High-Throughput aCGH Analysis using NGS Bravo Automation
- with Agilent HT Microarrays and SureTag HT Kit

For Blood, Cells, or Tissues

Protocol

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

Revision A0, April 2025

This protocol uses the NGS Bravo to automate liquid handling steps

For high-throughput aCGH analysis without Bravo automation, see Agilent publication G4132-90000

Notices

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In This Guide…

This guide describes the Agilent recommended protocol to analyze DNA copy number variations using Agilent high-throughput (HT) 60-mer oligonucleotide microarrays (16-pack or 24-pack) in an array-based Comparative Genomic Hybridization (aCGH) analysis. This protocol is specifically developed and optimized to enzymatically label DNA from blood, cells or tissues using the Agilent SureTag HT Complete Kit or SureTag HT Kit.

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.

2 About the NGS Bravo Option A and VWorks Software

This chapter describes how to use automated protocols on the NGS Bravo to assist with sample fragmentation, sample labeling, and hybridization preparation.

3 gDNA Quantitation and Quality Analysis

This chapter describes the Agilent recommended procedures for measuring the concentration of the gDNA samples and assessing the gDNA quality.

4 Sample Fragmentation

This chapter describes how to use automated protocols on the NGS Bravo to fragment gDNA and anneal random primers.

5 Sample Labeling

This chapter describes how to use automated protocols on the NGS Bravo to fluorescently label the gDNA samples with cyanine 3 and cyanine 5.

6 Microarray Processing

This chapter describes how to hybridize, wash and scan Agilent CGH and CGH+SNP microarrays and to extract data using the Agilent CytoGenomics software.

7 Troubleshooting

This chapter contains tips for troubleshooting potential issues with the HT aCGH protocol.

8 Reference

This chapter contains reference information and other helpful tips that pertain to this protocol.

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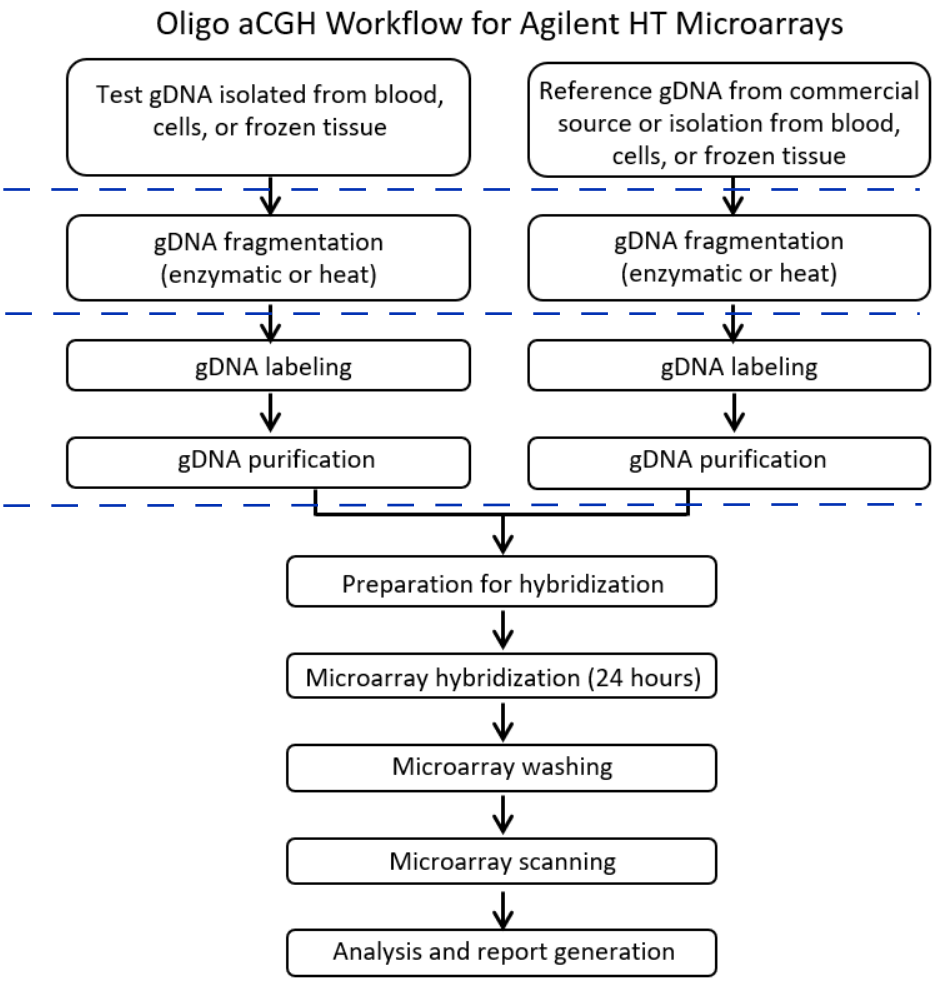
Content

1 Before You Begin

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

Workflow Overview and Considerations

The Agilent array-based Comparative Genomic Hybridization (aCGH) application uses a "two-color" process in which the test sample and reference sample are labeled with different dyes. This process allows for the identification of DNA copy number variations (CNV) — and copy-neutral Loss of Heterozygosity (cnLOH) or Uniparental Disomy if CGH+SNP microarrays are used — in the test sample relative to the reference sample.



"Before You Begin" on page 7

"Sample Fragmentation" on page 25

"Sample Labeling" on page 41

"Microarray Processing" on page 53

Figure 1 Workflow for sample preparation and HT microarray processing

The Agilent HT microarrays for aCGH are available in two different formats: 16-pack microarray slides with 16 microarrays/slide and 24-pack microarray slides with 24 microarrays/slide. The design options and aCGH protocol for the HT microarrays differ from those for the standard Agilent aCGH microarrays of smaller pack size. **Table 1** summarizes the differences in options and protocol specifics.

Table 1 Differences Between HT and Standard Microarrays

	Agilent HT Microarrays	Standard Agilent Microarrays
Format	16-pack, 24-pack	1-pack, 2-pack, 4-pack, 8-pack
Available Designs	Custom designs	Custom designs and multiple catalog designs
SNP Designs?	16-pack only	Yes
Labeled gDNA Purification Method	Magnetic SurePure Beads	Spin columns
Blocking Agent Concentration	40×	10×
SpeedVac Concentration Required?	No	Yes
Wash Buffer Incubations	Wash Buffer 1: 5 minutes Wash Buffer 2: 5 minutes	Wash Buffer 1: 5 minutes Wash Buffer 2: 1 minute
Supported Scanners	Agilent SureScan Microarray Scanner	Agilent SureScan Microarray Scanner or Agilent C Microarray Scanner
Supported Software	Agilent CytoGenomics 5.3 or higher, or Agilent Feature Extraction 12.2 or higher	Any version of Agilent CytoGenomics or Feature Extraction

Selecting a reference

The type of sample used as a reference is a matter of experimental choice, but commercially available gDNA, such as the Agilent **Human Reference DNA (Male and Female)** that is included in the SureTag HT Complete Kit, is a common choice. The Agilent Human Reference DNA may also be purchased separately as p/n 5190-3797 (Female) or p/n 5190-3796 (Male).

CGH HT microarrays

The following DNA samples are suitable reference samples for CGH HT microarrays.

1. Agilent Human Reference DNA (Female or Male) from the SureTag HT Complete Kit.
2. Promega Human Genomic DNA, p/n G1521 (Female) or p/n G1471 (Male).
3. Coriell Institute p/n NA18507, NA18517, NA12891, NA12878, or NA18579.
4. Your own reference DNA sample isolated from blood, cells, or tissue.

CGH+SNP HT microarrays

When you process CGH+SNP HT microarrays, you must use the Agilent **Human Reference DNA (Male and Female)** that is included in the SureTag HT Complete Kit as the reference.

Selecting a gDNA isolation method

This protocol is compatible with genomic DNA (gDNA) isolated from blood, cells, or tissues. High-quality gDNA is crucial to the success of an aCGH experiment. Use a gDNA isolation method that yields high-quality, intact gDNA with minimal degradation that is free of contaminants (carbohydrates, proteins, and traces of organic solvents). Agilent recommends the QIAGEN DNeasy Blood & Tissue Kit (QIAGEN p/n 69504), which calls for treating the samples with proteinase K.

If you are isolating your own reference sample gDNA, Agilent recommends using the same DNA isolation method as that used for the test sample.

Safety Notes

WARNING

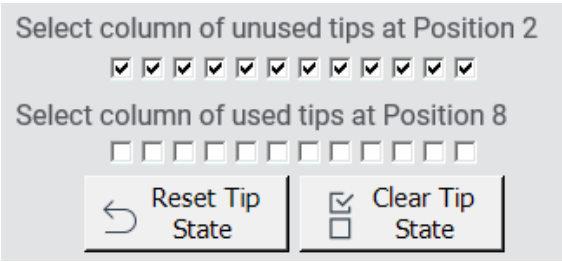
Wear appropriate personal protective equipment (PPE) when working in the laboratory.

For Agilent reagent safety information, consult the safety data sheets and any product hazard labels. Agilent safety data sheets are available at www.agilent.com.

Procedural Notes

- To prevent contamination of reagents by nucleases, always wear powder-free laboratory gloves, and use dedicated solutions and pipettes with nuclease-free aerosol-resistant tips.
- Maintain a clean work area.
- Avoid repeated freeze-thaw cycles of solutions containing gDNA or enzymes.
- When preparing frozen reagent stock solutions for use:
  1. Thaw the aliquot as quickly as possible without heating above room temperature.
  2. Mix briefly on a vortex mixer, and then spin in a microcentrifuge for 5 to 10 seconds to drive the contents off the walls and lid.
  3. Store on ice or in a cold block until use.
- Use a compression mat if compatible with your thermal cycler.
- In general, follow Biosafety Level 1 (BL1) safety rules.
- Agilent cannot guarantee microarray performance and does not provide technical support to those who use non-Agilent protocols to process Agilent microarrays.
- The automated protocols for this workflow require the NGS Bravo to pipette very low volumes of liquids. Make sure that the teachpoints for the Bravo platform are properly set to optimize pipetting accuracy.
- Some workflow steps require the rapid transfer of sample plates between the Bravo deck and a thermal cycler. Locate your thermal cycler in close proximity to the NGS Bravo to allow rapid and efficient plate transfer.
- Several workflow steps require that the sample plate be sealed using the PlateLoc thermal microplate sealer and then centrifuged to collect any dispersed liquid. To maximize efficiency, locate the centrifuge in close proximity to the NGS Bravo.
- The VWorks form includes a **Current Tip State** indicator (Protocol Parameter #8, shown below). Before running a protocol, verify that the indicator matches the configuration of unused tips in the tip box at Bravo Deck position 2.

  For a fresh tip box, containing 12 columns of tips, all positions of the **Current Tip State** unused tip indicator (top row of check boxes in the indicator) should be selected, as shown below. Clicking **Reset** selects all columns for position 2.



  Also verify that the used tip indicator (bottom row of check boxes in the indicator) matches the configuration of used tips in the tip box at Bravo Deck position 8. For an empty tip box, all positions of the **Current Tip State** used tip indicator should be cleared, as shown above. Clicking **Reset Tip State** clears all columns for position 8.

Materials Required

gDNA Samples

Table 2 lists the recommended input of gDNA sample for the workflow. The quantity is dependent on the array format (16-pack or 24-pack) and fragmentation method (enzymatic or heat).

Table 2 gDNA Input Requirements

Array format	Fragmentation method	gDNA sample input quantity
16-pack	Enzymatic	125 ng (in volume of 2.75 µL)
	Heat	125 ng (in volume of 5.75 µL)
24-pack	Enzymatic	162.5 ng (in volume of 2.75 µL)
	Heat	162.5 ng (in volume of 5.75 µL)

Refer to **"Selecting a reference"** and **"Selecting a gDNA isolation method"** on page 10 for further guidance on reference sample options and gDNA sample preparation.

