SurePrint G3 Custom CGH Microarray, 1x1M
G4124A	SurePrint G3 Custom CGH Microarray, 2x400K
G4125A	SurePrint G3 Custom CGH Microarray, 4x180K
G4126A	SurePrint G3 Custom CGH Microarray, 8x60K
G4882A*	SurePrint G3 Custom CGH+SNP Microarray, 1x1M
G4883A*	SurePrint G3 Custom CGH+SNP Microarray, 2x400K
G4884A*	SurePrint G3 Custom CGH+SNP Microarray, 4x180K
G4885A*	SurePrint G3 Custom CGH+SNP Microarray, 8x60K

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

Required Equipment

Table 7 Required equipment

Description	Company and part no.
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 1x244K, 2x105K, 4x44K or 8x15K, *or* for 1x1M, 2x400K, 4x180K or 8x60K	 Agilent p/n G2565BA or G2565CA Agilent p/n G2565CA
Hybridization Chamber, stainless	Agilent p/n G2534A
Hybridization Chamber gasket slides, 5-pack (20 and 100 packaging sizes are available) for 1x microarrays *or* for 2x microarrays *or* for 4x microarrays *or* for 8x 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	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 PP/PS *or* Manual Heat Sealer Peel-it-lite Foil (removable)	Agilent p/n G5402A and Agilent p/n 24210-001 Eppendorf p/n 951023078 and Eppendorf p/n 951023205
UV-Transilluminator with SYBR photographic filter	Alpha Innotech p/n Alphalmager 2000 or equivalent
Nuclease-free 1.5 mL microfuge tubes (sustainable at 95°C)	Ambion p/n AM12400 or equivalent

Table 7 Required equipment **(continued)**

Description	Company and part no.
Magnetic stir bar (×2 or ×4)†	Corning p/n 401435 or equivalent
Magnetic stir plate (×1 or ×3)†	Corning p/n 6795-410 or equivalent
Magnetic stir plate with heating element	Corning p/n 6795-420 or equivalent
Microcentrifuge	Eppendorf p/n 5430 or equivalent
Thermocycler with heated lid	Eppendorf p/n 950000015 or equivalent
Full skirted 96-well plate PCR plates compatible with Bravo platform and thermocycler	Eppendorf p/n 951020401
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‡	Invitrogen p/n G5300-01
E-Gel PowerBase v.4‡	Invitrogen p/n G6200-04
Qubit Fluorometer**	Invitrogen p/n Q32857
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
250 mL capacity slide-staining dish, with slide rack (×3 or ×5)†	Wheaton p/n 900200 *or* Thermo Shandon p/n 121
Circulating water baths or heat blocks set to 56°and 90°C (for DNA extraction)	
Ice bucket	
Clean forceps	
Powder-free gloves	
Sterile, nuclease-free aerosol barrier pipette tips	
Timer	

Table 7 Required equipment **(continued)**

Description	Company and part no.
Vacuum dessicator or N_2 purge box for slide storage	
Vortex mixer	

* Optional when processing arrays in environments in which ozone levels are 5 ppb or higher.

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

‡ Optional when Invitrogen E-gels are used.

** Optional.

Table 8 Required equipment for hybridization preparation for 1x microarrays.

Description	Company and part no.
Deep-well plates	ABgene p/n AB-0859
Eppendorf ThermoStat	Eppendorf p/n 022670204
Eppendorf Deep-Well Plate Block	Eppendorf p/n 022670565

Table 9 Required equipment for hybridization preparation for 2x microarrays.

Description	Company and part no.
Tall Chimney PCR plates	ABgene p/n AB-1184

Table 10 Required equipment for 4x and 8x microarrays.

Description	Company and part no.
Vacuum Concentrator	Thermo Scientific p/n DNA120-115 or equivalent

Table 11 Optional equipment for DNA extraction from tissue or FFPE samples.

Description	Company and part no.
Thermal shaker	Eppendorf Thermomixer p/n 022670000 or equivalent

Required Reagents

Table 12 Required reagents for gDNA isolation

Description	Company and part no.
Phosphate Buffered Saline pH 7.4 (PBS)	Amresco p/n E504-500ML
Clear E-Gel 18-Pak (1.2% agarose, no stain)	Invitrogen p/n G5518-01
SYBR Gold Nucleic Acid Gel Stain	Invitrogen p/n S11494
TrackIt 1 Kb DNA Ladder	Invitrogen p/n 10488-072
Quant-IT dsDNA BR Assay Kit, for use with the Qubit fluorometer (100 assays)*	Invitrogen p/n Q32850
Qiagen RNase A (100 mg/mL)	Qiagen p/n 19101
Qiagen DNeasy Blood & Tissue Kit	Qiagen p/n 69504
Qiagen Proteinase K (>600 mAU/mL, solution)	Qiagen p/n 19131
Sodium thiocyanate (NaSCN)†	Sigma 467871-50G
Ethanol (95% to 100% molecular biology grade)	Sigma p/n E7023-6x500ML
Tween 20†	Sigma p/n P9416-50ML

* Optional.

† Optional components if isolating DNA from FFPE samples.

Table 13 Required reagents for enzymatic sample prep and labeling

Description	Company and part no.
Agilent Genomic DNA Enzymatic Labeling Kit	Agilent p/n 5190-0449
AutoScreen-96A Well plates	GE Healthcare p/n 25-9005-98

Table 13 Required reagents for enzymatic sample prep and labeling (continued)

Description (continued)	Company and part no.
For possible use as a reference sample:	
Human Genomic DNA *or*	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 p/n NA18579
Mouse Genomic DNA *or*	Jackson Labs p/n 000664 (female and male)
Rat Genomic DNA	Harlan Sprague Dawley (custom)
Alu I (10 U/µL)*	Promega p/n R6281
Rsa I (10 U/µL)*	Promega p/n R6371
1 × TE (pH 8.0), Molecular grade	Promega p/n V6231
GenElute PCR Clean-Up Kit†	Sigma p/n NA1020
GenomePlex Complete Whole Genome Amplification Kit†	Sigma p/n WGA2

* Optional component recommended if doing a restriction digestion step when you process CGH microarrays. Required when you process SurePrint G3 CGH+SNP microarrays.

† Optional components recommended if using the Amplification Method for sample preparation.

Table 14 Required reagents for ULS sample prep and labeling*

Description	Company and part no.
Genomic DNA High-Throughput ULS Labeling Kit	Agilent p/n 5190-0450
Genomic DNA 96-well Purification Module	Agilent p/n 5190-0451
For possible use as a reference sample:	
Human Genomic DNA *or*	Promega p/n G1521 (female) or p/n G1471 (male)
Mouse Genomic DNA *or*	Jackson Labs p/n 000664 (female and male)
Rat Genomic DNA	Harlan Sprague Dawley (custom)

* ULS is not supported for CGH+SNP.

Table 15 Required reagents for hybridization and wash

Description	Company and part no.
Agilent Oligo aCGH Wash Buffer 1 and 2 set *or* Agilent Oligo aCGH Wash Buffer 1 (4 L) Agilent Oligo aCGH Wash Buffer 2 (4 L)	Agilent p/n 5188-5226 Agilent p/n 5188-5221 Agilent p/n 5188-5222
Stabilization and Drying Solution, 500 mL*	Agilent p/n 5185-5979
Agilent Oligo aCGH Hybridization Kit	Agilent p/n 5188-5220 (25) or p/n 5188-5380 (100)
Human Cot-1 DNA *or* Mouse Cot-1 DNA *or* Rat Hybloc	Invitrogen p/n 15279-011 or 15279-101 or Kreatech p/n EA-020 Invitrogen p/n 18440-016 Applied Genetics p/n RHB
Acetonitrile*	Sigma 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. See http://www.genomics.agilent.com.
- Refer to the Agilent Scanner and Feature Extraction manuals for minimum memory requirements and other specifications. Go to http://www.genomics.agilent.com.

2 DNA Isolation

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

Agilent's array-based Comparative Genomic Hybridization (aCGH) application uses a "two-color" process to measure DNA copy number changes 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.

This chapter describes Agilent's recommended procedure to isolate genomic DNA (gDNA) from blood, cells, frozen or FFPE tissues using the Qiagen DNeasy Blood & Tissue Kit (p/n 69504). 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 32.

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 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. Or you can genotype your own reference 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 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 (DLRSD). Different DNA isolation methods can create different quantitation artifacts, so a higher risk of assay noise exists when the experimental and reference DNA samples are isolated from different sources (for example, experimental DNA isolated from blood and reference DNA obtained from a Coriell cell line).

To minimize assay noise, especially when experimental and reference samples are isolated from different sources, measure the DNA concentration with both a spectrophotometer (e.g. Nanodrop) and a fluorometer (e.g. Qubit) to give you two independent methods of measurement.

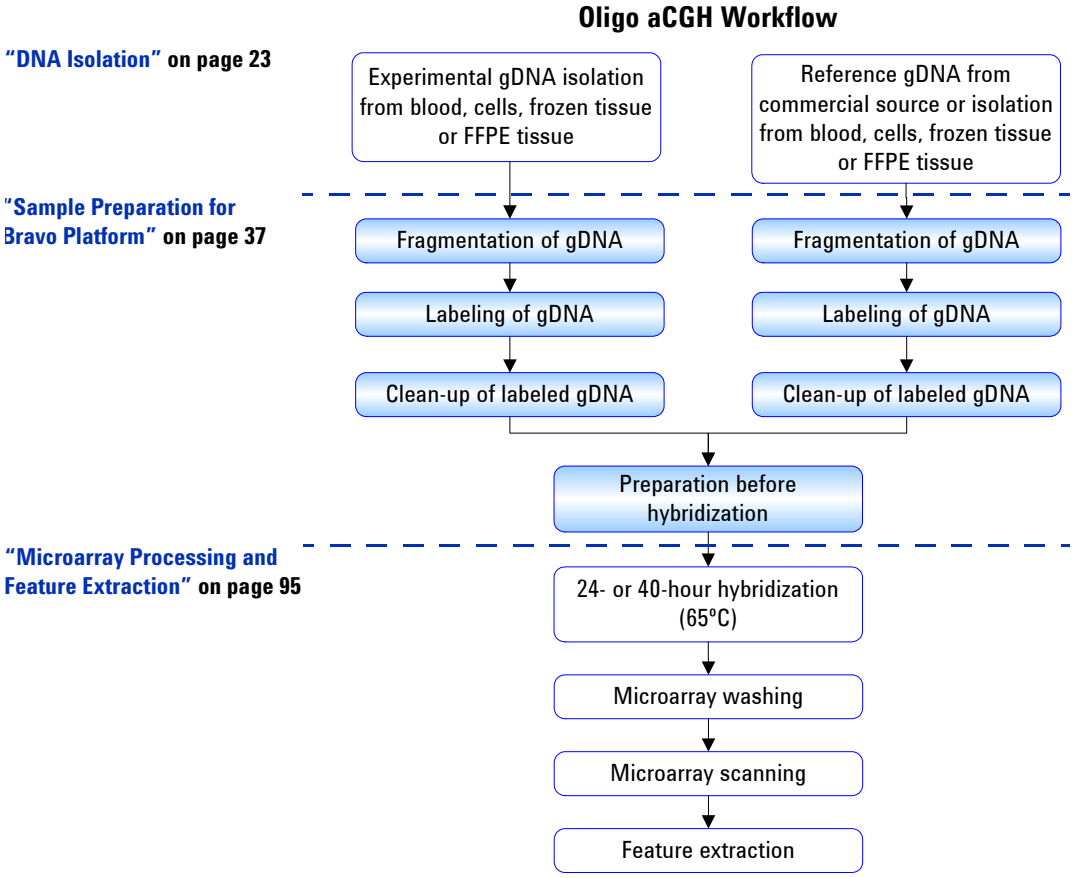

Figure 1 Workflow diagram for sample preparation and microarray processing. Steps that are in blue use the Bravo Automated Liquid Handling Platform.