Agilent HT Microarray Kits

The Agilent HT microarrays are available in 16-pack and 24-pack formats (i.e., 16 or 24 microarrays printed on each 1-inch × 3-inch glass slide). **Table 3** lists the Agilent part numbers for the HT microarrays.

Store microarray slides 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.

Table 3 Agilent HT Microarrays

Part Number	Description
G4132B	Custom CGH+SNP HT Microarray Slide, 16×25K
G4132A	Custom CGH HT Microarray Slide, 16×25K
G4133A	Custom CGH HT Microarray Slide, 24×13K

Required Agilent Equipment, Reagents, and Software

Table 4 lists the Agilent equipment, reagent kits, and software required for the aCGH workflow for Agilent HT microarrays.

Table 4 Required Agilent Equipment, Reagents, and Software

Description	Part number
Hybridization gasket slides	**For use with 16-pack microarrays:** Agilent p/n G2534-60019 (5 gasket slides) Agilent p/n G2534-60020 (20 gasket slides) Agilent p/n G2534-60021 (100 gasket slides) **For use with 24-pack microarrays:** Agilent p/n G2534-60022 (5 gasket slides) Agilent p/n G2534-60023 (20 gasket slides) Agilent p/n G2534-60024 (100 gasket slides)
Agilent SureScan Microarray Scanner Bundle	Agilent p/n G4900DA Additional slide holders: Agilent p/n G4900-60035
Hybridization Chamber, stainless	Agilent p/n G2534A
Agilent Hyb Station (recommended but not required)	Agilent p/n G5765A
Hybridization oven; temperature set at 67°C Calibrate the hybridization oven regularly for accuracy. Refer to *Agilent G2545A Hybridization Calibration Procedure* (publication G2545-90002).	Agilent p/n G2545A
Hybridization oven rotator for Agilent Microarray Hybridization Chambers	Agilent p/n G2530-60029
Agilent NGS Bravo Option A Contact Agilent Automation Solutions for more information: Customerservice.automation@agilent.com	Agilent p/n G5573AA (VWorks software version 13.1.0.1366)
Agilent PlateLoc Thermal Microplate Sealer	Agilent p/n G5585HA or G5585BA
Robotic Pipetting Tips (Sterile, Filtered, 250 µL)	Agilent p/n 19477-022
Agilent waste plates, 96 wells, 2 mL/square well	Agilent p/n 201240-100
SureTag HT Complete Kit (with Human Reference DNA), or SureTag HT Kit (without Human Reference DNA)*	Agilent p/n G9978A Agilent p/n G9978B
Oligo aCGH/ChIP-on-chip Wash Buffer Kit*	Agilent p/n 5188-5226
Oligo aCGH/ChIP-on-chip Hybridization Kit*	Agilent p/n 5188-5220 (minimum 24 slides) or p/n 5188-5380 (minimum 96 slides)
Human Cot-1 DNA (1.0 mg/mL)	Agilent p/n 5190-3393
Agilent CytoGenomics software, version 5.3 or higher, and compatible Windows PC system†	To download the latest version of Agilent CytoGenomics and review the system requirements, visit **www.agilent.com**.

* Kit contents and storage temperatures are listed in **"Reagent Kit Components"** on page 84.

† Agilent Feature Extraction software, version 12.2 or higher, can also support extraction of HT microarrays. See **Table 23** on page 54 for the appropriate QC metrics.

Other Required Materials

Table 5 lists the remaining required materials (reagents, equipment, and plasticware) needed to complete the aCGH workflow.

Table 5 Other required reagents, equipment, and plasticware

Description	Vendor and part number
Thermal cycler that is compatible with at least one of the supported thermal cycler plates listed in the row below.	Various suppliers
Thermal cycler plates compatible with the NGS Bravo and associated VWorks automation protocols	**Only the following thermal cycler plates are supported:** • 96-well ABI PCR half-skirted plates (MicroAmp Optical plates), Thermo Fisher Scientific p/n N8010560 • 96-well Agilent semi-skirted PCR plate, Agilent p/n 401334 • 96-well Eppendorf twin.tec half-skirted PCR plates, Eppendorf p/n 951020303 • 96-well Eppendorf twin.tec PCR plates (full-skirted), Eppendorf p/n 951020401 • 96-well Armadillo plates, Thermo Fisher Scientific p/n AB2396
Nuclease-free distilled water	Thermo Fisher Scientific p/n 10977-015 or equivalent
Ethanol, 100% (molecular biology grade)	Sigma-Aldrich p/n E7023-6×500ML or equivalent
1×TE buffer (pH 8.0), molecular grade	Promega p/n V6231 or equivalent
Microcentrifuge	Eppendorf p/n 5430 or equivalent
Processing plates, 96-well, full-skirted	**Only the following processing plate are supported:** 96-well Eppendorf twin.tec plates (Eppendorf p/n 951020401) OR 96-well Armadillo plates (Thermo Fisher Scientific p/n AB2396)
DNA LoBind Tubes, 1.5-mL PCR clean	Eppendorf p/n 022431021 or equivalent
Centrifuge for plates (if using 96-well PCR plates), *or* centrifuge compatible with tube strips (if using tube strips)	Eppendorf p/n 5810 or equivalent Corning p/n 6770 or equivalent
Storage bottle, 1 L, sterile	Nalgene 455-1000 or equivalent
P10, P20, P200 and P1000 pipettes	Pipetman P10, P20, P200, P1000 or equivalent
Optional: Multichannel pipette or adjustable spacer pipette (8-channel)	Rainin Pipet-Lite Multi Pipette, Integra Voyager adjustable tip spacing pipette, or equivalent
Ultra-pure, deionized water (e.g., Milli-Q ultrapure water)	Millipore or equivalent
1.5 L glass dish	Pyrex p/n 213-R or equivalent
Magnetic stir plate with heating element (×2)	Corning p/n 6795-420D or equivalent
Magnetic stir bar, 7.9 × 38.1 mm (×2)	VWR p/n 58948-150 or equivalent
250 mL capacity slide-staining dishes with lids (×3) and a slide rack	Wheaton p/n 900200 *or* Thermo Fisher Scientific p/n 121
Incubator or water bath set to 37°C (for warming Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2)	Various suppliers
Vortex mixer with variable speed	VWR p/n 97043-562 or Fisher Scientific p/n 02-215-365

Table 5 Other required reagents, equipment, and plasticware (continued)

Description	Vendor and part number
Conical tubes, sterile, 15-mL and 50-mL	Thermo Fisher Scientific p/n 352059 (15-mL) and p/n 352070 (50-mL), or equivalent
Lint-free, disposable laboratory wipes (e.g., KimWipes wipers)	Various suppliers
Ice bucket	Various suppliers
Clean forceps	Various suppliers
Powder-free gloves	Various suppliers
Sterile, nuclease-free aerosol barrier pipette tips	Various suppliers
Timer	Various suppliers
Vacuum desiccator or N2 purge box for slide storage	Various suppliers
Tube racks	Various suppliers
Thermometer	Various suppliers

Recommended Materials for gDNA Quantitation and Quality Analysis

Table 6 lists the materials used only in the recommended assays for gDNA quantitation and quality analysis described in **Chapter 3**. These assay are performed prior to starting an aCGH protocol with the Agilent HT microarrays. If you choose to use alternative assays for gDNA analysis, then the materials listed in **Table 6** may not be needed.

Table 6 Recommended materials

Description	Vendor and part number
Qubit dsDNA BR Assay Kit*	Thermo Fisher Scientific p/n Q32850
Qubit 4 Fluorometer (or other Qubit Fluorometer model)	Thermo Fisher Scientific p/n Q33226
0.5-mL PCR tubes, thin wall	Thermo Fisher Scientific p/n Q32856 *or* VWR p/n 10011-830
UV-VIS spectrophotometer	NanoDrop 8000, or equivalent
E-Gel Opener	Thermo Fisher Scientific p/n G530001
E-Gel Simple Runner Electrophoresis Device	Thermo Fisher Scientific p/n G8000
E-Gel General Purpose Agarose Gels, 1.2%	Thermo Fisher Scientific p/n G501801
TrackIt 1 Kb Plus DNA Ladder	Thermo Fisher Scientific p/n 10488085
SYBR Gold Nucleic Acid Gel Stain	Thermo Fisher Scientific p/n S11494
SYBR photographic filter	Thermo Fisher Scientific p/n S7569
UV-Transilluminator with SYBR photographic filter	Alpha Innotech p/n AlphaImager 2000 or equivalent

* Kit contents and storage temperatures are listed in **"Reagent Kit Components"** on page 84.

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2 About the NGS Bravo Option A and VWorks Software

This chapter describes how to use automated protocols on the NGS Bravo to assist with sample fragmentation, sample labeling, and hybridization preparation.

After hybridization preparation, the remainder of the workflow must be performed manually (i.e., without the use of NGS Bravo automation).

About the Bravo Platform

CAUTION Before you begin, make sure that you have read and understand operating, maintenance, and safety instructions for using the Bravo platform and additional devices and software. Refer to the user guides listed in **Table 7**.

Table 7 Agilent NGS Bravo User Guide reference information

Device	User Guide part number
Bravo Platform	SD-V1000376 (previously G5562-90000)
VWorks Software (version 13.1.0.1366)	G5415-90068
PlateLoc Thermal Microplate Sealer	G5585-90010

Bravo Platform Deck

The Bravo platform is a versatile liquid handler with a nine plate-location platform deck. Use **Figure 2** to familiarize yourself with the location numbering convention on the Bravo platform deck.

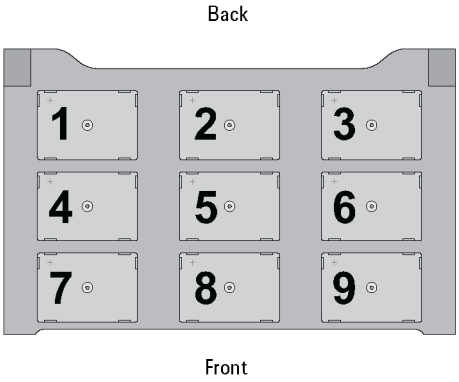


Figure 2 Bravo platform deck

Setting the Temperature of Bravo Deck Heat Blocks

Bravo deck positions 4 and 6 are equipped with Inheco heat blocks, used to incubate sample plates at defined temperatures during the run. Runs that include high- (85°C) or low- (4°C) temperature incubation steps may be expedited by pre-setting the temperature of the affected block before starting the run.

Bravo deck heat block temperatures may be changed using the Inheco Multi TEC Control device touchscreen as described in the steps below. See **Table 8** for designations of the heat block-containing Bravo deck positions on the Multi TEC control device.

Table 8 Inheco Multi TEC Control touchscreen designations

Bravo Deck Position	Designation on Inheco Multi TEC Control Screen
4	CPAC 2 1
6	CPAC 2 2

1 Using the arrow buttons, select the appropriate block (CPAC 2 block 1 or CPAC 2 block 2). See panel A in **Figure 3**.

2 To set the temperature of the selected block, press the SET button. See panel B in **Figure 3**.

3 Using the numeral pad, enter the desired temperature. The entered temperature appears in the top, left rectangle. Once the correct temperature is displayed, press the rectangle to enter the temperature. See panel C in **Figure 3**.

4 Press the Temp button until the new temperature is displayed on the SET button and until the Temp button is darkened, indicating that the selected heat block is heating or cooling to the new temperature setting. The current temperature of the block is indicated in the center of the display. See panel D in **Figure 3**.