Blood, Cells or Frozen Tissues

This section describes Agilent's recommended procedure to isolate genomic DNA (gDNA) from blood, cells or frozen tissues using the Qiagen DNeasy Blood & Tissue Kit (p/n 69504).

Table 16 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*
1x microarray	1500	500	1000
2x microarray	1000	500	1000
4x microarray	500	500	1000
8x microarray	250	200	500

* You can use more gDNA, but the gDNA needs to be at a higher concentration. See Table 18, Table 19 and Table 20 on page 39 for required concentrations.

Step 1. gDNA Extraction

1. Equilibrate a thermomixer to 55°C and heat block or water bath to 56°C.
2. For blood with nonnucleated erythrocytes (mammals):
   a. Put 20 µL proteinase K (supplied with Qiagen DNeasy Blood & Tissue Kit) into the bottom of a 1.5 mL microfuge tube.
   b. Add 50 to 100 µL anticoagulated blood.
   c. Add enough 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 proteinase K (supplied with Qiagen DNeasy Blood & Tissue Kit) into the bottom of a 1.5 mL microfuge tube.
   b. Add 5 to 10 µL anticoagulated blood.
   c. Add enough PBS to make a total volume of 220 µL.
   d. Go to step 7.

4 For cells:

   a Spin a maximum of $5x10^6$ cells in a centrifuge for 5 minutes at 300 x g. Resuspend the pellet in 200 µL PBS.

   b Add 20 µL proteinase K (supplied with Qiagen DNeasy Blood & Tissue Kit).

   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 microfuge tube.

   b Add 180 µL Buffer ATL (supplied with Qiagen DNeasy Blood & Tissue Kit).

   c Add 20 µL proteinase K (supplied).

   d Mix well on a vortex mixer.

   e Incubate in a thermomixer at 55°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 x 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 PBS to make a total volume of 220 µL.

   c Add 20 µL proteinase K (supplied with Qiagen DNeasy Blood & Tissue Kit).

7 Add 4 µL 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 x g to drive the contents off the walls and lid.

8 Add 200 µL Buffer AL (supplied) 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 x g to drive the contents off the walls and lid.

9 Add 200 µL 100% ethanol to each sample, and mix thoroughly on a vortex mixer. Spin in a microcentrifuge for 30 seconds at 6,000 x 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 (supplied). Spin in a centrifuge at 6,000 x g for 1 minute. Discard the flow-through and collection tube. Put the DNeasy Mini spin column in a new 2 mL collection tube (supplied).

11 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.

12 Add 500 µL Buffer AW1 onto the column, and spin in a microcentrifuge for 1 minute at 6,000 x g. Discard the flow-through and collection tube. Put the DNeasy Mini spin column in a new 2 mL collection tube (supplied).

13 For Enzymatic labeling: Before using for the first time, prepare Buffer AW2 by adding 100% ethanol to the Buffer AW2 bottle (supplied; 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 nuclease-free water.

CAUTION

For ULS labeling: Do *not* use Buffer AW2 supplied with the Qiagen 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 x 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 microcentrifuge tube, and pipette the amount below directly onto the center of the DNeasy column membrane.

- For ULS labeling: 200 µL of nuclease free water
- For Enzymatic labeling: 200 µL of Buffer AE (supplied)

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

18 Repeat elution with nuclease-free 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 32 for details on how to isolate gDNA from FFPE tissues.

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

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.

UV-VIS Spectrophotometry

1. In the Nanodrop program menu, select **Nucleic Acid Measurement**, then select **Sample Type** to be **DNA-50**.
2. Use 1.5 µL of nuclease-free water (for ULS labeling) or elution buffer (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

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

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.

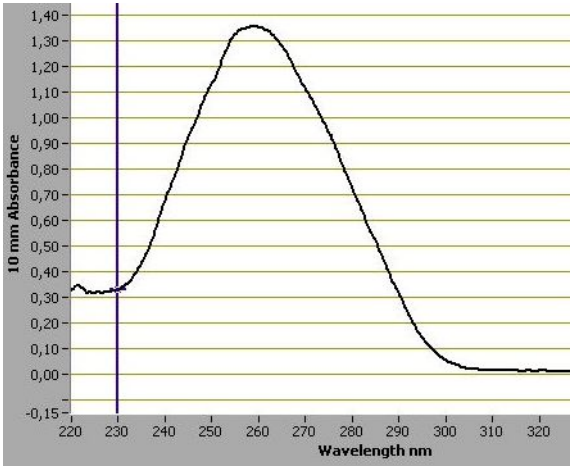

Figure 2 Typical spectrum of pure DNA

Agarose Gel Electrophoresis

1. Load 20 ng gDNA for each sample in a volume of 10 µL nuclease-free water in the well of a single-comb 1.2% Clear E-Gel. (You do not need to add loading buffer in this system).
2. As a control, load 20 ng of commercial Human Genomic DNA in a volume of 10 µL nuclease free water in one of the wells of the E-Gel.
3. Mix 5 µL TrackIt 1 Kb DNA Ladder with 95 µL 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 nuclease-free water) in a plastic tray for 15 minutes.
7. Visualize the gel on the UV-transilluminator using a SYBR Gold photographic filter.

FFPE Tissues

This section describes Agilent's recommended procedure to isolate genomic DNA (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 Qiagen DNeasy Blood & Tissue Kit (p/n 69504). Determine the number of FFPE sections needed for your experiment based on the estimates summarized in Table 17. One 20 micron FFPE section containing 1 cm^2 of tissue is estimated to generate a minimal yield of 500 ng of gDNA.

For more information about CGH experiments on FFPE samples, please refer to the application note "Copy Number Analysis of Archival FFPE Tumor Samples by Oligo Array CGH" (p/n 5989-7120EN) available from the Agilent Web site at www.agilent.com/chem/dnaapplications. Note that the CGH+SNP platform does not support FFPE samples.

Table 17 Estimated number of 20 micron FFPE sections needed per microarray

Microarray format	gDNA input amount requirement (ng)	Estimated number of 20 micron FFPE sections
1x microarray	2000	4 to 5
2x microarray	1000	3
4x microarray	500	2
8x microarray	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 nuclease-free microfuge tube.
3. Prepare 10% Tween 20, by adding 100 µL Tween 20 to 900 µL of nuclease-free water. The solution can be prepared in advance and stored up to 6 months at room temperature.
4. Add 480 µL PBS and 20 µL 10% Tween 20 to the FFPE sections in the 1.5 mL nuclease-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 x 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 x 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 NaSCN to 123 mL of nuclease free water. The solution can be prepared in advance and stored up to 1 month at room temperature.
13. Add 400 µL 1M 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. Shake at 450 rpm.

Step 2. Proteinase K Treatment

1. Equilibrate a thermomixer to 55°C.
2. Transfer the sample tube to a microcentrifuge. Spin for 20 minutes at 10,000 x g.
3. Remove and discard the supernatant without disturbing the pellet.
4. Add 400 µL PBS to the pellet and vortex briefly.
5. Spin again for 20 minutes at 10,000 x g in a microcentrifuge.
6. Remove and discard the supernatant without disturbing the pellet.
7. Add 360 µL of Qiagen buffer ATL (supplied with Qiagen DNeasy Blood & Tissue Kit).
8. Add 40 µL proteinase K (supplied), mix well on a vortex mixer, and incubate overnight in a thermomixer at 55°C shaking at 450 rpm.
9. Transfer the sample tube to a microcentrifuge. Spin for 30 seconds at 6,000 x 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 55°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 x 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 55°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 x 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 x g to drive the contents off the walls and lid.
4. Add 400 µL Buffer AL (supplied), 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 x 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 x g to drive the contents off the walls and lid.
6. Put two DNeasy Mini spin columns in two clean 2 mL collection tubes (supplied). 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 x g. Discard the flow-through and collection tube. Put the DNeasy Mini spin columns in fresh 2 mL collection tubes (supplied).
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 x g. Discard the flow-through and collection tube. Put the DNeasy Mini spin columns in fresh 2 mL collection tubes (supplied).

10 Prepare a fresh 80% ethanol solution by adding 40 mL 100% ethanol to 10 mL nuclease-free water.

CAUTION Do *not* use Buffer AW2 supplied with the Qiagen 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 80% ethanol onto each column, and spin in a microcentrifuge for 3 minutes at 20,000 x 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 microcentrifuge tube, and add 50 µL of nuclease free 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 x 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 30.

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.

CAUTION

Use equal amounts of gDNA for both the experimental and reference channels. The required gDNA input amount and concentration depends on the microarray format, labeling method and DNA source used (see Table 16 and Table 17).

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 water and requantitate to make sure quantitation is accurate.

Step 1. gDNA Dilution

- Dilute the gDNA with nuclease-free water to the concentration listed in Table 18, Table 19, and Table 20.

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 18, Table 19, and Table 20, concentrate the sample using a concentrator (such as Speed Vac) to dryness and resuspend in water to the final concentration listed above.

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

Table 18 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
1x microarray (non-FFPE samples)	100	17	15
1x microarray (FFPE samples)	133.5	17	15
2x microarray	100	12	10
4x microarray	100	7	5
8x microarray	50	7	5

* If you want to use more gDNA than what is listed in Table 16 on page 26, use gDNA that is at a higher concentration than is listed in this table.

Table 19 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
1x, 2x and 4x microarray	100	7	5
8x microarray	50	6	4

* If you want to use more gDNA than what is listed in Table 16 on page 26, use gDNA that is at a higher concentration than is listed in this table.

Table 20 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
1x, 2x and 4x microarray	50	22	20
8x microarray	71.4	9	7

* If you want to use more gDNA than what is listed in Table 16 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 plate (Eppendorf p/n 951020401), 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 18 on page 38, or Table 19 or Table 20 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.

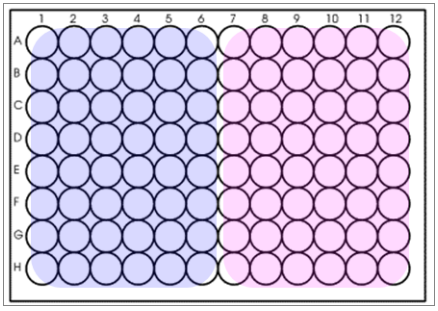

Figure 3 96-well layout of gDNA before labeling. Columns 1 through 6 contain 48 experimental gDNA samples (blue). Columns 7 through 12 contain 48 reference gDNA samples (pink).

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 DNA 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 (p/n 5190-0450) uses a non-enzymatic procedure to differentially label gDNA samples with fluorescent dyes. The kit contains two-color labeling reaction reagents sufficient for:

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

You also need to order the Genomic DNA 96-well Purification Module (p/n 5190-0451) 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 will need 2 Bravo Platform CPAC Ultraflat heated/cooled Deck Pads and the Riser option on the Bravo platform (Agilent p/n G5409A Option 002).