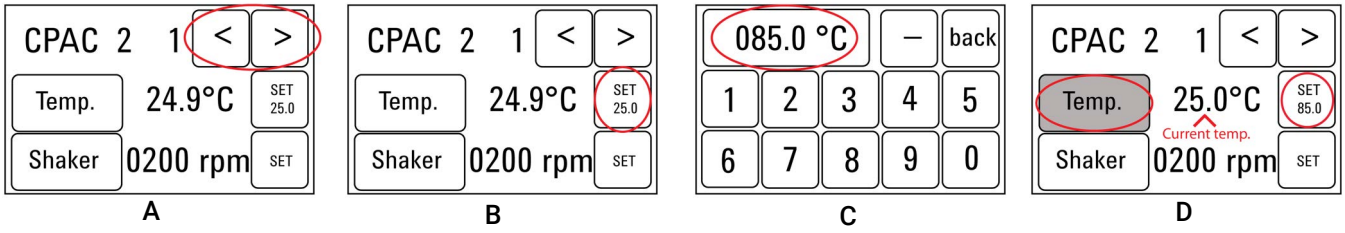


Figure 3 The steps for setting the temperature of Bravo deck heat blocks

Setting the Temperature of Bravo Deck Position 9 Using the ThermoCube Device

Bravo deck position 9 is equipped with a ThermoCube thermoelectric temperature control system, used to incubate components at a defined temperature during the run. During protocols that require temperature control at position 9, you will be instructed to start and set the temperature of the ThermoCube device before starting the run.

ThermoCube temperature settings are modified using the control panel (LCD display screen and four input buttons) on the front panel of the device using the following steps.

1 Turn on the ThermoCube and wait for the LCD screen to display **TEMP**.

2 Press the **UP** or **DOWN** button to change **SET TEMP 1** to the required set point.

3 Press the **START** button.

The ThermoCube then initiates temperature control of Bravo deck position 9 at the displayed set point.

About the VWorks Software Form for High-Throughput aCGH

The VWorks automation control software, included with your Agilent NGS Bravo Option A, allows you to control the integrated devices using a PC. The VWorks form *Agilent_SureTagHT_aCGH* allows you to set up and run the automated protocols that support the sample fragmentation, sample labeling, and hybridization preparation steps of the high-throughput aCGH workflow.

NOTE

The instructions in this manual are compatible with VWorks software version 13.1.0.1366.

If you have questions about VWorks version compatibility, please contact service.automation@agilent.com.

Logging in to the VWorks software

1. Double-click the VWorks icon for the *Agilent_SureTagHT_aCGH* VWorks form shortcut on the Windows desktop to start the VWorks software.
2. If User Authentication dialog is not visible, click **Log in** on the VWorks window toolbar.
3. In the User Authentication dialog, type your VWorks user name and password, and click **OK**. (If no user account is set up, contact the administrator.)

Automation Protocols used in the Workflow

Table 9 Overview of VWorks protocols

Workflow Step	Description	VWorks Protocols Used for NGS Bravo automation
Enzymatic DNA fragmentation*	Shears DNA samples using enzymatic fragmentation	00 EnzymaticFragmentation_SureTagHT
Primer annealing	Anneals random primers to fragmented DNA	01 AnnealRandomPrimers_SureTagHT
Labeling of gDNA with fluorescent dyes	Adds the appropriate Labeling Master Mix (Cy3 or Cy5) to the fragmented gDNA samples	02 Labeling_SureTagHT
Purification of labeled gDNA	Purifies the fluorescently labeled gDNA samples using SurePure Beads	03 PurifyLabeledgDNA_SureTagHT
Set up of hybridization reactions	Combines each test sample with its corresponding reference sample and adds the required volume of Hybridization Master Mix	04 HybSetup_SureTagHT

* The workflow also supports heat fragmentation. In such workflows, the heat fragmentation is performed as part of the primer annealing incubation. See **"Heat Fragmentation"** on page 35 for instructions.

3 gDNA Quantitation and Quality Analysis

This chapter describes the Agilent recommended procedures for measuring the concentration of the gDNA samples and assessing the gDNA quality.

Accurate assessment of gDNA quantity and quality is crucial to the success of an aCGH experiment. Inaccurate DNA quantitation can lead to different DNA inputs into the test sample and reference sample labeling reactions, which increases assay noise (as measured by the DLRSD or LogRatioImbalance QC metric). 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.

Assess gDNA Quantity and Quality

Agilent recommends the following assays for measuring gDNA concentration and quality. Instructions for the recommended assays are provided in this chapter.

Fluorometry: Use the Qubit dsDNA BR Assay Kit to measure the concentration of double-stranded DNA by fluorometry. ***This is the preferred method for assessing sample quantity.***

UV-VIS spectrophotometry: Use the NanoDrop 8000 UV-VIS Spectrophotometer (or equivalent) to assess gDNA concentration and purity.

Agarose gel electrophoresis: Run a sample of the gDNA on an agarose gel to assess gDNA intactness and the average molecular weight for each sample.

For assessment of gDNA quantity, Agilent recommends using a fluorometric method (such as Qubit) that is highly selective for double-stranded DNA, thus minimizing assay noise. A NanoDrop spectrophotometer can be used to assess gDNA purity.

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

Fluorometry

NOTE

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

This procedure uses reagents from the Qubit dsDNA BR Assay Kit. See **"Qubit dsDNA BR Assay Kit"** on page 84 for a list of kit components.

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

1. Set up 0.5-mL PCR tubes for the two standards plus the number of samples you are processing.
2. Make a Qubit working solution.

   For each standard and sample to be quantified, mix the components in **Table 10** together on a vortex mixer for 2 to 3 seconds.

Table 10 Qubit working solution

Component	Amount
Qubit dsDNA BR Reagent	1 µL
Qubit dsDNA BR Buffer	199 µL

3. Load 190 µL of Qubit working solution into the two 0.5-mL PCR tubes labeled for the standards.
4. Load 180 to 199 µL of Qubit working solution into the tubes labeled for your samples.

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

6 Add 1 to 20 µL of your DNA sample to the appropriate tubes to bring the final volume to 200 µL.

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

8 Incubate the tubes at room temperature for 2 minutes.

- To calibrate the Qubit Fluorometer:
  - **a** On the home screen of the Qubit, use the up or down arrow to select **dsDNA Broad Range Assay** as assay type, and then press **GO**. The standard screen is automatically displayed.
  - **b** Select **Run new calibration**, and then press **GO**.
  - **c** Insert the tube with the first standard into the Qubit Fluorometer, close the lid and press **GO**. After the reading is done, remove the standard.
  - **d** Insert the tube with the second standard into the Qubit Fluorometer, close the lid, and press **GO**. After the reading is done remove the standard.

  The calibration is complete after the second standard has been read.
- To measure sample concentration:
  - **a** After the calibration is complete, insert a sample and press **GO**.
  - **b** When the measurement is complete (approximately 5 seconds later), make a note of the reading.
  - **c** The result is displayed on the screen. The number displayed is the concentration of the nucleic acid in the assay tube.
  - **d** Remove the sample from the instrument, insert the next sample, and press **GO**.
  - **e** Repeat sample readings until all samples have been read.
  - **f** Calculate the concentration of your original sample.

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

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

where

QF value = the value given by the Qubit Fluorometer

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

UV-VIS spectrophotometry

This procedure uses the NanoDrop 8000 UV-VIS Spectrophotometer system.

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

2 Blank the instrument using 1.5 µL of the gDNA storage buffer that was used.

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 follows.

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

4 Record the $\mathbf{A_{260}/A_{280}}$ and $\mathbf{A_{260}/A_{230}}$ ratios. 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 4**. The ideal 260/230 ratio for pure DNA is >1.8. 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.

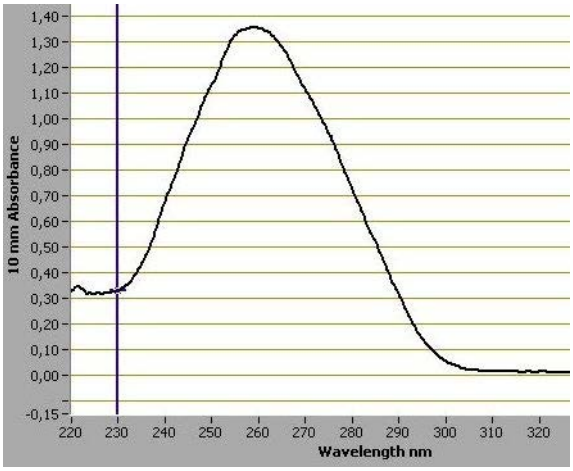


Figure 4 Typical spectrum of pure DNA

Agarose gel electrophoresis

This procedure uses the E-Gel agarose gel system and SYBR Gold Nucleic Acid Gel Stain.

1 Load 20 ng of gDNA for each sample in 10 µL of nuclease-free distilled water in the well of a single-comb E-Gel General Purpose Agarose Gel, 1.2%. (You do not need to add loading buffer in this system).

2 As a control, load 20 ng of human gDNA (e.g., Human Reference DNA from the SureTag HT Complete Kit or another source of commercial human gDNA) in 10 µL of nuclease-free distilled water in one of the wells of the E-Gel.

3 Mix 5 µL of TrackIt 1 Kb Plus DNA Ladder with 95 µL of nuclease-free distilled 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 the manufacturer's instructions.

5 Open the gel cassette with an E-Gel Opener as described in the manufacturer'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 distilled water) in a plastic tray for 15 minutes.

7 Visualize the gel on the UV-transilluminator using a SYBR photographic filter. Intact gDNA should appear as a compact, high-molecular weight band with no lower molecular weight smears.

4 Sample Fragmentation

This chapter describes how to use automated protocols on the NGS Bravo to fragment gDNA and anneal random primers.

Select between the following methods for DNA fragmentation.

- **"Enzymatic Fragmentation"** on page 26 – Fragments DNA using restriction digestion. This method is compatible with both CGH and CGH+SNP HT microarrays.
- **"Heat Fragmentation"** on page 35 – Fragments DNA by heating it to 98°C. This method is compatible with CGH HT microarrays, but is not suitable for use with CGH+SNP HT microarrays.

Enzymatic Fragmentation

This procedure uses restriction digestion enzymes and other reagents included in the SureTag HT Complete Kit and SureTag HT Kit. See **"Agilent SureTag HT Complete Kit and SureTag HT Kit"** on page 84 for a list of kit components.

The enzymatic fragmentation reactions require either 125 ng (for 16-pack microarrays) or 162.5 ng (for 24-pack microarrays) of gDNA sample as input. Before you begin, make sure you have enough gDNA for each sample to proceed.

Step 1. Fragment the gDNA samples with digestion enzymes

This step uses NGS Bravo automation protocol **00 EnzymaticFragmentation_SureTagHT** to prepare the enzymatic fragmentation reaction plate. Once the plate is prepared, transfer the plate from the Bravo deck to the pre-programmed thermal cycler to perform enzymatic fragmentation of the gDNA samples.

Prepare the thermal cycler and NGS Bravo

1 Pre-program the thermal cycler for the fragmentation reactions using the program in **Table 11**. Set the heated lid to 105°C. If your thermal cycler requires entering a reaction volume setting, enter 7 µL.

Table 11 Thermal cycler program for enzymatic fragmentation

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

2 Pre-set the temperature of Bravo deck position 4 and Bravo deck position 6 to 4°C using the Inheco Multi TEC control touchscreen.

Refer to **"Setting the Temperature of Bravo Deck Heat Blocks"** on page 18.

3 Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.

Refer to **"Setting the Temperature of Bravo Deck Position 9 Using the ThermoCube Device"** on page 19.

Prepare the gDNA sample source plate

4 For each test and reference gDNA sample, prepare a diluted stock of the appropriate concentration using **Nuclease Free Water**. See **Table 12** for the gDNA concentration required. Keep the diluted gDNA samples on ice.