The processing of CGH+SNP microarrays requires the restriction digestion of the gDNA with AluI and RsaI prior to labeling, to enable the genotyping of SNPs located in the enzymes' recognition sites for the detection of LOH/UPD.

The Agilent Genomic DNA Enzymatic Labeling Kit (Agilent p/n 5190-0449) uses random primers and the exo-Klenow fragment to differentially label genomic DNA samples with fluorescent-labeled nucleotides. The kit contains sufficient two-color labeling reaction agents for:

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

You also need to order the AutoScreen-96A Well plates (GE Healthcare p/n 25-9005-98) 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.

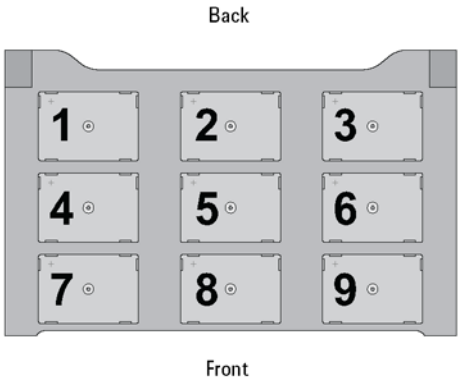

Figure 4 Bravo platform deck

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* (G5409-90004) and the *VWorks Software User Guide* (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, then click **Continue**.

1x1M or 1x244K microarrays	1
2x400K or 2x105K microarrays	2
4x180K or 4x44K microarrays	4
8x60K or 8x15K 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 76.

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 thermocycler, the Bravo platform adds the Labeling Master Mix to the fragmented gDNA, then the reactions occur in a thermocycler.

NOTE Do not fragment gDNA isolated from FFPE tissues.

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 working plate (Eppendorf p/n 951020401)
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 21 through Table 25.

Table 21 Multiplier to calculate the volume of Labeling Master Mix (for 1x microarrays using non-FFPE samples)*

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

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

Table 22 Multiplier to calculate the volume of Labeling Master Mix (for 1x microarrays using FFPE samples)*

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

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

Table 23 Multiplier to calculate the volume of Labeling Master Mix (for 2x microarrays using non-FFPE and FFPE samples)*

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

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

Table 24 Multiplier to calculate the volume of Labeling Master Mix (for 4x microarrays using non-FFPE and FFPE samples)*

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

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

Table 25 Multiplier to calculate the volume of Labeling Master Mix (for 8x microarrays using non-FFPE and FFPE samples)*

Components	Per reaction (µL)	Total volume needed	Volume to add per well of 384 deep well plate
Nuclease-free water	3.75	(3.75 µL x N) + 24 µL	
ULS-Cy3 or ULS-Cy5	0.25	(0.25 µL x N) + 1.6 µL	
10 x labeling solution	1	(1 µL x N) + 6.4 µL	
Final volume of Labeling Master Mix	**5**	**(5 µL x N) + 32 µL**	**((5 µL x 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 21 through Table 25 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.

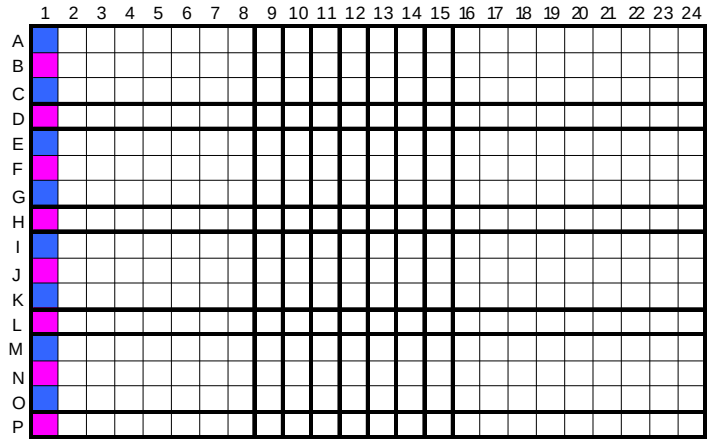

Figure 5 384 deep well plate for ULS labeling. Well plate contains Cy5 Labeling Master Mix (blue) in quadrant 1 and Cy3 Labeling Master Mix (pink) in quadrant 3.

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 thermocycler.

e Program the thermocycler according to Table 26 and run the program.

Table 26 DNA fragmentation using a thermocycler

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 thermocycler.
- **d** Program the thermocycler according to Table 27 and run the program.

Table 27 DNA labeling using a thermocycler

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 Agilent 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 nuclease-free 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 x 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 x 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 4x and 8x 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 x g to collect the purified labeled gDNA. For 1x and 2x microarrays the volume per sample will be approximately 19 µL. For 4x and 8x 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 55.

12 For 8x 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 Genomic DNA 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**, then select **Sample Type** to be **DNA-50**.
2. Use 1.5 µL of 1x labeling solution (dilute 10x 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:

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

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

   *pmol dyes per µg genomic DNA

   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

$$\text{Yield (µg)} = \frac{\text{DNAconcentration (ng/µL)} \cdot \text{Sample Volume (µL)}}{1000 \text{ ng/µg}}$$

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 Genomic DNA 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 thermocycler, and the Bravo platform adds the Agilent-CGHblock.

Bravo Platform Protocol Used

HybridizationPrepProtocol_ULS

Initial Deck Layout

Location	1x microarray	2x microarray	4x and 8x 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 • 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 plate (ABgene p/n AB-1184) placed in Eppendorf plate (p/n 951020401)	
7	Deep-well plate (ABgene p/n AB-0859)		
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 100X Blocking Agent:

   a Add 135 µL of nuclease-free water to the vial containing lyophilized 10X CGH Blocking Agent (supplied with Agilent Oligo aCGH 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 "10X" and write "100X". For ULS labeled samples, you will be making and using 100X of the blocking agent.

   The 100X 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 28 Multiplier to calculate the volume of Hybridization Master Mix for 1x microarrays, non-FFPE and FFPE samples*

Components	Per reaction (µL)	Total volume needed
Nuclease-free water	39.8	(39.8 µL x N) + 157 µL
Cot-1 DNA (1.0 mg/mL)†	50	(50 µL x N) + 197.2 µL
Agilent 100X Blocking Agent‡	5.2	(5.2 µL x N) + 20.5 µL
Agilent 2X Hi-RPM Hybridization Buffer†	260	(260 µL x N) + 1025.4 µL
Final Volume of Hybridization Master Mix	**355**	**(355 µL x 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 from the appropriate species.

‡ Supplied with Agilent Oligo aCGH Hybridization Kit

Table 29 Multiplier to calculate the volume of Hybridization Master Mix for 2x microarrays, non-FFPE and FFPE samples*

Components	Per reaction	Total volume needed
Nuclease-free water	2.4	(2.4 µL x N) + 21 µL
Cot-1 DNA (1.0 mg/mL)†	25	(25 µL x N) + 218.8 µL
Agilent 100X Blocking Agent‡	2.6	(2.6 µL x N) + 22.8 µL
Agilent 2X Hi-RPM Hybridization Buffer†	130	(130 µL x N) + 1137.5 µL
Final Volume of Hybridization Master Mix	**160**	**(160 µL x 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 from the appropriate species.

‡ Supplied with Agilent Oligo aCGH Hybridization Kit

Table 30 Multiplier to calculate the volume of Hybridization Master Mix for 4x microarrays, non-FFPE and FFPE samples*

Components	Per reaction (µL)	Total volume needed
Cot-1 DNA (1.0 mg/mL)†	5	(5 µL x N) + 114.7 µL
Agilent 100X Blocking Agent‡	1	(1 µL x N) + 23.0 µL
Agilent 2X Hi-RPM Hybridization Buffer†	55	(55 µL x N) + 1262.3 µL
Final Volume of Hybridization Master Mix	**61**	**(61 µL x 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 from the appropriate species.

‡ Supplied with Agilent Oligo aCGH Hybridization Kit

Table 31 Multiplier to calculate the volume of Hybridization Master Mix for 8x microarrays, non-FFPE and FFPE samples*

Components	Per reaction (µL)	Total volume needed
Cot-1 DNA (1.0 mg/mL)†	2	(2 µL x N) + 112 µL
Agilent 100X Blocking Agent‡	0.5	(0.5 µL x N) + 28 µL
Agilent 2X Hi-RPM Hybridization Buffer†	22.5	(22.5 µL x N) + 1260 µL
Final Volume of Hybridization Master Mix	**25**	**(25 µL x 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 from the appropriate species.

‡ Supplied with Agilent Oligo aCGH Hybridization Kit

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

4 Use Table 32 to calculate how much Agilent-CGHblock (supplied with the ULS Labeling Kit) to use.

Table 32 Multiplier to calculate volume of Agilent-CGHblock*

Format	Total volume needed
1x	(130 µL x N) + 1400 µL
2x	(65 µL x N) + 1400 µL
4x	(27 µL x N) + 1400 µL
8x	(11 µL x 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 1x and 2x microarrays, the Bravo platform transfers the hybridization master mix to the empty deep-well or tall-chimney plates. Continue at step 8.

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

8 For 1x and 2x 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 thermocycler.

b Program the thermocycler according to Table 33 and run the program.

For the 1x microarray deep-well plates, use an incubator that is compatible with deep-well plates, such as the Eppendorf ThermoStat plus (Eppendorf p/n 022670204 with deep-well plate block p/n 022670565).

Table 33 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 and Feature Extraction."

Enzymatic Labeling for CGH

Follow the procedure in this section if you are using Enzymatic labeling for CGH.

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 thermocycler, the Bravo platform adds the Labeling Master Mix to the fragmented gDNA, and the labeling reactions are done in a thermocycler.

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 working plate (Eppendorf p/n 951020401)
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 34 to calculate how much Random Primers (supplied with the Agilent Genomic DNA Enzymatic Labeling Kit) to use.

Table 34 Multiplier to calculate volume of Random Primer*

Format	Total volume needed	Volume to add per well of 384 deep well plate
1x, 2x or 4x	(5 µL x N x 2) + 32 µL	((5 µL x N x 2) + 24 µL)/8
8x	(2.5 µL x N x 2) + 32 µL	((2.5 µL x N x 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 34 for volumes. Add the Random Primers 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.

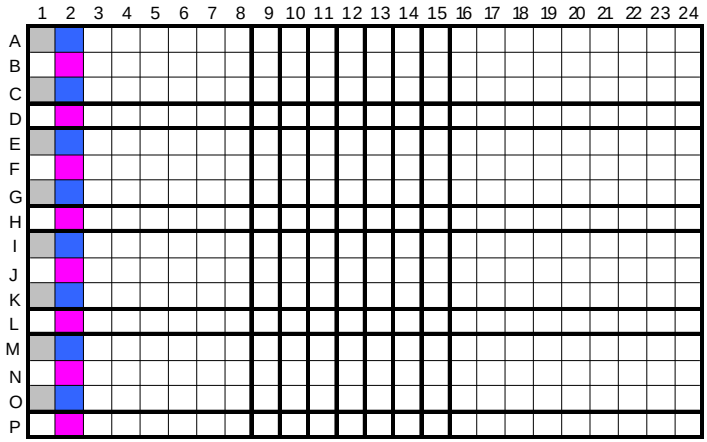

Figure 6 384 deep well plate for Enzymatic labeling containing Random Primer (gray) in quadrant 1, Cy5 Labeling Master Mix (blue) in quadrant 2 and Cy3 Labeling Master Mix (pink) in quadrant 4

4 Prepare one Cy3 and one Cy5 Labeling Master Mix by mixing the components in Table 35 and Table 36, based on your microarray format.

5 In a 384 deep well plate:

- Refer to Table 35 and Table 36 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.

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.

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

NOTE Protect the 5X Buffer from light. Although exposure to light does not affect the performance of the 5X Buffer, the buffer can change to a yellow color.

Table 35 Multiplier to calculate the volume of Labeling Master Mix (for 1x, 2x and 4x microarrays)*

Component	Per reaction (µL)	Total volumes needed	Volume to add per well of 384 deep well plate
Nuclease-free water	1	(1 µL x N) + 2.1 µL	
5X Buffer	5	(5 µL x N) + 10.7 µL	
10X dNTP Mix†	5	(5 µL x N) + 10.7 µL	
Cyanine 3-dUTP (1.0 mM) *or* Cyanine 5-dUTP (1.0 mM)	3	(3 µL x N) + 6.4 µL	
Exo-Klenow fragment	1	(1 µL x N) + 2.1 µL	
Final volume of Labeling Master Mix	**15**	**(15 µL x N) + 32 µL**	**((15 µL x 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 5X dNTP mix

Table 36 Multiplier to calculate the volume of Labeling Master Mix (for 8x microarrays)

Component	Per reaction (µL)	Total volumes needed	Volume to add per well of 384 deep well plate
Nuclease-free water	9	(9 µL x N) + 15.6 µL	
5X Buffer	5	(5 µL x N) + 8.6 µL	
10X dNTP Mix	2.5	(2.5 µL x N) + 4.3 µL	
Cyanine 3-dUTP (1.0 mM) *or* Cyanine 5-dUTP (1.0 mM)	1.5	(1.5 µL x N) + 2.6 µL	
Exo-Klenow fragment	0.5	(0.5 µL x N) + 0.9 µL	
Final volume of Labeling Master Mix	**18.5**	**(18.5 µL x N) + 32 µL**	**((18.5 µL x N) + 24 µL)/8**

7 Start the FragmentationLabelingProtocol_Enz protocol run.

The Bravo platform adds the Random Primers 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.

8 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 thermocycler.

e Program the thermocycler according to Table 37 and run the program.

Table 37 DNA fragmentation using a thermocycler

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

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

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

11 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.

12 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 thermocycler.
- **d** Program the thermocycler according to Table 38 and run the program.

Table 38 DNA labeling using a thermocycler

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

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

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

15 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 nuclease-free 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 96-well PCR plate (Eppendorf p/n 951020401) 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 x 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 x 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 x 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 69.

14 For 8x 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 water.

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

15 Keep the plate at 4°C in the dark until ready for "Step 3. Preparation of Enzymatic Labeled Genomic DNA for Hybridization" on page 71.

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**, 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:

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

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

   *pmol dyes per µg genomic DNA

   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

$$\text{Yield (µg)} = \frac{\text{DNAconcentration (ng/µL)} \cdot \text{Sample Volume (µL)}}{1000 \text{ ng/µg}}$$

Refer to Table 39 for expected yield of labeled genomic DNA and specific activity after labeling and clean-up, when starting with high quality genomic DNA.

Table 39 Expected Yield and Specific Activity after Labeling and Clean-up

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	4.5 to 5.5	15 to 25	15 to 20
0.2*	2.5 to 3	15 to 25	15 to 20

* 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 121.

Step 3. Preparation of Enzymatic Labeled Genomic DNA 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 thermocycler.

Bravo Platform Protocol Used

HybridizationPrepProtocol_Enz

Initial Deck Layout

Location	1x microarray	2x microarray	4x and 8x 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 plate (ABgene p/n AB-1184) placed in Eppendorf plate (p/n 951020401)	
7	Deep-well plate (ABgene p/n AB-0859)		
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 10X Blocking Agent:

   a Add 1350 µL of nuclease-free water to the vial containing lyophilized 10X CGH Blocking Agent (supplied with Agilent Oligo aCGH 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 10X 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 40 Multiplier to calculate the volume of Hybridization Master Mix for 1x microarrays*

Component	Per reaction (µL)	Total volume needed in column 1 if N <= 32	Additional volume needed in column 2 if N > 32
1X TE	123	(123 µL x N) + 355.1 µL	(123 µL x (N-32)) + 355.1 µL
Cot-1 DNA (1.0 mg/mL)†	50	(50 µL x N) + 144.3 µL	(50 µL x (N-32)) + 144.3 µL
Agilent 10X Blocking Agent‡	52	(52 µL x N) + 150.1 µL	(52 µL x (N-32)) + 150.1 µL
Agilent 2X Hi-RPM Hybridization Buffer†	260	(260 µL x N) + 750.5 µL	(260 µL x (N-32)) + 750.5 µL
Final Volume of Hybridization Master Mix	**485**	**(485 µL x N) + 1400 µL**	**(485 µL x (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 from the appropriate species.

‡ Supplied with Agilent Oligo aCGH Hybridization Kit

Table 41 Multiplier to calculate the volume of Hybridization Master Mix for 2x microarrays

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

* Use Cot-1 DNA from the appropriate species.

† Supplied with Agilent Oligo aCGH Hybridization Kit

Table 42 Multiplier to calculate the volume of Hybridization Master Mix for 4x microarrays

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

* Use Cot-1 DNA from the appropriate species.

† Supplied with Agilent Oligo aCGH Hybridization Kit

Table 43 Multiplier to calculate the volume of Hybridization Master Mix for 8x microarrays

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

* Use Cot-1 DNA from the appropriate species.

† Supplied with Agilent Oligo aCGH Hybridization Kit

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

- For 1x and ≤ 32 samples, 2x, 4x and 8x: add to column 1.
- For 1x and > 32 samples: add to column 1 and 2.

4 Start the HybridizationPrepProtocol_Enz protocol run.

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

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

5 For 1x and 2x 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 from location 5, or the PCR plate from location 8 to a thermocycler. Program the thermocycler according to Table 44 and run the program.

For the 1x microarray deep-well plates, use an incubator that is compatible with deep-well plates, such as the Eppendorf ThermoStat plus (Eppendorf p/n 022670204 with deep-well plate block p/n 022670565).

Table 44 DNA preparation before hybridization

Step	Temperature	Time
Step 1	95°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.

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 gDNA is denatured in the presence of random primers in a thermocycler, the Bravo platform adds the Labeling Master Mix to the digested gDNA, and the labeling reactions are done in a thermocycler.

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 working plate (Eppendorf p/n 951020401)
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 45 and Table 46, based on your microarray format.

Table 45 Multiplier to calculate the volume of Restriction Digestion Master Mix (for 1x, 2x and 4x 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 x N x 2) + 11.7	
10x Reaction buffer C	2.6	(2.6 µL x N x 2) + 13.9	
Acetylated BSA	0.2	(0.2 µL x N x 2) + 1.1	
AluI	0.5	(0.5 µL x N x 2) + 2.7	
RsaI	0.5	(0.5 µL x N x 2) + 2.7	
Final volume of Restriction Digestion Master Mix	**6**	**(6 µL x N x 2) + 32**	**((6 µL x N x 2) + 24 µL)/8**

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

Table 46 Multiplier to calculate the volume of Restriction Digestion Master Mix (for 8x 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 x N x 2) + 21.9	
10x Reaction buffer C	1.3	(1.3 µL x N x 2) + 6.9	
Acetylated BSA	0.1	(0.1 µL x N x 2) + 0.5	
AluI	0.25	(0.25 µL x N x 2) + 1.3	
RsaI	0.25	(0.25 µL x N x 2) + 1.3	
Final volume of Restriction Digestion Master Mix	**6**	**(6 µL x N x 2) + 32**	**((6 µL x N x 2) + 24 µL)/8**

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.

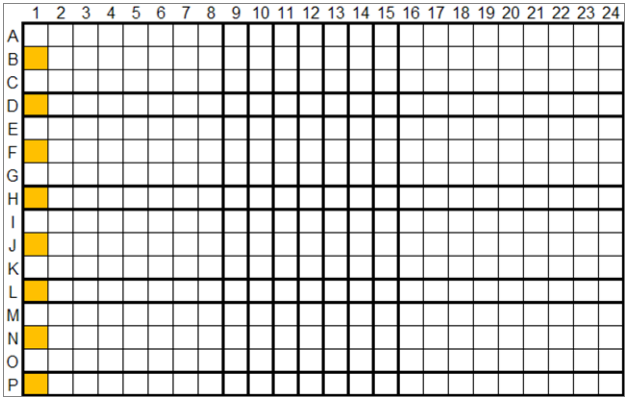

Figure 7 384 deep well plate for CGH+SNP Enzymatic Labeling containing Restriction Digestion Master Mix in quadrant 3

4 Spin the plate in a centrifuge for 30 seconds at 6,000 x 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 thermocycler.

e Program the thermocycler according to Table 47 and run the program.

Table 47 DNA restriction digestion using a thermocycler

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

8 When the incubation is finished, remove the plate from the thermocycler and spin the plate in a centrifuge for 30 seconds at 6,000 x 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 Primers and Labeling Master Mix.

9 Use Table 48 to calculate how much Random Primers (supplied with the Agilent Genomic DNA Enzymatic Labeling Kit) to use.

Table 48 Multiplier to calculate volume of Random Primer*

Format	Total volume needed	Volume to add per well of 384 deep well plate
1x, 2x or 4x	(5 µL x N x 2) + 32 µL	((5 µL x N x 2) + 24 µL)/8
8x	(2.5 µL x N x 2) + 32 µL	((2.5 µL x N x 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 48 for volumes. Add the Random Primers 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.

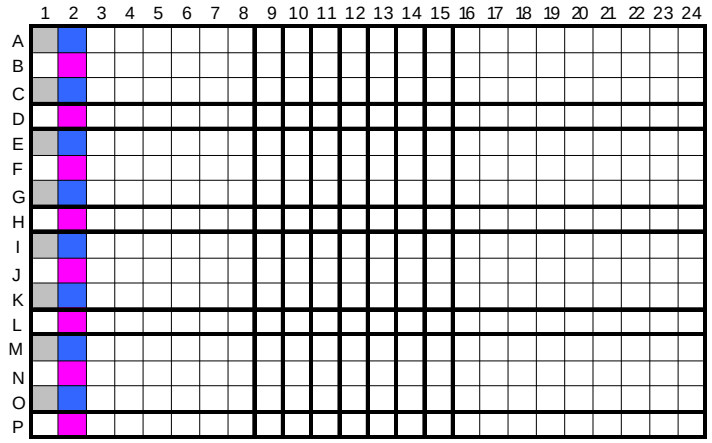

Figure 8 384 deep well plate for Enzymatic labeling containing Random Primer (gray) in quadrant 1, Cy5 Labeling Master Mix (blue) in quadrant 2 and Cy3 Labeling Master Mix (pink) in quadrant 4

11 Prepare one Cy3 and one Cy5 Labeling Master Mix by mixing the components in Table 49 and Table 50, based on your microarray format.