Table 12 Concentration of diluted gDNA samples based on microarray format

Microarray format	Concentration of diluted gDNA
16-pack microarrays	45.45 ng/µL
24-pack microarrays	59.09 ng/µL

5 Add 2.75 µL of diluted gDNA sample to the wells of a processing plate (Eppendorf twin.tec or Armadillo) using the following configuration (depicted in **Figure 5**).

- Add the samples that you intend to label with cyanine 3 (Cy3) to the wells in columns 1 through 6.
- Add the samples that you intend to label with cyanine 5 (Cy5) to the wells in columns 7 through 12. Spin the plate briefly then keep on ice.

Typically, reference samples are labeled with Cy3 and test samples are labeled with Cy5.

NOTE

Important notes for preparing the gDNA sample source plate

- You can load as many as 48 test samples and 48 reference samples (i.e., 48 sample pairs). For greatest efficiency of reagent use, plan experiments for 48 samples pairs. This configuration uses all 96 wells of the plate.
- If you have fewer than 48 sample pairs, load the samples that you intend to label with Cy3 starting with column 1, well A1. Load the samples that you intend to label with Cy5 starting with column 7, well A7. Load an equal number of reference samples and test samples. Do not load partial columns.
- During preparation of the hybridization reactions, each Cy3-labeled sample in columns 1–6 is combined with the Cy5-labeled sample at the corresponding well location in columns 7–12. For example, the Cy3-labeled sample in well A1 is combined with the Cy5-labeled sample in well A7.

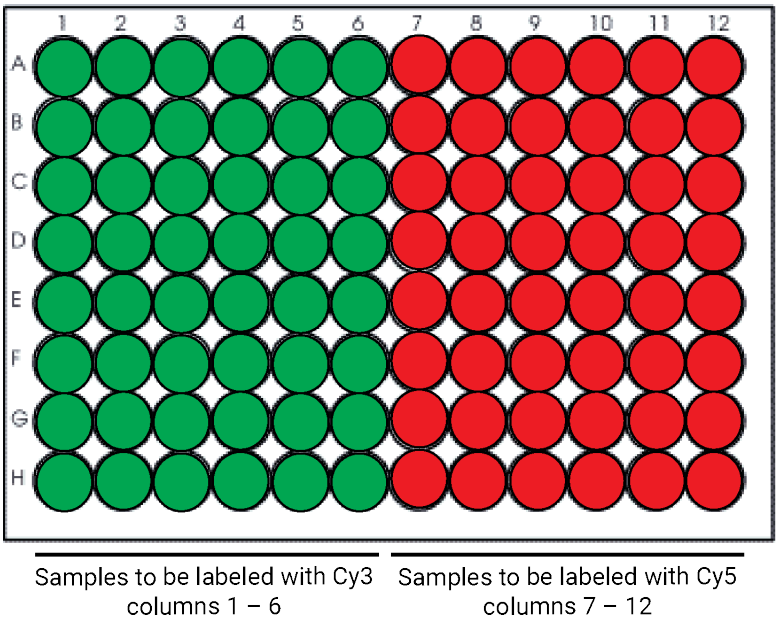


Figure 5 Configuration of the gDNA sample source plate. Columns 1–6 contain samples to be labeled with Cy3 (green). Columns 7–12 contain samples to be labeled with Cy5 (red).

Prepare the Enzymatic Fragmentation Master Mix source plate

6 If you have not already prepared a diluted working solution of 2 µg/µL BSA, then prepare that solution as follows.

   a Transfer 12.5 µL of the supplied 10 µg/µL **BSA** stock solution to a fresh, nuclease-free tube.

   b Add 50 µL of **Nuclease Free Water** to the tube. Mix on a vortex mixer for 5 seconds, then spin briefly. Keep on ice.

   The diluted BSA working solution is 2 µg/µL.

   c After preparing the master mix in **step 7**, keep the unused portion of the diluted BSA working solution at –20°C for future assays.

7 Prepare the appropriate volume of Enzymatic Fragmentation Master Mix by combining the components in **Table 13** in a 1.5-mL tube. Mix on a vortex mixer for 5 seconds, then spin briefly. Keep on ice.

   Table 13 lists the volumes required per reaction as well as the required volumes (including excess) based on the number of columns of sample pairs on the plate. Each sample pair consists of an individual reference sample and an individual test sample. Thus, 6 columns of sample pairs is a full 96-well plate.

Table 13 Enzymatic Fragmentation Master Mix preparation for 1 to 6 columns of sample pairs

Component	Volume per reaction	Volume Based on Number of Columns of Sample Pairs (1 to 6 Columns)					
		1 Column	2 Columns	3 Columns	4 Columns	5 Columns	6 Columns
Nuclease Free Water	1.85 µL	62.9 µL	94.4 µL	125.8 µL	157.3 µL	188.7 µL	220.2 µL
10× Restriction Enzyme Buffer	0.65 µL	22.1 µL	33.2 µL	44.2 µL	55.3 µL	66.3 µL	77.4 µL
Diluted BSA (2 µg/µL) prepared in **step 6**	0.25 µL	8.5 µL	12.8 µL	17.0 µL	21.3 µL	25.5 µL	29.8 µL
Alu I	0.125 µL	4.3 µL	6.4 µL	8.5 µL	10.6 µL	12.8 µL	14.9 µL
Rsa I	0.125 µL	4.3 µL	6.4 µL	8.5 µL	10.6 µL	12.8 µL	14.9 µL
Total Volume	**3.0 µL**	**102.1 µL**	**153.2 µL**	**204.0 µL**	**255.1 µL**	**306.1 µL**	**357.2 µL**

8 In a processing plate (Eppendorf twin.tec or Armadillo) prepare the Enzymatic Fragmentation Master Mix source plate for the run as indicated in **Table 14**. Add the indicated volume of master mix to all wells of column 1 of the plate. Keep the master mix source plate on ice during the aliquoting steps. The final configuration of the master mix source plate is shown in **Figure 6**.

Table 14 Enzymatic Fragmentation Master Mix source plate for 1 to 6 columns of sample pairs

Master Mix Solution	Position on Source Plate	Volume per well Based on Number of Columns of Sample Pairs (1 to 6 Columns)					
		1 Column	2 Columns	3 Columns	4 Columns	5 Columns	6 Columns
Enzymatic Fragmentation Master Mix	Column 1 (A1-H1)	12.0 µL	18.0 µL	24.0 µL	30.0 µL	36.0 µL	42.0 µL

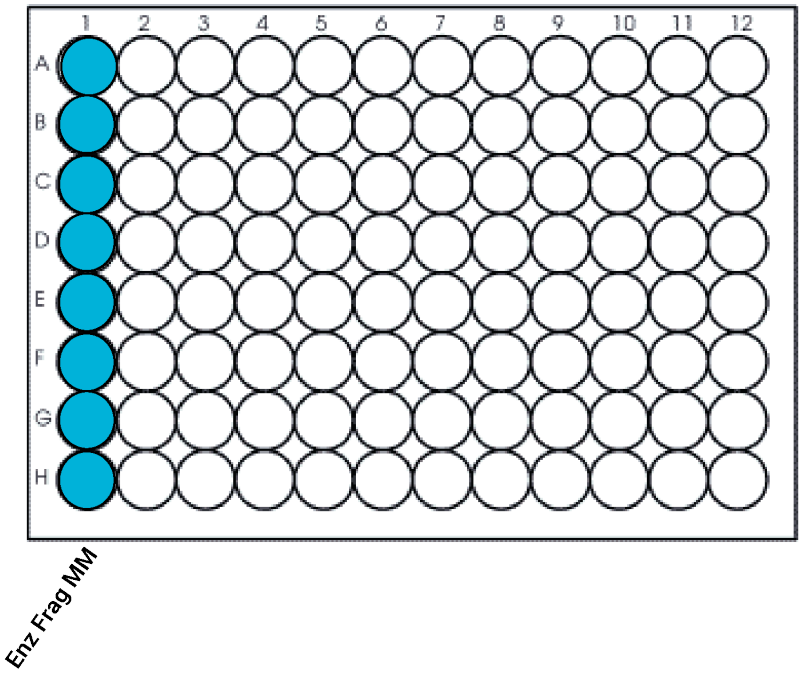


Figure 6 Configuration of the Enzymatic Fragmentation Master Mix source plate for protocol **00 EnzymaticFragmentation_SureTagHT**. The wells of column 1 contain the appropriate volume of Enzymatic Fragmentation Master Mix (Enz Frag MM).

Load the NGS Bravo and run the protocol

9 On the VWorks form, under **Protocol Parameters**, make selections for steps 1 through 5. Refer to **Table 15** for details.

Table 15 Protocol parameters for Enzymatic Fragmentation protocol

Protocol Parameter Step	Selection
1	Select protocol **00 EnzymaticFragmentation_SureTagHT.**
2	Select the type of plate for the thermal cycler.
3	Select the type of processing plate (Eppendorf twin.tec or Armadillo)
4	Selection not required for this protocol.
5	Select the number of columns of sample pairs in the gDNA sample source plate. A full plate (6 columns of test samples and 6 columns of reference samples) is 6 columns of sample pairs.

10 Click **Display Initial Bravo Deck Setup**.

The Bravo Deck Setup diagram displays the required Bravo deck configuration for the protocol.

11 Load the Bravo deck according to the Bravo Deck Setup diagram. The setup is also displayed in **Table 16**.

Table 16 Initial Bravo deck configuration for Enzymatic Fragmentation protocol

Bravo deck location	Content
2	New tip box
4	Empty thermal cycler plate
6	gDNA sample source plate prepared on **page 26**
8	Empty tip box
9	Enzymatic Fragmentation Master Mix source plate prepared on **page 28**

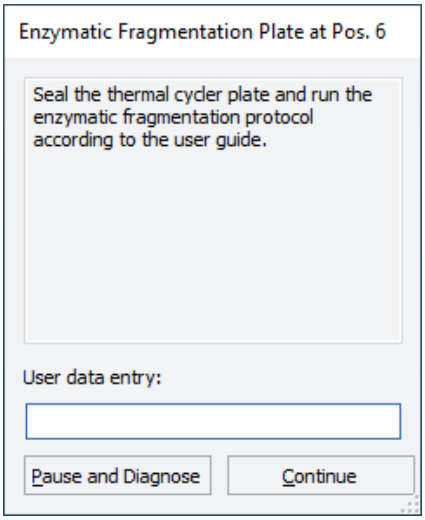
12 Update the **Current Tip State** indicator on the form to match the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively.

13 Click **Run Selected Protocol**.

The NGS Bravo executes the run, setting up the enzymatic fragmentation reactions in the thermal cycler plate.

Running the protocol takes approximately 5 minutes.

14 When you see the prompt below, remove the thermal cycler plate from position 6 of the Bravo deck. Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec. Click **Continue** in the prompt.



15 Centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal and to eliminate air bubbles.

16 Immediately place the plate in the thermal cycler. Close the lid and run the thermal cycler program in **Table 11**.

17 From the Bravo deck, remove the plate that was used as the Enzymatic Fragmentation Master Mix source plate from position 9 and set it aside. You can use this same plate again as the Random Primers source plate in the next automation protocol.

18 When the thermal cycler program is complete, remove the thermal cycler plate containing the fragmented gDNA samples from the thermal cycler. Spin the plate briefly then keep on ice. Continue to **"Step 2. Anneal the Random Primers"** or store the plate for later processing.

The locations of the fragmented gDNA samples in the thermal cycler plate match the locations of samples in the gDNA source plate.

Stopping Point At this point, you can store the plate of fragmented gDNA samples at –20°C for up to 30 days.