12 In the 384 deep well plate:

- Refer to Table 49 and Table 50 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.

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.

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

NOTE Protect the 5X Buffer from light. Although exposure to light does not affect the performance of the 5X Buffer, the buffer can change to a yellow color.

Table 49 Multiplier to calculate the volume of Labeling Master Mix (for 1x, 2x and 4x microarrays)*

Component	Per reaction (µL)	Total volumes needed	Volume to add per well of 384 deep well plate
5X Buffer	10	(10 µL x N) + 16.8 µL	
10X dNTP Mix†	5	(5 µL x N) + 8.4 µL	
Cyanine 3-dUTP (1.0 mM) *or* Cyanine 5-dUTP (1.0 mM)	3	(3 µL x N) + 5.1 µL	
Exo-Klenow fragment	1	(1 µL x N) + 1.7 µL	
Final volume of Labeling Master Mix	**19**	**(19 µL x N) + 32 µL**	**((19 µL x 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 5X dNTP mix

Table 50 Multiplier to calculate the volume of Labeling Master Mix (for 8x microarrays)

Component	Per reaction (µL)	Total volumes needed	Volume to add per well of 384 deep well plate
5X Buffer	5	(5 µL x N) + 16.8 µL	
10X dNTP Mix	2.5	(2.5 µL x N) + 8.4 µL	
Cyanine 3-dUTP (1.0 mM) *or* Cyanine 5-dUTP (1.0 mM)	1.5	(1.5 µL x N) + 5.1 µL	
Exo-Klenow fragment	0.5	(0.5 µL x N) + 1.7 µL	
Final volume of Labeling Master Mix	**9.5**	**(9.5 µL x N) + 32 µL**	**((9.5 µL x N) + 24 µL)/8**

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

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

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

16 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 thermocycler.

d Program the thermocycler according to Table 51 and run the program.

Table 51 DNA denaturation using a thermocycler

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

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

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

19 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.

20 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 thermocycler.

d Program the thermocycler according to Table 52 and run the program.

Table 52 DNA labeling using a thermocycler

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

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

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

23 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

CGH+SNP_PurificationProtocol_Enz

Initial Deck Layout

Location	Content
1	Full tip box
4	Agilent reservoir containing nuclease-free 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 96-well PCR plate (Eppendorf p/n 951020401) 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 x 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 x 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 x g to collect the purified labeled gDNA in the sample collection plate. The volume per sample will be ~19 µL.

   For 1x, 2x, or 4x array 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 x 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 87.

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

- For 8x microarrays, concentrate to 8 µL. If needed, concentrate the labeled samples to dryness and resuspend in 8 µL.
- For 4x microarrays, concentrate to 24.5 µL. If needed, concentrate the labeled samples to dryness and resuspend in 24.5 µL water.

  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 becomes difficult to resuspend.

17 Keep the plate at 4°C in the dark until ready for "Step 3. Preparation of Enzymatic Labeled Genomic DNA for Hybridization" on page 89.

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**, 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:

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

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

   *pmol dyes per µg genomic DNA

   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

$$\text{Yield (µg)} = \frac{\text{DNAconcentration (ng/µL)} \cdot \text{Sample Volume (µL)}}{1000 \text{ ng/µg}}$$

Refer to Table 53 for expected yield of labeled genomic DNA and specific activity after labeling and clean-up, when starting with high quality genomic DNA.

Table 53 Expected Yield and Specific Activity after Labeling and Clean-up

Input gDNA (µg)	Yield (µg)	Specific Activity of Cyanine-3 Labeled Sample (pmol/µg)	Specific Activity of Cyanine-5 Labeled Sample (pmol/µg)
1	7.5 to 10	12 to 22	10 to 20
0.5*	2.5 to 4.5	15 to 20	12 to 17

* 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 121.

Step 3. Preparation of Enzymatic Labeled Genomic DNA 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 thermocycler.

Bravo Platform Protocol Used

CGH+SNP_HybridizationPrepProtocol_Enz

Initial Deck Layout

Location	1x microarray	2x microarray	4x and 8x 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 plate (ABgene p/n AB-1184) placed in Eppendorf plate (p/n 951020401)	
7	Deep-well plate (ABgene p/n AB-0859)		
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 100X Blocking Agent:

   a Add 135 µL of nuclease-free water to the vial containing lyophilized 10X CGH Blocking Agent (supplied with Agilent Oligo aCGH 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 "10X" and write "100X". For labeled samples that will hybridize to CGH+SNP arrays, you make and use 100X of the blocking agent.

   The 100X 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 54 Multiplier to calculate the volume of Hybridization Master Mix for 1x microarrays*

Component	Per reaction (µL)	Total volume needed in column 1 if N <= 32	Additional volume needed in column 2 if N > 32
1X TE	131.8	(131.8 µL x N) + 412.8 µL	(131.8 µL x (N-32)) + 412.8 µL
Cot-1 DNA (1.0 mg/mL)†	50	(50 µL x N) + 156.6 µL	(50 µL x (N-32)) + 156.6 µL
Agilent 100X Blocking Agent‡	5.2	(5.2 µL x N) + 16.3 µL	(5.2 µL x (N-32)) + 16.3 µL
Agilent 2X Hi-RPM Hybridization Buffer†	260	(260 µL x N) + 814.3 µL	(260 µL x (N-32)) + 814.3 µL
Final Volume of Hybridization Master Mix	**447**	**(447 µL x N) + 1400 µL**	**(447 µL x (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 from the appropriate species.

‡ Supplied with Agilent Oligo aCGH Hybridization Kit

Table 55 Multiplier to calculate the volume of Hybridization Master Mix for 2x microarrays

Component	Per reaction (µL)	Total volume needed
1X TE	29.4	(29.4 µL x N) + 220.1 µL
Cot-1 DNA (1.0 mg/mL)*	25	(25 µL x N) + 187.2 µL
Agilent 100X Blocking Agent†	2.6	(2.6 µL x N) + 19.5 µL
Agilent 2X Hi-RPM Hybridization Buffer†	130	(130 µL x N) + 973.3 µL
Final Volume of Hybridization Master Mix	**187**	**(187 µL x N) + 1400 µL**

* Use Cot-1 DNA from the appropriate species.

† Supplied with Agilent Oligo aCGH Hybridization Kit

Table 56 Multiplier to calculate the volume of Hybridization Master Mix for 4x microarrays

Component	Per reaction (µL)	Total volume needed
Cot-1 DNA (1.0 mg/mL)*	5	(5 µL x N) + 114.6 µL
Agilent 100X Blocking Agent†	1.1	(1.1 µL x N) + 25.2 µL
Agilent 2X Hi-RPM Hybridization Buffer†	55	(55 µL x N) + 1260.2 µL
Final Volume of Hybridization Master Mix	**61.1**	**(61.1 µL x N) + 1400 µL**

* Use Cot-1 DNA from the appropriate species.

† Supplied with Agilent Oligo aCGH Hybridization Kit

Table 57 Multiplier to calculate the volume of Hybridization Master Mix for 8x microarrays

Component	Per reaction (µL)	Total volume needed
1X TE	4.05	(4.05 µL x N) + 195.5 µL
Cot-1 DNA (1.0 mg/mL)*	2	(2 µL x N) + 96.6 µL
Agilent 100X Blocking Agent†	0.45	(0.45 µL x N) + 21.7 µL
Agilent 2X Hi-RPM Hybridization Buffer†	22.5	(22.5 µL x N) + 1086.2 µL
Final Volume of Hybridization Master Mix	**29**	**(29 µL x N) + 1400 µL**

* Use Cot-1 DNA from the appropriate species.

† Supplied with Agilent Oligo aCGH Hybridization Kit

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

- For 1x and ≤ 32 samples, 2x, 4x and 8x: add to column 1.
- For 1x and > 32 samples: add to column 1 and 2.

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

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

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

5 For 1x and 2x 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 from location 5, or the PCR plate from location 8 to a thermocycler. Program the thermocycler according to Table 58 and run the program.

For the 1x microarray deep-well plates, use an incubator that is compatible with deep-well plates, such as the Eppendorf ThermoStat plus (Eppendorf p/n 022670204 with deep-well plate block p/n 022670565).

Table 58 DNA preparation before hybridization

Step	Temperature	Time
Step 1	95°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 and Feature Extraction

Microarray processing consists of hybridization, washing, and scanning.

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

Step 1. Microarray Hybridization

NOTE

Familiarize yourself with the assembly and disassembly instructions for use with the Agilent microarray hybridization chamber and gasket slides. Please refer to the Agilent Microarray Hybridization Chamber User Guide (G2534-90001) for in-depth instructions on how to load samples, assemble and disassemble chambers, as well as other helpful tips. This user guide can be downloaded from the Agilent Web site at www.agilent.com/chem/dnamanuals-protocols.

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 glass slide.

The hybridization sample mixture is applied directly to the gasket slide and not to the microarray slide. Then the active side of the microarray slide is put 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.

Hybridization Assembly

1. Load a clean gasket slide into the Agilent SureHyb chamber base with the gasket label facing up and aligned with the rectangular section of the chamber base. Ensure that the gasket slide is flush with the chamber base and is not ajar.
2. Slowly dispense 490 µL (for 1x microarray), 245 µL (for 2x microarray), 100 µL (for 4x microarray) or 40 µL (for 8x microarray) of hybridization sample mixture onto the gasket well in a "drag and dispense" manner. For multi-pack microarray formats (i.e. 2x, 4x or 8x microarray), load all gasket wells before you load the microarray slide. For multi-pack formats, refer to "Agilent Microarray Layout and Orientation" on page 131

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, thermocycler or in an oven.

3 Put a microarray slide "active side" down onto the gasket slide, so the numeric barcode side is facing up and the "Agilent"-labeled barcode is facing down. Assess that the sandwich-pair is properly aligned.

4 Put the SureHyb chamber cover onto the sandwiched slides and slide the clamp assembly onto both pieces.

5 Hand-tighten the clamp firmly onto the chamber.

6 Vertically rotate the assembled chamber to wet the slides and assess the mobility of the bubbles. Tap the assembly on a hard surface if necessary to move stationary bubbles.

7 Put assembled slide chamber in the rotator rack in a hybridization oven set to 65°C. Set your hybridization rotator to rotate at 20 rpm.

8 Hybridize at 65°C:

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

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

Step 2. Wash Preparation

NOTE

Cyanine 5 has been shown to be sensitive to ozone degradation. Ozone levels as low as 5 ppb (approximately 10 µg/m^3) can affect Cyanine 5 signal and compromise microarray results. The Agilent Stabilization and Drying Solution and the Ozone-Barrier Slide Cover are designed to protect against ozone-induced degradation of Cyanine dyes. Note that the Ozone-Barrier Slide covers are compatible with the slide holders of the Agilent B and C model scanners only.

Another option to address ozone-induced Cyanine-5 degradation is to use Carbon Loaded Non-woven Filters to remove ozone from the air. These filters can be installed in either your HVAC system, or as part of small Ozone Controlled Enclosures. These free-standing enclosures can be installed either on a lab bench or as a walk-in room within your lab. These products are available through filter suppliers listed in Agilent Technical Note 5989-0875EN.