Step 2. Anneal the Random Primers

This step uses NGS Bravo automation protocol **01 AnnealRandomPrimers_SureTagHT** to prepare the primer annealing reaction plate. Once the plate is prepared, transfer the plate from the Bravo deck to the pre-programmed thermal cycler to anneal the Random Primers to the fragmented gDNA samples.

Prepare the thermal cycler and NGS Bravo

1 Pre-program the thermal cycler for random primer annealing using the program in **Table 17**. Set the heated lid to 105°C. If your thermal cycler requires entering a reaction volume setting, enter 9 µL.

Table 17 Thermal cycler program for random primer annealing

Step	Temperature	Time
Step 1	98°C	3 minutes
Step 2	4°C	5 minutes
Step 3	4°C	Hold

2 Pre-set the temperature of Bravo deck position 6 to 4°C using the Inheco Multi TEC control touchscreen.

3 Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.

Prepare the Random Primers Mix source plate

4 Prepare the appropriate volume of Random Primers Mix by combining the components in **Table 18** in a 1.5-mL tube. Mix on a vortex mixer for 5 seconds, then spin briefly. Keep on ice.

Table 18 lists the volumes required per reaction as well as the required volumes (including excess) based on the number of columns of sample pairs on the plate. Each sample pair consists of an individual reference sample and an individual test sample. Thus, 6 columns of sample pairs is a full 96-well plate.

Table 18 Random Primers Mix preparation for 1 to 6 columns of sample pairs

Component	Volume per reaction	Volume Based on Number of Columns of Sample Pairs (1 to 6 Columns)					
		1 Column	2 Columns	3 Columns	4 Columns	5 Columns	6 Columns
Nuclease Free Water	0.75 µL	19.1 µL	31.9 µL	44.6 µL	57.4 µL	70.1 µL	89.3 µL
Random Primers	1.25 µL	31.9µL	53.1 µL	74.4 µL	95.6 µL	116.9 µL	148.8 µL
Total Volume	**2.0 µL**	**51.0 µL**	**85.0 µL**	**119.0 µL**	**153.0 µL**	**187.0 µL**	**238.1 µL**

5 In a processing plate (Eppendorf twin.tec or Armadillo) prepare the Random Primers Mix source plate for the run as indicated in **Table 19**. Add the indicated volume of Random Primers Mix to all wells of column 2 of the plate. Keep the source plate on ice during the aliquoting steps. The final configuration of the source plate is shown in **Figure 7**.

You can use the same processing plate that was used for the Fragmentation Master Mix (if that protocol was performed the same day).

Table 19 Random Primers Mix source plate for 1 to 6 columns of sample pairs

Solution	Position on Source Plate	Volume per well Based on Number of Columns of Sample Pairs (1 to 6 Columns)					
		1 Column	2 Columns	3 Columns	4 Columns	5 Columns	6 Columns
Random Primers Mix	Column 2 (A2-H2)	6.0 µL	10.0 µL	14.0 µL	18.0 µL	22.0 µL	28.0 µL

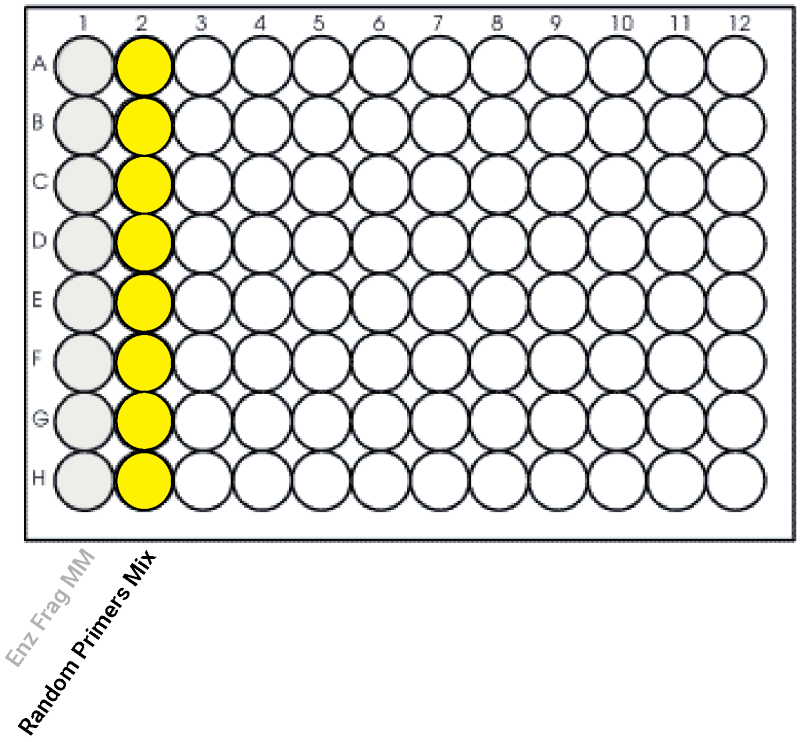


Figure 7 Configuration of the Random Primers Mix source plate for protocol **01 AnnealRandomPrimers_SureTagHT**. The wells of column 2 contain the appropriate volume of Random Primers Mix. The master mix dispensed into column 1 during a previous protocol is shown in light shading.

Load the NGS Bravo and run the protocol

6 On the VWorks form, under **Protocol Parameters**, make selections for steps 1 through 5. Refer to **Table 20** for details.

Table 20 Protocol parameters for Random Primer Annealing protocol

Protocol Parameter Step	Selection
1	Select **01 AnnealRandomPrimers_SureTagHT.**
2	Select the type of plate for the thermal cycler.
3	Select the type of processing plate (Eppendorf twin.tec or Armadillo)
4	Selection not required for this protocol.
5	Select the number of columns of sample pairs in the fragmented gDNA sample plate. A full plate (6 columns of test samples and 6 columns of reference samples) is 6 columns of sample pairs.

7 Click **Display Initial Bravo Deck Setup**.

The Bravo Deck Setup diagram displays the required Bravo deck configuration for the protocol.

8 Load the Bravo deck according to the Bravo Deck Setup diagram. The setup is also displayed in **Table 21**.

Table 21 Initial Bravo deck configuration for Random Primer Annealing protocol

Bravo deck location	Content
2	New tip box
6	Thermal cycler plate (seated in red aluminum insert) containing enzymatically fragmented gDNA samples
8	Empty tip box
9	Random Primers source plate prepared on **page 31**

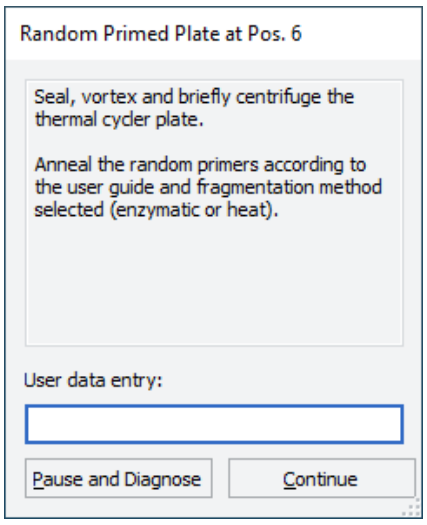
9 Update the **Current Tip State** indicator on the form to match the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively.

10 Click **Run Selected Protocol**.

The NGS Bravo executes the run, adding Random Primers Mix to the fragmented gDNA samples in the thermal cycler plate.

Running the protocol takes approximately 10 minutes.

11 When you see the prompt below, remove the thermal cycler plate from position 6 of the Bravo deck. Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec. Click **Continue** in the prompt.



12 Centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal and to eliminate air bubbles.

13 Immediately place the plate in the thermal cycler. Close the lid and run the thermal cycler program in **Table 17**.

14 From the Bravo deck, remove the plate that was used as the Random Primers source plate from position 9 and set it aside. You can use this same plate again for addition of the Labeling Master Mixes in the next automation protocol.

15 When the thermal cycler program is complete, remove the plate from the thermal cycler. Spin the plate briefly then keep on ice. The plate contains fragmented gDNA samples with Random Primers annealed. Continue to **Chapter 5**, "Sample Labeling," starting on page 41.

Heat Fragmentation

The heat fragmentation procedure uses the **01 AnnealRandomPrimers_SureTagHT** automation protocol to combine the gDNA samples with Random Primers and prepare the heat fragmentation reaction plate. Once the plate is prepared, transfer the plate from the Bravo deck to the pre-programmed thermal cycler to fragment the gDNA samples with heat while also annealing the Random Primers to the fragmented gDNA.

CAUTION Do not use heat fragmentation if you are processing CGH+SNP HT microarrays. Use the enzymatic fragmentation procedure provided on **page 26** to fragment gDNA using restriction digestion.

This procedure uses the **Nuclease Free Water** and **Random Primers** included in the SureTag HT Complete Kit and SureTag HT Kit. See **"Agilent SureTag HT Complete Kit and SureTag HT Kit"** on page 84 for a list of kit components.

The heat fragmentation reactions require either 125 ng (for 16-pack microarrays) or 162.5 ng (for 24-pack microarrays) of gDNA sample as input. Before you begin, make sure you have enough gDNA for each sample to proceed.

Prepare the thermal cycler and NGS Bravo

1 Pre-program the thermal cycler for the heat fragmentation reactions using the program in **Table 22**. Set the heated lid to 105°C. If your thermal cycler requires entering a reaction volume setting, enter 9 µL.

Table 22 Thermal cycler program for heat fragmentation and random primer annealing

Step	Temperature	Time
Step 1	98°C	10 minutes
Step 2	4°C	5 minutes
Step 3	4°C	Hold

2 Pre-set the temperature of Bravo deck position 4 and Bravo deck position 6 to 4°C using the Inheco Multi TEC control touchscreen.

Refer to **"Setting the Temperature of Bravo Deck Heat Blocks"** on page 18.

3 Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.

Refer to **"Setting the Temperature of Bravo Deck Position 9 Using the ThermoCube Device"** on page 19.

Prepare the gDNA sample source plate

4 For each test and reference gDNA sample, prepare a diluted stock of the appropriate concentration using **Nuclease Free Water**. See **Table 23** for the gDNA concentration required. Keep the diluted gDNA samples on ice.

Table 23 Concentration of diluted gDNA samples based on microarray format

Microarray format	Concentration of diluted gDNA
16-pack microarrays	21.74 ng/µL
24-pack microarrays	28.26 ng/µL

5 Add 5.75 µL of diluted gDNA sample to the wells of a thermal cycler plate using the following configuration (depicted in **Figure 8**).

- Add the samples that you intend to label with cyanine 3 (Cy3) to the wells in columns 1 through 6.
- Add the samples that you intend to label with cyanine 5 (Cy5) to the wells in columns 7 through 12. Spin the plate briefly then keep on ice.

Typically, reference samples are labeled with Cy3 and test samples are labeled with Cy5.

NOTE

Important notes for preparing the gDNA sample source plate

- You can load as many as 48 test samples and 48 reference samples (i.e., 48 sample pairs). For greatest efficiency of reagent use, plan experiments for 48 samples pairs. This configuration uses all 96 wells of the plate.
- If you have fewer than 48 sample pairs, load the samples that you intend to label with Cy3 starting with column 1, well A1. Load the samples that you intend to label with Cy5 starting with column 7, well A7. Load an equal number of reference samples and test samples. Do not load partial columns.
- During preparation of the hybridization reactions, each Cy3-labeled sample in columns 1–6 is combined with the Cy5-labeled sample at the corresponding well location in columns 7–12. For example, the Cy3-labeled sample in well A1 is combined with the Cy5-labeled sample in well A7.

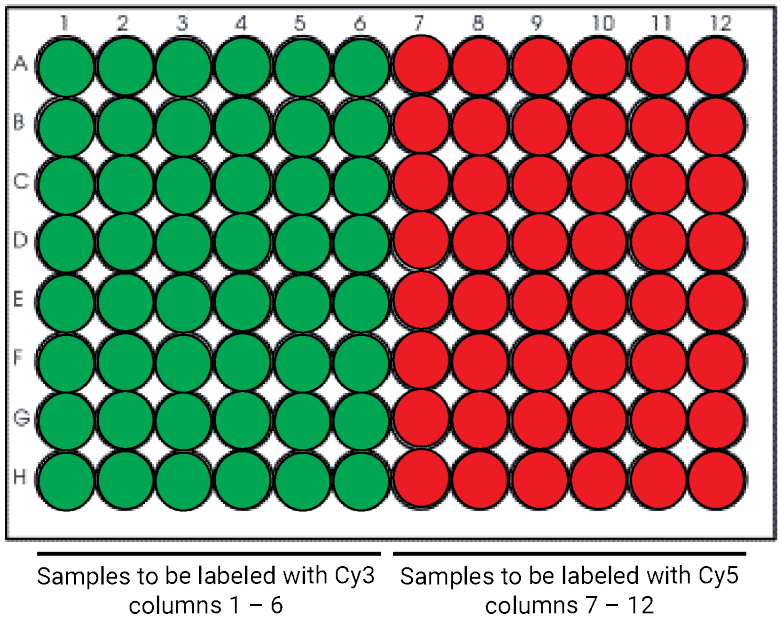


Figure 8 Configuration of the gDNA sample source plate. Columns 1–6 contain samples to be labeled with Cy3 (green). Columns 7–12 contain samples to be labeled with Cy5 (red).

Prepare the Random Primers Mix source plate for heat fragmentation

6 Prepare the appropriate volume of Random Primers Mix by combining the components in **Table 24** in a 1.5-mL tube. Mix on a vortex mixer for 5 seconds, then spin briefly. Keep on ice.

Table 24 lists the volumes required per reaction as well as the required volumes (including excess) based on the number of columns of sample pairs on the plate. Each sample pair consists of an individual reference sample and an individual test sample. Thus, 6 columns of sample pairs is a full 96-well plate.

Table 24 Random Primers Mix preparation for 1 to 6 columns of sample pairs

Component	Volume per reaction	Volume Based on Number of Columns of Sample Pairs (1 to 6 Columns)					
		1 Column	2 Columns	3 Columns	4 Columns	5 Columns	6 Columns
Nuclease Free Water	0.75 µL	19.1 µL	31.9 µL	44.6 µL	57.4 µL	70.1 µL	89.3 µL
Random Primers	1.25 µL	31.9µL	53.1 µL	74.4 µL	95.6 µL	116.9 µL	148.8 µL
Total Volume	**2.0 µL**	**51.0 µL**	**85.0 µL**	**119.0 µL**	**153.0 µL**	**187.0 µL**	**238.1 µL**

7 In a processing plate (Eppendorf twin.tec or Armadillo) prepare the Random Primers source plate for the run as indicated in **Table 25**. Add the indicated volume of Random Primers Mix to all wells of column 2 of the plate. Keep the source plate on ice during the aliquoting steps. The final configuration of the source plate is shown in **Figure 9**.

Table 25 Random Primers Mix source plate for 1 to 6 columns of sample pairs

Solution	Position on Source Plate	Volume per well Based on Number of Columns of Sample Pairs (1 to 6 Columns)					
		1 Column	2 Columns	3 Columns	4 Columns	5 Columns	6 Columns
Random Primers Mix	Column 2 (A2-H2)	6.0 µL	10.0 µL	14.0 µL	18.0 µL	22.0 µL	28.0 µL

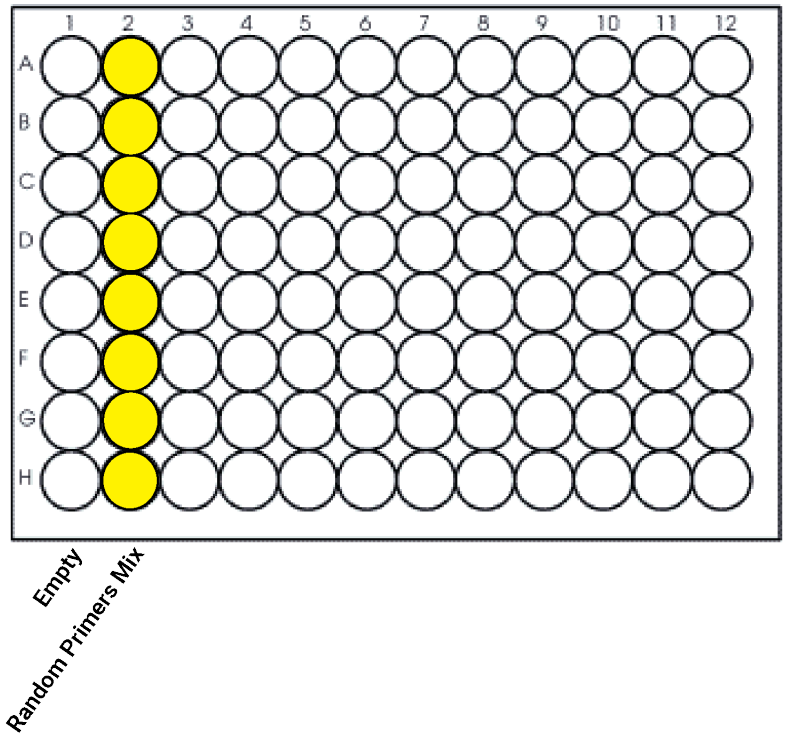


Figure 9 Configuration of the Random Primers Mix source plate for protocol **01 AnnealRandomPrimers_SureTagHT**. The wells of column 2 contain the appropriate volume of Random Primers Mix. The wells of column 1 are empty.

Load the NGS Bravo and run the protocol

8 On the VWorks form, under **Protocol Parameters**, make selections for steps 1 through 5. Refer to **Table 26** for details.

Table 26 Protocol parameters for Random Primer Annealing protocol

Protocol Parameter Step	Selection
1	Select **01 AnnealRandomPrimers_SureTagHT.**
2	Select the type of plate for the thermal cycler.
3	Select the type of processing plate (Eppendorf twin.tec or Armadillo)
4	Selection not required for this protocol.
5	Select the number of columns of sample pairs in the gDNA sample plate. A full plate (6 columns of test samples and 6 columns of reference samples) is 6 columns of sample pairs.

9 Click **Display Initial Bravo Deck Setup**.

The Bravo Deck Setup diagram displays the required Bravo deck configuration for the protocol.

10 Load the Bravo deck according to the Bravo Deck Setup diagram. The setup is also displayed in **Table 27**.

Table 27 Initial Bravo deck configuration for Random Primer Annealing protocol

Bravo deck location	Content
2	New tip box
6	gDNA sample source plate
8	Empty tip box
9	Random Primers source plate prepared on **page 37**

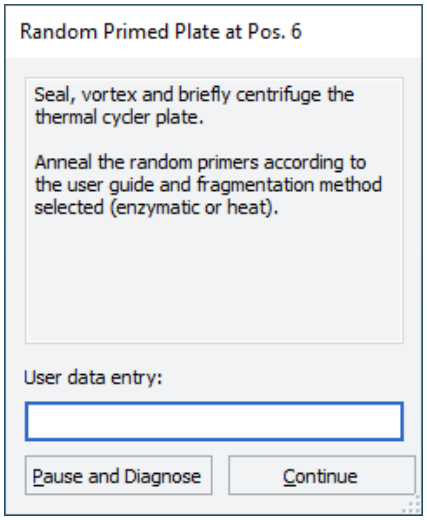
11 Update the **Current Tip State** indicator on the form to match the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively.

12 Click **Run Selected Protocol**.

The NGS Bravo executes the run, adding Random Primers Mix to the gDNA samples in the thermal cycler plate.

Running the protocol takes approximately 10 minutes.

13 When you see the prompt below, remove the thermal cycler plate from position 6 of the Bravo deck. Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec. Click **Continue** in the prompt.



14 Centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal and to eliminate air bubbles.

15 Immediately place the plate in the thermal cycler. Close the lid and run the thermal cycler program in **Table 22**.

16 From the Bravo deck, remove the plate that was used as the Random Primers source plate from position 9 and set it aside. You can use this same plate again for addition of the Labeling Master Mixes in the next automation protocol.

17 When the thermal cycler program is complete, remove the plate from the thermal cycler. Spin the plate briefly then keep on ice. The plate contains heat fragmented gDNA samples with Random Primers annealed. Continue to **Chapter 5**, "Sample Labeling," starting on page 41.

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5 Sample Labeling

This chapter describes how to use automated protocols on the NGS Bravo to fluorescently label the gDNA samples with cyanine 3 and cyanine 5.

The Agilent SureTag HT Complete Kit and SureTag HT Kit contain sufficient two-color labeling reaction reagents for:

- Six 16-pack microarrays *or*
- Four 24-pack microarrays

The kit uses random primers and the Exo(-) Klenow fragment to differentially label gDNA samples with fluorescent-labeled nucleotides (Cyanine 5-dUTP and Cyanine 3-dUTP). The test sample is labeled with one dye while the reference sample is labeled with the other dye. The “polarity” of the sample labeling is a matter of experimental choice. Typically, the test sample is labeled with cyanine 5 and the reference with cyanine 3.

Step 1. Label gDNA with Fluorescent Dyes (Cyanine 3 and Cyanine 5)

This step uses NGS Bravo automation protocol **02 Labeling_SureTagHT** to combine the fragmented gDNA samples with the appropriate Labeling Master Mix. Once the plate is prepared, transfer the plate from the Bravo deck to the pre-programmed thermal cycler to fluorescently label the gDNA samples.

This procedure uses reagents included in the SureTag HT Complete Kit and SureTag HT Kit. See **"Agilent SureTag HT Complete Kit and SureTag HT Kit"** on page 84 for a list of kit components.

NOTE

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

Prepare the thermal cycler and NGS Bravo

1 Pre-program the thermal cycler for the labeling reactions using the program in **Table 28**. If your thermal cycler requires entering a reaction volume setting, enter 12.5 µL.

Table 28 Thermal cycling program for DNA labeling

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

2 Pre-set the temperature of Bravo deck position 6 to 4°C using the Inheco Multi TEC control touchscreen, as described in Setting the Temperature of Bravo Deck Heat Blocks.

3 Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck.

4 Place a red aluminum insert at Bravo deck position 6.

Prepare the Labeling Master Mixes source plate

5 Prepare two Labeling Master Mixes (one with Cy3 dye and one with Cy5 dye) by combining the components in **Table 29** in a 1.5-mL tube. Mix each master mix on a vortex mixer for 5 seconds, then spin briefly. Keep on ice.

Table 29 lists the volumes required per reaction as well as the required volumes (including excess) based on the number of columns of sample pairs. ***One of the master mixes is to be used for the references samples, while the other is to be used for the test samples.***

Table 29 Cy3/Cy5 Labeling Master Mixes preparation - one with Cyanine 3-dUTP and one with Cyanine 5-dUTP

Component	Volume per reaction	Volume Based on Number of Columns of Sample Pairs (1 to 6 Columns)					
		1 Column	2 Columns	3 Columns	4 Columns	5 Columns	6 Columns
5× Reaction Buffer	2.5 µL	42.5 µL	63.8 µL	85.0 µL	106.3 µL	127.5 µL	148.8 µL
10× dNTPs	1.25 µL	21.3 µL	31.9 µL	42.5 µL	53.1 µL	63.8 µL	74.4 µL
Cyanine 3-dUTP *or* **Cyanine 5-dUTP**	0.75 µL	12.8 µL	19.1 µL	25.5 µL	31.9 µL	38.3 µL	44.6 µL
Exo (-) Klenow	0.25 µL	4.3 µL	6.4 µL	8.5 µL	10.6 µL	12.8 µL	14.9 µL
Total volume	**4.75 µL**	**80.9 µL**	**121.1 µL**	**161.5 µL**	**201.9 µL**	**242.4 µL**	**282.7 µL**

6 In a processing plate (Eppendorf twin.tec or Armadillo) prepare the Labeling Master Mixes source plate for the run as indicated in **Table 30**. Keep the master mix source plate on ice during the aliquoting steps. The final configuration of the master mix source plate is shown in **Figure 10**.