Before you begin, determine which wash procedure to use:

Table 59 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 102	No
> 5 ppb < 10 ppb	"Wash Procedure A (without Stabilization and Drying Solution)" on page 102	Yes
> 10 ppb	"Wash Procedure B (with Stabilization and Drying Solution)" on page 105	Yes

Equipment Preparation

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 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.

Cleaning with Milli-Q Water Wash

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

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

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

c Repeat step a and step b until all traces of contaminating material are removed.

Cleaning with Acetonitrile Wash (Wash Procedure B Only)

Acetonitrile wash removes any remaining residue of Agilent 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.**

a Add the slide rack and stir bar to the slide-staining dish, and transfer to a magnetic stir plate.

b Fill the slide-staining dish with 100% acetonitrile.

c Turn on the magnetic stir plate and adjust the speed to a setting of 4 (medium speed).

d Wash for 5 minutes at room temperature.

e Discard the acetonitrile as is appropriate for your site.

f Repeat step a through step e.

g Air dry everything in the vented fume hood.

h Continue with the Milli-Q water wash as previously instructed.

Prewarming Oligo aCGH Wash Buffer 2 (Overnight)

The temperature of Oligo aCGH Wash Buffer 2 must be at 37°C for optimal performance.

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

2 Put a slide-staining dish into a 1.5 L glass dish three-fourths filled with milli-Q water and warm to 37°C by storing overnight in an incubator set to 37°C.

Prewarming Stabilization and Drying Solution (Wash Procedure B Only)

The Agilent 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 array performance.

WARNING **The Agilent 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 3. Microarray Washing

Before you begin, determine which wash procedure to use:

Table 60 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 102	No
> 5 ppb < 10 ppb	"Wash Procedure A (without Stabilization and Drying Solution)" on page 102	Yes
> 10 ppb	"Wash Procedure B (with Stabilization and Drying Solution)" on page 105	Yes

Wash Procedure A (without Stabilization and Drying Solution)

Always use fresh Oligo aCGH Wash Buffer 1 and Oligo aCGH Wash Buffer 2 for each wash group (up to five slides).

Table 61 lists the wash conditions for the Wash Procedure A without Stabilization and Drying Solution.

Table 61 Wash conditions

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

1 Completely fill slide-staining dish #1 with Oligo aCGH Wash Buffer 1 at room temperature.

2 Put a slide rack into slide-staining dish #2. Add a magnetic stir bar. Fill slide-staining dish #2 with enough Oligo aCGH Wash Buffer 1 at room temperature to cover the slide rack. Put this dish on a magnetic stir plate.

3 Put the prewarmed 1.5 L glass dish filled with water and containing slide-staining dish #3 on a magnetic stir plate with heating element. Fill the

slide-staining dish #3 approximately three-fourths full with Oligo aCGH Wash Buffer 2 (warmed to 37°C). Add a magnetic stir bar. Turn on the heating element and maintain temperature of Oligo aCGH Wash Buffer 2 at 37°C; monitor using a thermometer.

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

5 Prepare the hybridization chamber disassembly.

   a Put the hybridization chamber assembly on a flat surface and loosen the thumbscrew, turning counter-clockwise.

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

   c With gloved fingers, remove the array-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 array-gasket sandwich into slide-staining dish #1 containing Oligo aCGH Wash Buffer 1.

6 With the sandwich completely submerged in Oligo aCGH Wash Buffer 1, pry the sandwich open from the barcode end only. Do this by slipping one of the blunt ends of the forceps between the slides and then gently twist the forceps to separate the slides. Let the gasket slide drop to the bottom of the staining dish. Remove the microarray slide, grasp it from the upper corners with thumb and forefinger, and quickly put into slide rack in the slide-staining dish #2 containing Oligo aCGH Wash Buffer 1 at room temperature. Minimize exposure of the slide to air. *Touch only the barcode portion of the microarray slide or its edges!*

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

8 When all slides in the group are put into the slide rack in slide-staining dish #2, stir using setting 4 for 5 minutes. Adjust the setting to get good but not vigorous mixing.

9 Transfer slide rack to slide-staining dish #3 containing Oligo aCGH Wash Buffer 2 at 37°C, and stir using setting 4 for 1 minute.

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

11 Discard used Oligo aCGH Wash Buffer 1 and Oligo aCGH Wash Buffer 2.

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

13 Put the slides in a slide holder:

- In environments in which the ozone level exceeds 5 ppb, immediately put the slides with Agilent barcode facing up in a slide holder. Make sure that the slide is not caught up on any corner. Put an ozone-barrier slide cover on top of the array as shown in Figure 9.

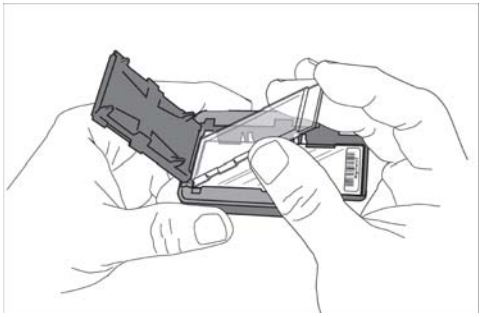

Figure 9 Inserting the ozone-barrier slide cover

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

14 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.

Wash Procedure B (with Stabilization and Drying Solution)

Cy5 is susceptible to degradation by ozone. Use this wash procedure if the ozone level exceeds 10 ppb in your laboratory.

Always use fresh Oligo aCGH Wash Buffer 1 and Oligo aCGH 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 62 lists the wash conditions for the Wash Procedure B with Stabilization and Drying Solution.

Table 62 Wash conditions

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

1 Completely fill slide-staining dish #1 with Oligo aCGH Wash Buffer 1 at room temperature.

2 Put a slide rack into slide-staining dish #2. Add a magnetic stir bar. Fill slide-staining dish #2 with enough Oligo aCGH Wash Buffer 1 at room temperature to cover the slide rack. Put this dish on a magnetic stir plate.

3 Put the prewarmed 1.5 L glass dish filled with water and containing slide-staining dish #3 on a magnetic stir plate with heating element. Fill the

slide-staining dish #3 approximately three-fourths full with Oligo aCGH Wash Buffer 2 (warmed to 37°C). Add a magnetic stir bar. Turn on the heating element and maintain temperature of Oligo aCGH Wash Buffer 2 at 37°C; monitor using a thermometer.

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

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

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

7 Prepare the hybridization chamber disassembly.

   a Put the hybridization chamber assembly on a flat surface and loosen the thumbscrew, turning counter-clockwise.

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

   c With gloved fingers, remove the array-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 array-gasket sandwich into slide-staining dish #1 containing Oligo aCGH Wash Buffer 1.

8 With the sandwich completely submerged in Oligo aCGH Wash Buffer 1, pry the sandwich open from the barcode end only. Do this by slipping one of the blunt ends of the forceps between the slides and then gently twist the forceps to separate the slides. Let the gasket slide drop to the bottom of the staining dish. Remove the microarray slide, grasp it from the upper corners with thumb and forefinger, and quickly put into slide rack in the slide-staining dish #2 containing Oligo aCGH Wash Buffer 1 at room temperature. Minimize exposure of the slide to air. *Touch only the barcode portion of the microarray slide or its edges!*

9 Repeat step 6 through step 8 for up to four additional slides in the group. A maximum of five disassembly procedures yielding five microarray slides is advised at one time in order to facilitate uniform washing.

10 When all slides in the group are placed into the slide rack in slide-staining dish #2, stir using setting 4 for 5 minutes. Adjust the setting to get good but not vigorous mixing.

11 Transfer slide rack to slide-staining dish #3 containing Oligo aCGH Wash Buffer 2 at 37°C, and stir using setting 4 for 1 minute.

12 Remove the slide rack from Oligo aCGH Wash Buffer 2 and tilt the rack slightly to minimize wash buffer carry-over. Quickly transfer the slide rack to slide-staining dish #4 containing acetonitrile, and stir using setting 4 for 10 seconds.

13 Transfer slide rack to slide-staining dish #5 filled with Stabilization and Drying Solution, and stir using setting 4 for 30 seconds.

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

15 Discard used Oligo aCGH Wash Buffer 1 and Oligo aCGH 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 water.

16 Repeat step 1 through step 15 for the next group of five slides using fresh Oligo aCGH Wash Buffer 1 and Oligo aCGH Wash Buffer 2 prewarmed to 37°C.

17 Immediately put the slides with Agilent barcode facing up in a slide holder with an ozone-barrier slide cover on top of the array as shown in Figure 9 on page 104.

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

19 Dispose of acetonitrile and Stabilization and Drying Solution as flammable solvents.

Step 4. Microarray Scanning using Agilent C, B or A Scanner or GenePix Scanner

Agilent C Scanner Settings

An Agilent C-scanner and Agilent Scanner Control software v8.3 or higher is required for 1x1M, 2x400K, 4x180K and 8x60K density microarrays and is optional for 1x244K, 2x105K, 4x44K and 8x15K density microarrays.

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 Agilent G3_CGH** for 1x1M, 2x400K, 4x180K and 8x60K microarrays. Select **Profile Agilent HD_CGH** for 1x244K, 2x105K, 4x44K and 8x15K microarrays.
4. Verify that
   - **Slide ID** is set to **<Auto Detect>.**
   - **Channels** is set to **R+G**
   - **Scan region** is set to **Agilent HD (61 × 21.6 mm)**.
   - **Resolution (µm)** is set to **3 µm** for 1x1M, 2x400K, 4x180K and 8x60K microarrays, and **5 µm** for 1x244K, 2x105K, 4x44K and 8x15K microarrays.
   - **Tiff** is set to **16 bit**
   - **R PMT** is set to **100%**.
   - **G PMT** is set to **100%**.
   - **XDR** is set to **<No XDR>**.
   - **Output Path Browse** is set for desired location.
5. Verify that the Scanner status in the main window says Scanner Ready.
6. 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.

Agilent A and B Scanner Settings

Agilent Scanner Control software v7.0.03 is recommended for 5 µm scans of 1x, 2x, 4x and 8x density microarrays.

1 Assemble slides into appropriate slide holders:
   - For version B and A slide holders, put slide into slide holder, with or without the ozone-barrier slide cover, with Agilent barcode facing up.
   - For version A slide holders, check that slides are seated parallel to the bottom of the slide holder.

2 Put assembled slide holders into scanner carousel.

3 Verify Default Scan Settings (click **Settings > Modify Default Settings**).
   - **Scan region** is set to **Scan Area (61 × 21.6 mm)**.
   - **Scan resolution (µm)** is set to **5** for 1x244K, 2x105K, 4x44K and 8x15K microarrays.
   - **Dye channel** is set to **Red & Green**.
   - **Green PMT** is set to **100%**.
   - **Red PMT** is set to **100%**.

4 Select settings for the automatic file naming.
   - **Prefix1** is set to **Instrument Serial Number**.
   - **Prefix2** is set to **Array Barcode**.

5 Clear the **eXtended Dynamic Range** check box.

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.

GenePix Scanner Settings

Agilent 1x244K, 2x105K, 4x44K and 8x15K CGH microarrays require 5 µm scan resolution, which is only supported in the GenePix 4000B scanner.

Agilent 1x1M, 2x400K, 4x180K and 8x60K CGH microarrays require 3 µm scan resolution, which is not supported in the GenePix 4000B scanner.