- You can use the same processing plate that was used as the master mix source plate in the previous automation protocol (if that protocol was performed the same day).

Table 30 Labeling Master Mix source plate for 1 to 6 columns of sample pairs

Master Mix Solution	Position on Source Plate	Volume per well Based on Number of Columns of Sample Pairs (1 to 6 Columns)					
		1 Column	2 Columns	3 Columns	4 Columns	5 Columns	6 Columns
Cy3 Labeling Master Mix	Column 3 (A3-H3)	9.5 µL	14.0 µL	19.0 µL	23.5 µL	28.5 µL	33.0 µL
Cy5 Labeling Master Mix	Column 4 (A4-H4)	9.5 µL	14.0 µL	19.0 µL	23.5 µL	28.5 µL	33.0 µL

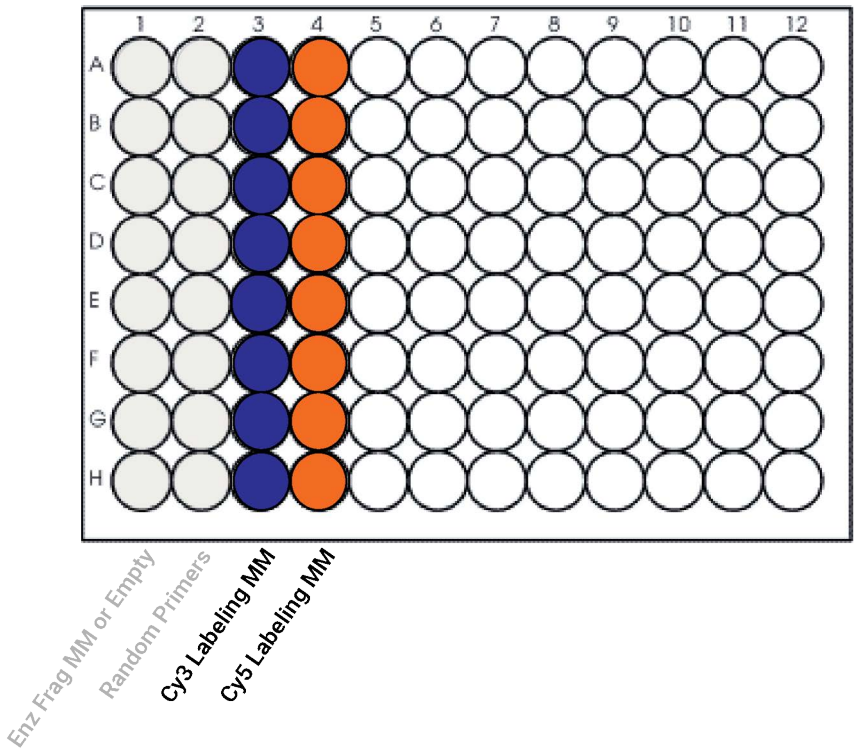


Figure 10 Configuration of the Labeling Master Mixes source plate for protocol **02 Labeling_SureTagHT**. The wells of column 3 contain Cy3 Labeling Master Mix and the wells of column 4 contain Cy5 Labeling Master Mix. The solutions dispensed into columns 1 and 2 during previous protocols are shown in light shading.

Load the NGS Bravo and run the protocol

7 On the VWorks form, under **Protocol Parameters**, make selections for steps 1 through 5. Refer to **Table 31** for details.

Table 31 Protocol parameters for Labeling protocol

Protocol Parameter Step	Selection
1	Select **02 Labeling_SureTagHT.**
2	Select the type of plate for the thermal cycler.
3	Select the type of processing plate (Eppendorf twin.tec or Armadillo)
4	Selection not required for this protocol.
5	Select the number of columns of sample pairs in the gDNA sample plate. A full plate (6 columns of test samples and 6 columns of reference samples) is 6 columns of sample pairs.

8 Click **Display Initial Bravo Deck Setup**.

The Bravo Deck Setup diagram displays the required Bravo deck configuration for the protocol.

9 Load the Bravo deck according to the Bravo Deck Setup diagram. The setup is also displayed in **Table 32**.

Table 32 Initial Bravo deck configuration for Labeling protocol

Bravo deck location	Content
2	New tip box
6	Thermal cycler plate (seated in red aluminum insert) of fragmented gDNA samples with Random Primers annealed
8	Empty tip box
9	Labeling Master Mixes source plate prepared on **page 42**

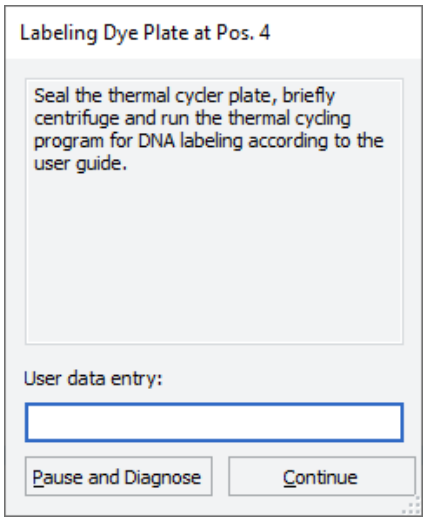
10 Update the **Current Tip State** indicator on the form to match the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively.

11 Click **Run Selected Protocol**.

The NGS Bravo executes the run, adding the appropriate Labeling Master Mix to the plate of fragmented gDNA samples. The Cy3 Labeling Master Mix is added to the samples in columns 1–6 and the Cy5 Labeling Master Mix is added to the samples in columns 7–12.

Running the protocol takes approximately 15 minutes.

12 When you see the prompt below, remove the thermal cycler plate from position 4 of the Bravo deck. Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing setting of 165°C and 1.0 seconds. Click **Continue** in the prompt.



13 Centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal and to eliminate air bubbles.

14 Immediately place the plate in the thermal cycler. Close the lid and run the thermal cycler program in **Table 28**.

15 From the Bravo deck, remove the plate that was used as the Labeling Master Mixes source plate from position 9 and set it aside. You can use this same plate again as the Hybridization Master Mix source plate in a later automation protocol.

16 When the thermal cycler program is complete, remove the plate containing the fluorescently labeled gDNA samples from the thermal cycler. Spin the plate briefly then keep on ice. Continue to **"Step 2. Purify Labeled gDNA"** or store the plate for later processing.

Stopping Point At this point, you can store the labeled gDNA samples at –20°C for up to 30 days. Protect the samples from light during storage.

Step 2. Purify Labeled gDNA

This step uses NGS Bravo automation protocol **03 PurifyLabeledgDNA_SureTagHT** to purify the fluorescently labeled gDNA samples using SurePure Beads.

This procedure uses reagents included in the SureTag HT Complete Kit and SureTag HT Kit. See **"Agilent SureTag HT Complete Kit and SureTag HT Kit"** on page 84 for a list of kit components.

Prepare the reagent reservoirs and NGS Bravo

1 Prepare an Agilent deep well reservoir containing 50 mL of freshly prepared 80% ethanol.

The 80% ethanol is used as a wash buffer in the purification procedure.

2 Prepare an Agilent deep well reservoir containing 50 mL of 1× TE buffer. Keep covered until use.

This reservoir is added to the Bravo deck when prompted during the automation protocol.

3 Pre-set the temperature of Bravo deck position 4 to 4°C and Bravo deck position 6 to 45°C using the Inheco Multi TEC control touchscreen.

4 Place a red aluminum insert at Bravo deck position 4.

Prepare the SurePure Beads Master Mix source plate

5 Prepare the appropriate volume of SurePure Beads Master Mix by combining the components in **Table 33** in an appropriately sized conical tube. Mix on a vortex mixer for 5 seconds, then spin briefly. Keep on ice.

Table 33 lists the volumes required per reaction as well as the required volumes (including excess) based on the number of columns of sample pairs on the plate. Each sample pair consists of an individual reference sample and an individual test sample. Thus, 6 columns of sample pairs is a full 96-well plate.

Table 33 SurePure Beads Master Mix preparation for 1 to 6 columns of sample pairs

Component	Volume per reaction	Volume Based on Number of Columns of Sample Pairs (1 to 6 Columns)					
		1 Column	2 Columns	3 Columns	4 Columns	5 Columns	6 Columns
SureTag HT Buffer	30.0 µL	592.3 µL	1151.6 µL	1645.2 µL	2221.0 µL	2714.5 µL	3290.3 µL
100% ethanol	120.0 µL	2369.0 µL	4606.5 µL	6580.6 µL	8883.9 µL	10858.1 µL	13161.3 µL
SurePure Beads	5.0 µL	98.7 µL	191.9 µL	274.2 µL	370.2 µL	452.4 µL	548.4 µL
Total Volume	**155.0 µL**	**3060.0 µL**	**5950.0 µL**	**8500.0 µL**	**11475.1 µL**	**14025.0 µL**	**17000.0 µL**

6 In a clean Agilent waste plate (p/n 201240-100) prepare the SurePure Beads Master Mix source plate for the run as indicated in **Table 34**. Mix the tube of SurePure Beads Master Mix on a vortex mixer for 5 seconds, then spin briefly. Add the indicated volume of SurePure Beads

Master Mix to all wells of column 1 of the plate. Keep the source plate on ice during the aliquoting steps. The final configuration of the source plate is shown in **Figure 11**.

Table 34 SurePure Beads Master Mix source plate for 1 to 6 columns of sample pairs

Solution	Position on Source Plate	Volume per well Based on Number of Columns of Sample Pairs (1 to 6 Columns)					
		1 Column	2 Columns	3 Columns	4 Columns	5 Columns	6 Columns
SurePure Beads Master Mix	Column 1 (A1-H1)	360 µL	700 µL	1000 µL	1350 µL	1650 µL	2000 µL

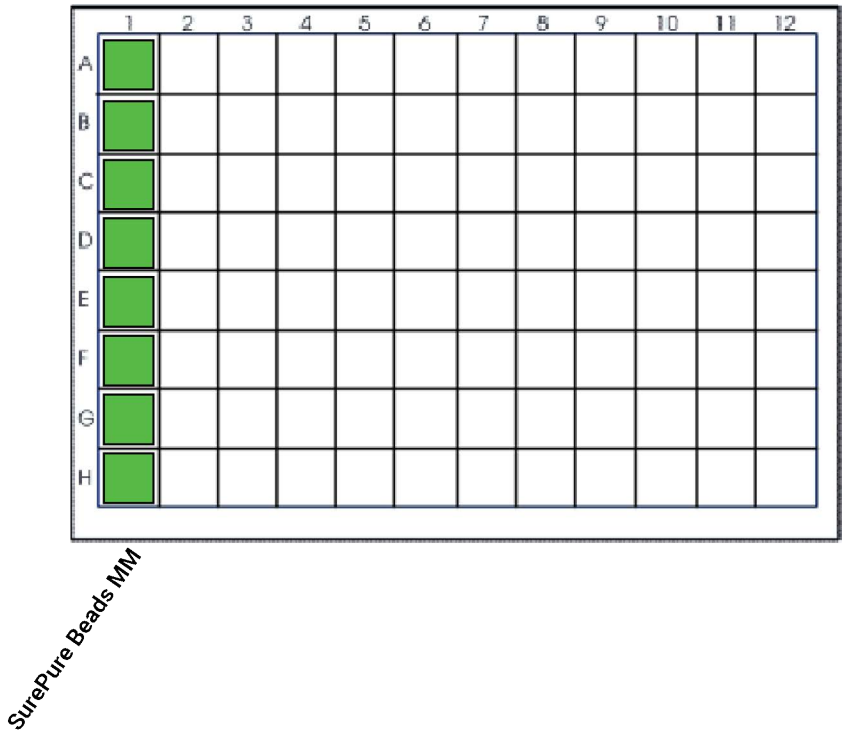


Figure 11 Configuration of the SurePure Beads Master Mix source plate for protocol **03 PurifyLabeled gDNA_SureTagHT**. The square wells of column 1 contain the appropriate volume of SurePure Beads Master Mix.