- Refer to the manufacturer's user guide for appropriate scanner settings.
- Refer to "Agilent Microarray Layout and Orientation" on page 131 for appropriate slide layout and orientation in GenePix scanner.

Step 5. Data Extraction using Feature Extraction Software

The Feature Extraction software v10.10 or higher supports extraction of microarray TIFF images (.tif) of Agilent CGH+SNP microarrays scanned on the Agilent C Scanner.

The Feature Extraction software v10.5 or higher supports extraction of microarray TIFF images (.tif) of Agilent CGH microarrays scanned on the Agilent C Scanner but does *not* support extraction of Agilent CGH microarrays on the GenePix 4000B scanner.

The Feature Extraction software v9.5 supports extraction of microarray TIFF images (.tif) of Agilent CGH microarrays scanned on the Agilent B Scanner and GenePix 4000B Scanner.

Feature Extraction version 10.7 or higher can automatically download Grid Templates, protocols and QC metrics (QCMT) from eArray if configured appropriately. See "Automatic Download from eArray" on page 115 for configuration.

Figure 10 shows an example of an Agilent SurePrint G3 1x1M CGH microarray image opened in Feature Extraction software v10.5 in both full and zoomed view.

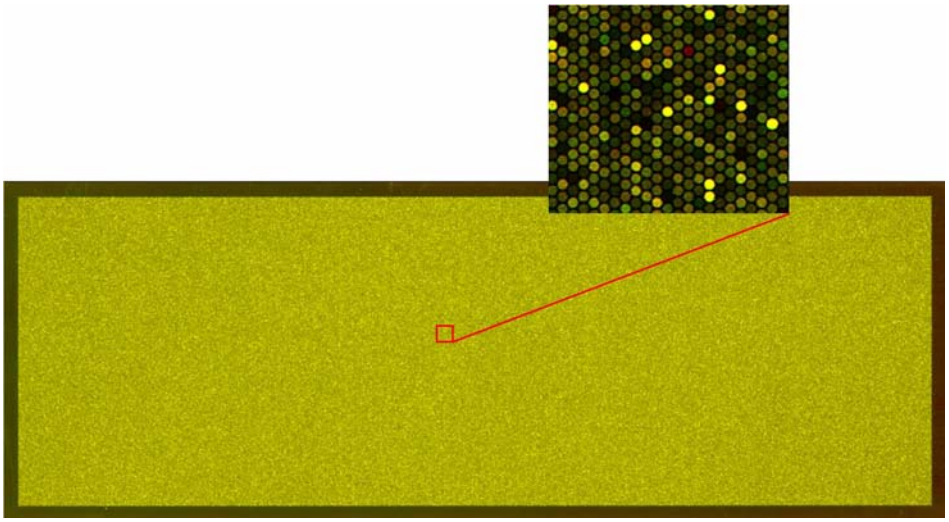

Figure 10 Agilent SurePrint G3 1x1M CGH microarray shown in red and green channels: full and zoomed view

1 Open the Agilent Feature Extraction program.

2 Add the images (.tif) to be extracted to the Feature Extraction Project.

   a Click **Add New Extraction Set(s)** icon on the toolbar or right-click the Project Explorer and select **Add Extraction...**

   You can also drag the image (.tif) from the desktop to the Feature Extraction project pane.

   b Browse to the location of the .tif files, select the .tif file(s) and click **Open**. To select multiple files, use the **Shift** or **Ctrl** key when selecting.

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

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

To access the Feature Extraction Grid Template properties, double-click on the grid template in the Grid Template Browser.

3 Set Feature Extraction Project Properties.

a Select the **Project Properties** tab.

b In the **General** section, enter your name in the Operator field.

c In all other sections, verify that at least the following default settings as shown in Figure 11 below are selected.

d For Feature Extraction 9.5, in the **Other** section, select **CGH_QCMT_Feb08**.

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

QC metrics updates are available automatically from eArray if configured appropriately. See "Automatic Download from eArray" on page 115 for configuration.

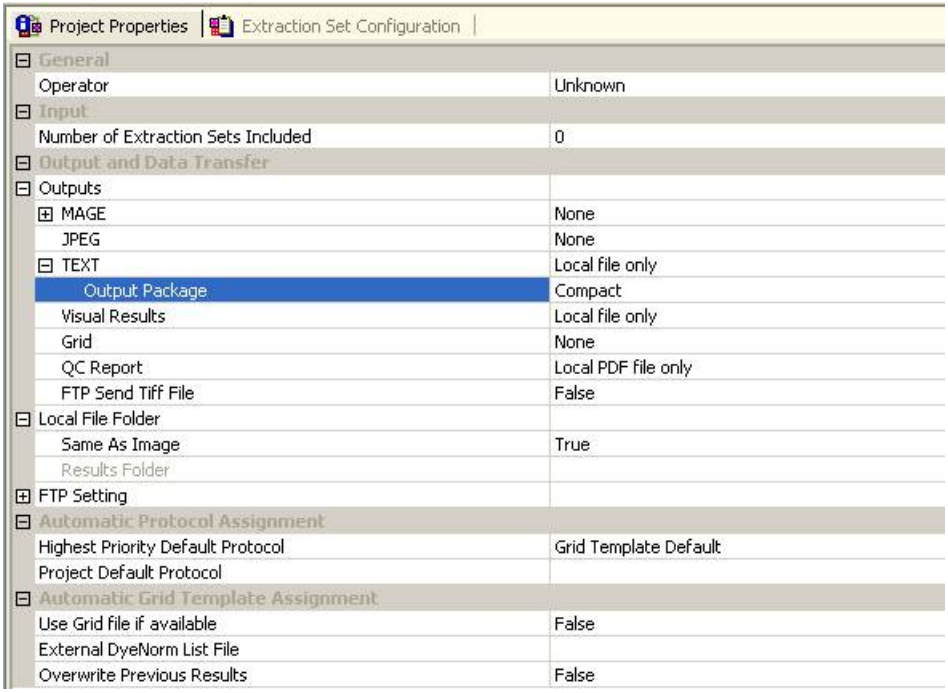

Figure 11 Default settings in Feature Extraction 10.5

4 Check the Extraction Set Configuration.

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

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

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

   To update to the latest grid templates via Online Update, right-click **Grid Template Browser** and select **Online Update**. You can also download the latest grid templates from Agilent Web site at www.agilent.com/chem/downloaddesignfiles. After downloading, you must add the grid templates to the Grid Template Browser.

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

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

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

   Protocols are also available automatically from eArray if configured appropriately. See "Automatic Download from eArray" on page 115 for configuration.

5 Save the Feature Extraction Project (.fep) by selecting **File > Save As** and browse for desired location.

6 Verify that the icons for the image files in the Feature Extraction Project Window no longer have a red X through them. A red X through the icon indicates that an extraction protocol was not selected or that the Grid Template is not in the database. If needed, reselect the extraction protocol for that image file.

7 Select **Project > Start Extracting**.

8 After the extraction is completed successfully, view the QC report for each extraction set by double-clicking the QC Report link in the **Summary Report** tab. Determine whether the grid has been properly placed by inspecting **Spot Finding of the Four Corners of the Array**. See Figure 13.

Automatic Download from eArray

Feature Extraction version 10.7 or higher can automatically download Grid Templates, protocols and QC metrics (QCM or QCMT). To set this up, in the eArray Login Setting dialog box, under **Advanced Options**, click **Use eArray server during extraction**. See Figure 12.

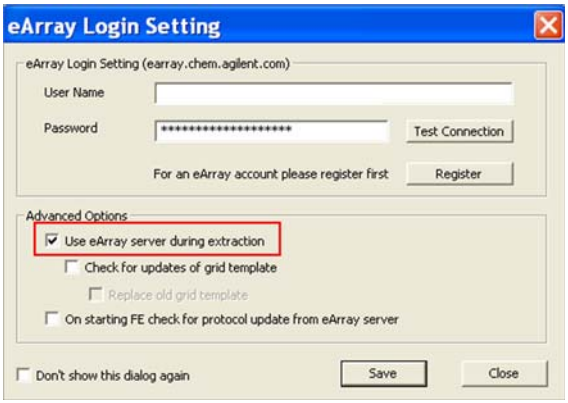

Figure 12 eArray Login Setting. You can mark the other two check boxes under Advanced Options if you want to get update of grid templates already in the database or to get protocol updates. See the Feature Extraction user guide for more information.

Microarray QC Metrics

These metrics are only appropriate for samples analyzed with Agilent CGH microarrays by following the standard operational procedures provided in this user guide. These metrics are exported to a table in the Feature Extraction QC report (in Feature Extraction 9.5, select **CGH_QCMT_Feb08** in Project Properties before extraction) and in Genomic Workbench. They can be used to assess the relative data quality from a set of microarrays in an experiment. In some cases, they can indicate potential processing errors that have occurred or suggest that the data from particular microarrays might be compromised. Many factors can influence the range of these metrics, including the microarray format (1x, 2x, 4x or 8x), 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 DLRSpread is <0.2.

Table 63 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
DLRSpread	<0.2	<0.3	<0.4

Table 64 QC metric thresholds for Enzymatic labeling

Metric	Excellent	Good	Poor
BGNoise	< 5	5 to 10	> 10
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
DLRSpread	< 0.2	0.2 to 0.3	> 0.3

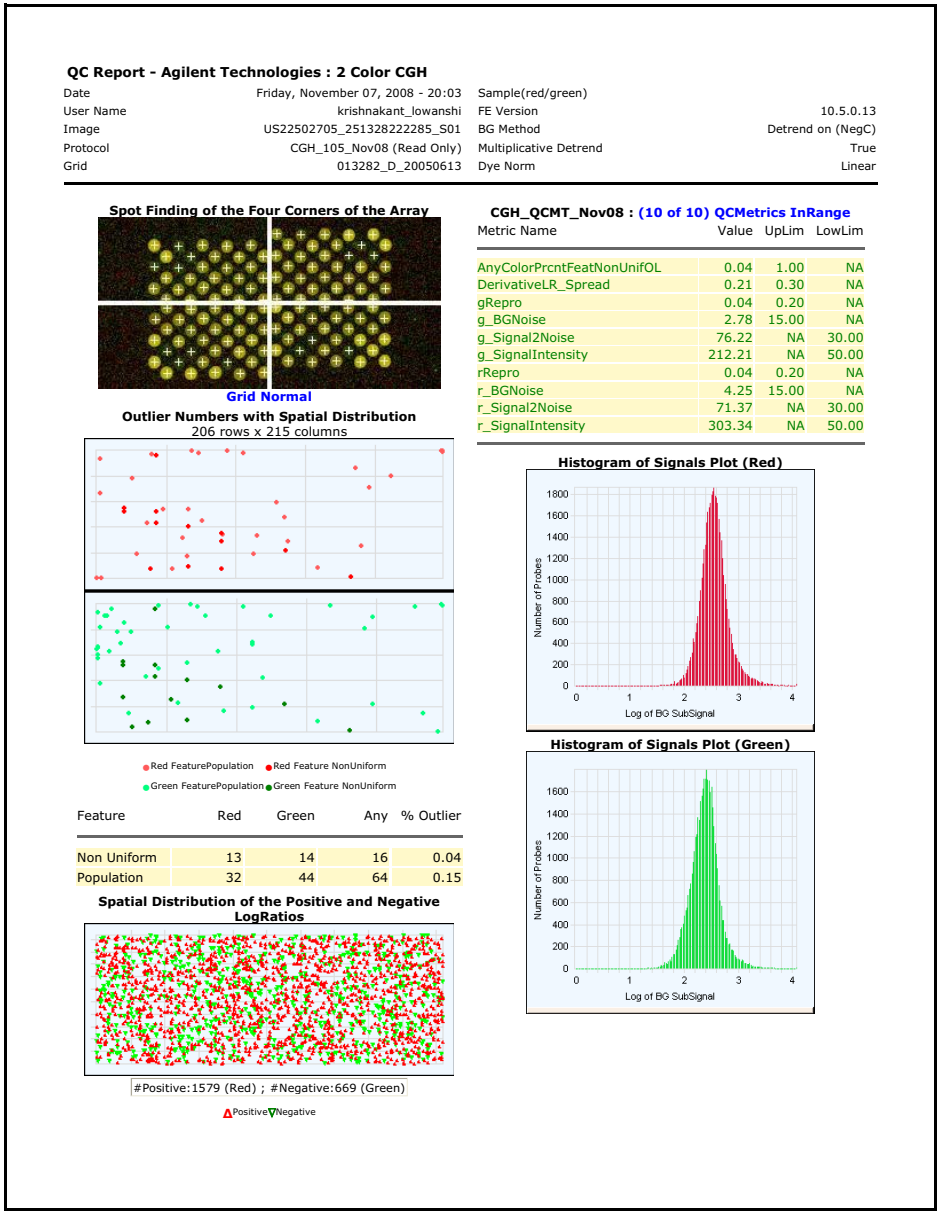

Figure 13 CGH QC report generated from Feature Extraction software v10.5, page 1

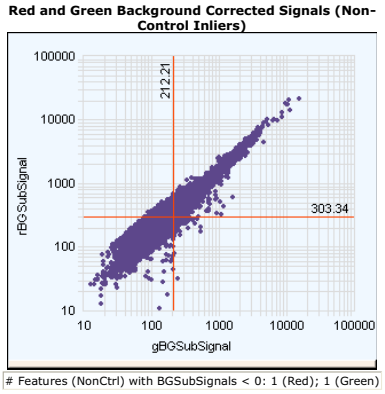

Figure 14 CGH QC report generated from Feature Extraction software v10.5, page 2

6 Troubleshooting

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

For troubleshooting information for the Bravo platform, refer to the *Bravo Platform User Guide* (G5409-90004) and the *VWorks Software User Guide* (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 (which would inhibit enzyme). 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 55, page 69 or page 87.

- ✔ 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 will label proteins that compete for dye. Make sure that the DNA is free of proteins. See "DNA Isolation" on page 23.

If you have poor sample quality due to residual RNA

The input amount of DNA for the experimental labeling reaction must be the same as for the reference sample labeling reaction. RNA absorbs at the same wavelength as DNA, which in an RNA-contaminated sample results 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 will label RNA that will hybridize to the array.

If you get poor sample quality due to degradation

For non-FFPE samples: On a 1 to 1.5% agarose gel, intact genomic DNA 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, or evaporation (use a thermocycler 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 labeling reaction must be the same as for the reference sample labeling reaction. 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 and re-quantitate 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. Verify the DNA concentration with an independent method. If you used a spectrophotometer (e.g. Nanodrop) for the initial measurement, also use a fluorometer (e.g. 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 4°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 thermocycler with heated lid.
- ✔ Evaporation can be a problem when you process samples in 96-well plates. Use a plate heat sealer (Eppendorf p/n 951023078) 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 84 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 hyb 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 oven and Wash 2 temperature.
- ✔ Check that Wash 2 was not accidentally used instead of Wash 1.
- ✔ Wash and scan slides in an ozone controlled environment (<5 ppb), such as an ozone tent.
- ✔ Use small batches that can be washed and scanned in about 40 minutes to minimize exposure to air.
- ✔ Use the Agilent Ozone-Barrier Slide Cover (p/n G2505-60550).
- ✔ Use the Stabilization and Drying Solution as described in "Wash Procedure B (with Stabilization and Drying Solution)" on page 105.

If you have high BGNoise values

High BGNoise can cause lower signal-to-noise values (see Table 63 or Table 64 on page 117 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 array image for visible non-uniformities. High BGNoise is often introduced during the washes.

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

✔ If needed, wash the slides with acetonitrile:

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

If you have poor reproducibility

Poor reproducibility (see Table 63 or Table 64 on page 117 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.

Supporting User Guides

If you are a first-time user of Agilent's oligo microarray system, please refer to the following user guides for detailed descriptions and operation recommendations for each of the hardware and software components used in the Oligo aCGH application workflow.

The user guides can be downloaded from the Agilent Web site at www.agilent.com/chem/dnamanuals-protocols.

- Agilent Microarray Hybridization Chamber User Guide (p/n G2534-90001)
- Agilent Ozone-Barrier Slide Cover User Guide (p/n G2505-90050)
- Agilent Technical Note "Improving microarray results by preventing ozone-mediated fluorescent signal degradation" (p/n 5989-0875EN)
- Agilent G2545A Hybridization Oven User Manual (p/n G2545-80001)
- Agilent G2565AA and G2565BA Microarray Scanner System User Manual
- Agilent G2565CA Microarray Scanner System User Manual
- Agilent Microarray Format Technical Drawings with Tolerances (p/n G4502-90001)
- Agilent Feature Extraction Software Quick Start Guide
- Agilent Feature Extraction Software User Guide
- Agilent Feature Extraction Software Reference Guide
- Bravo Automated Liquid Handling Platform User Guide (p/n G5409-90004)
- VWorks Automation Control Setup Guide (p/n G5415-90003)
- VWorks Automation Control User Guide (p/n G5415-90002)
- VWorks Software Quick Start (p/n G5415-90004)

Agilent Microarray Layout and Orientation

Agilent oligo microarray (1 microarray/slide format) as imaged on the Agilent microarray scanner (G2565CA)

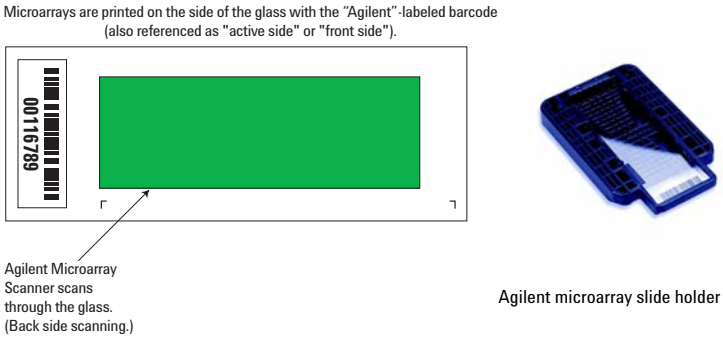

Figure 15 Agilent microarray slide and slide holder

Agilent oligo microarray formats and the resulting "microarray design files" are based on how the Agilent microarray scanner images 1-inch × 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 upside down. In this orientation, the "active side" containing the microarray is protected from potential damage by fingerprints and other elements. Once securely placed, the numeric barcode, "non-active side" of the slide is visible.

Figure 15 depicts how the Agilent microarray scanner reads the microarrays and how this relates to the "microarray design files" that Agilent generates during the manufacturing process of its *in situ*-synthesized oligonucleotide microarrays. Thus, if you have a scanner that reads microarrays from the "front side" of the glass slide, the collection of microarray data points will be different in relation to the "microarray design files" supplied with the Agilent oligo microarray kit you purchased. Therefore, please take a moment to become familiar with the microarray layouts for each of the Agilent oligo microarrays and the layout information as it pertains to scanning using a "front side" scanner.

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 16).

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

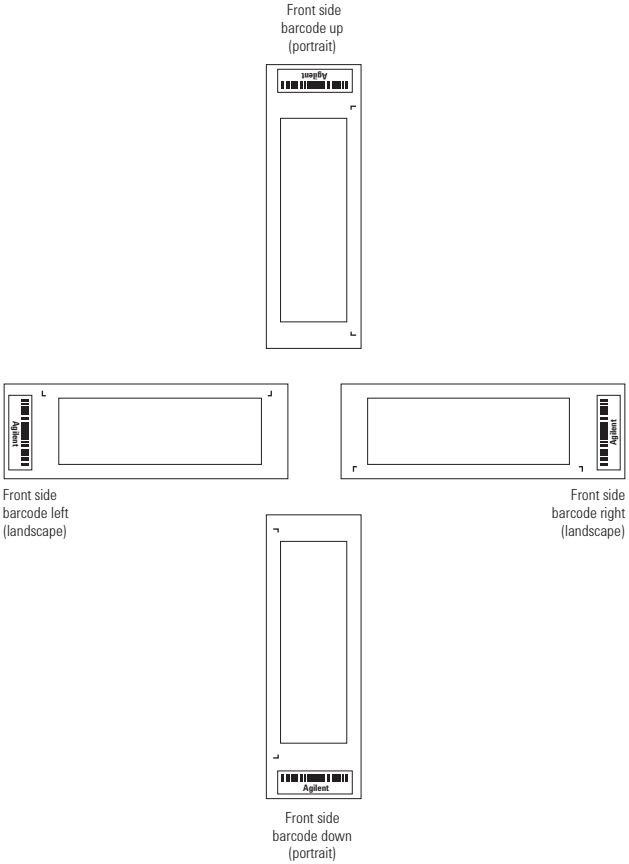

Figure 16 Microarray slide orientation

Array/Sample tracking on a 8-pack array slide

Use the form below to make notes to track your samples on a 8-pack array slide. The Agilent scanner scans through the bottom surface of the array, so if the gasket is loaded “as a book” (top row first and left to right), the array orientation will come out in the same way.

Arrays

	Array 1_1	Array 1_2	Array 1_3	Array 1_4
L A B E L	**Sample:** _____ _____ _____	**Sample:** _____ _____ _____	**Sample:** _____ _____ _____	**Sample:** _____ _____ _____
	Sample: _____ _____ _____	**Sample:** _____ _____ _____	**Sample:** _____ _____ _____	**Sample:** _____ _____ _____
	Array 2_1	**Array 2_2**	**Array 2_3**	**Array 2_4**

Barcode Number _____

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2 User may publish only fifty (50) probe-sequences in any single Publication.

3 User or its permitted publisher, including without limitation the investigator, scientists, and/or researchers, hereby agrees to add a statement in a prominent location within such publication; provided User publishes the specific design file number that Agilent shall provide, stating the following: ANY PUBLICATION OR PUBLIC DISCLOSURE OF OLIGONUCLEOTIDE SEQUENCES FOR USE WITH MICROARRAYS MAY ONLY BE REPRODUCED OR MANUFACTURED FOR YOU BY AGILENT TECHNOLOGIES, INC. BY REFERENCING THE CATALOG MICROARRAY PRODUCT NUMBER OR THE CUSTOM MICROARRAY DESIGN IDENTIFICATION NUMBER SUPPLIED BY YOU BY AGILENT.

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2 This Agreement constitutes the entire and exclusive agreement of the parties regarding the subject matter of this Agreement. This Agreement may not be varied.

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In This Book

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

Version 1.1, November 2010

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