Load the NGS Bravo and run the protocol.

7 On the VWorks form, under **Protocol Parameters**, make selections for steps 1 through 5. Refer to **Table 35** for details.

Table 35 Protocol parameters for Labeled gDNA Purification protocol

Protocol Parameter Step	Selection
1	Select **03 PurifyLabeledgDNA_SureTagHT.**
2	Select the type of plate for the thermal cycler.
3	Select the type of processing plate (Eppendorf twin.tec or Armadillo)
4	Selection not required for this protocol.
5	Select the number of columns of sample pairs in the gDNA sample plate. A full plate (6 columns of test samples and 6 columns of reference samples) is 6 columns of sample pairs.

8 Click **Display Initial Bravo Deck Setup**.

The Bravo Deck Setup diagram displays the required Bravo deck configuration for the protocol.

9 Load the Bravo deck according to the Bravo Deck Setup diagram. The setup is also displayed in **Table 36**.

Table 36 Initial Bravo deck configuration for Labeled gDNA Purification protocol

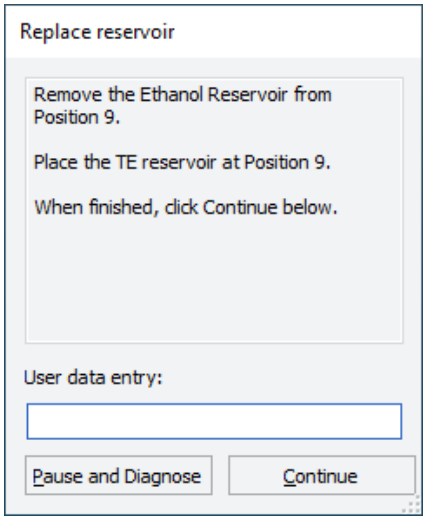
Bravo deck location	Content
1	SurePure Beads Master Mix source plate (Agilent 2 mL square well waste plate) prepared on **page 46**
2	New tip box
3	Empty processing plate (used for collection of purified labeled gDNA samples)
4	Thermal cycler plate (seated in red aluminum insert) containing labeled gDNA samples
6	Empty processing plate (used for bead processing)
8	Empty tip box
9	80% ethanol reservoir prepared on **page 46**

10 Update the **Current Tip State** indicator on the form to match the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively.

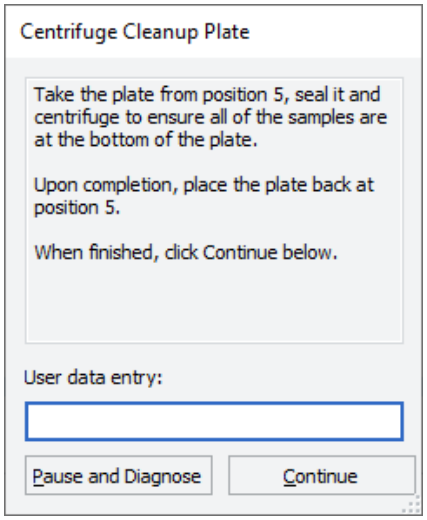
11 Click **Run Selected Protocol**.

The NGS Bravo executes the run, mixing the fluorescently labeled gDNA samples with the SurePure Beads Master Mix. Running the protocol takes approximately 45 minutes.

12 When you see the **Replace reservoir** prompt, shown below, remove the reservoir of 80% ethanol from Bravo deck position 9 and replace with the reservoir of 1× TE buffer prepared on **page 46**. Click **Continue** in the prompt.

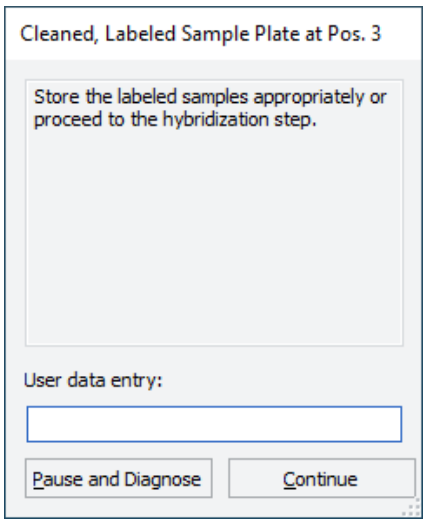


13 When you see the **Centrifuge Cleanup Plate** prompt, shown below, remove the processing plate from Bravo deck position 5. Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing setting of 165°C and 1.0 seconds, then centrifuge the plate for 30 seconds. After centrifugation, remove the plate seal and put the plate back on Bravo deck position 5. Click **Continue** in the prompt.



The NGS Bravo executes the remainder of the run, eluting the purified, fluorescently labeled gDNA samples from the beads and transferring the samples to the processing plate.

14 When you see the prompt below, remove the plate from position 3 of the Bravo deck. This plate contains the purified, labeled gDNA samples. Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing setting of 165°C and 1.0 seconds. Click **Continue** in the prompt.



15 Centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal and to eliminate air bubbles.

16 Continue to **"Step 3. Determine Yield and Specific Activity"** on page 50 or store the plate for later processing.

Stopping Point At this point, you can store the purified labeled gDNA samples at –20°C for up to 30 days. Protect the samples from light during storage.

Step 3. Determine Yield and Specific Activity

Use the NanoDrop 8000 UV-VIS Spectrophotometer to measure yield and specific activity.

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

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

3 Use 1 µL of purified labeled gDNA for quantitation. Measure the absorbance at A_{260nm} (DNA), A_{550nm} (cyanine 3), and A_{650nm} (cyanine 5).

NOTE The purified labeled gDNA samples may contain residual SurePure Beads that can interfere with the absorbance measurements. Before removing the 1-µL aliquots from the sample plate, make sure that any residual beads are collected at the bottom of the wells and take care to avoid pipetting the beads. You may use a magnet for this purpose.

4 Calculate the Specific Activity of the labeled gDNA:

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

*pmol dyes per µg gDNA

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

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

Refer to **Table 37** for expected yield of labeled gDNA and specific activity after labeling and purification, when starting with high quality gDNA.

Table 37 Expected Yield and Specific Activity after Labeling and Clean-up

Measurement	Expected result
Yield	2.0 to 4.5 µg per reaction*
Specific Activity of Cyanine 3 and Cyanine 5 Labeled Sample	20–50 pmol/µg

* If the average yield is outside of this range, Agilent recommends repeating the sample fragmentation and sample labeling steps with a fresh aliquot of the DNA sample.

The cyanine 3 and cyanine 5 yields after labeling should be the same or nearly the same (within 10%). If not, refer to **"Troubleshooting"** on page 77.

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6 Microarray Processing

This chapter describes how to hybridize, wash and scan Agilent CGH and CGH+SNP microarrays and to extract data using the Agilent CytoGenomics software.

Preparing the samples for hybridization uses an automated protocol on the NGS Bravo. After hybridization preparation, the remainder of the workflow must be performed manually (i.e., without the use of NGS Bravo automation).

Hybridization

Step 1. Reconstitute the aCGH Blocking Agent

Refer to **"Step 1. Reconstitute the aCGH Blocking Agent"** on page 36 of **Chapter 5** for instructions on reconstituting the aCGH Blocking Agilent. Then continue to **"Step 2. Prepare labeled gDNA for hybridization"**, below.

Step 2. Prepare labeled gDNA for hybridization

Prepare the thermal cycler and NGS Bravo

1 Pre-program the thermal cycler for the hybridization reactions using the program in **Table 38**. If your thermal cycler requires entering a reaction volume setting, enter either 24.5 µL (if using 16-pack arrays) or 17.2 µL (if using 24-pack arrays).

Table 38 Thermal cycling program for hybridization preparation

Step	Temperature	Time
Step 1	98°C	3 minutes
Step 2	37°C	30 minutes
Step 3	37°C	hold (do not allow hold to last longer than 1 hour; see CAUTION on **page 58**)

2 Pre-set the temperature of Bravo deck position 4 and Bravo deck position 6 to 4°C using the Inheco Multi TEC control touchscreen.

3 Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 30 mL of 80% ethanol.

4 Place a red aluminum insert at Bravo deck position 6.

Prepare the Hybridization Master Mix source plate

5 Prepare the appropriate volume of Hybridization Master Mix by combining the components in either **Table 39** (if using 16-pack arrays) or **Table 40** (if using 24-pack arrays) in a 1.5-mL tube. Mix well by pipetting up and down. Spin briefly and keep on ice.

Table 39 and **Table 40** list the volumes required per reaction as well as the required volumes (including excess) based on the number of columns of sample pairs on the plate. Each sample pair consists of an individual reference sample and an individual test sample. Thus, 6 columns of sample pairs is a full 96-well plate.

Table 39 Hybridization Master Mix preparation for 16-pack arrays - 1 to 6 columns of sample pairs

Component	Volume per reaction	Volume Based on Number of Columns of Sample Pairs (1 to 6 Columns)					
		1 Column	2 Columns	3 Columns	4 Columns	5 Columns	6 Columns
Nuclease-free water	2.51 µL	37.7 µL	59.7 µL	81.8 µL	110.4 µL	132.5 µL	154.5 µL
Cot-1 DNA (1.0 mg/mL)	1.11 µL	16.7 µL	26.4 µL	36.2 µL	48.8 µL	58.6 µL	68.3 µL
aCGH Blocking Agent, 40×	0.61 µL	9.2 µL	14.5 µL	19.9 µL	26.8 µL	32.2 µL	37.6 µL
2× HI-RPM Hybridization Buffer	12.2 µL	183.0 µL	290.3 µL	397.6 µL	536.5 µL	643.8 µL	751.1 µL
Total Volume	**16.43 µL**	**246.6 µL**	**390.9 µL**	**535.5 µL**	**722.5 µL**	**867.1 µL**	**1011.5 µL**

Table 40 Hybridization Master Mix preparation for 24-pack arrays - 1 to 6 columns of sample pairs

Component	Volume per reaction	Volume (µL) Based on Number of Columns of Sample Pairs (1 to 6 Columns)					
		1 Column	2 Columns	3 Columns	4 Columns	5 Columns	6 Columns
Cot-1 DNA (1.0 mg/mL)	0.78 µL	15.1 µL	22.1 µL	29.0 µL	39.4 µL	46.3 µL	53.3 µL
aCGH Blocking Agent, 40×	0.43 µL	8.3 µL	12.2 µL	16.0 µL	21.7 µL	25.5 µL	29.4 µL
2× HI-RPM Hybridization Buffer	8.59 µL	166.1 µL	242.9 µL	319.6 µL	433.6 µL	510.4 µL	587.1 µL
Total Volume	**9.8 µL**	**189.5 µL**	**277.2 µL**	**364.6 µL**	**494.7 µL**	**582.2 µL**	**669.8 µL**

6 Prepare the Hybridization Master Mix source plate for the run as indicated in **Table 41** (if using 16-pack arrays) or **Table 42** (if using 24-pack arrays). Keep the master mix source plate on ice during the aliquoting steps. The final configuration of the master mix source plate is shown in **Figure 12**.

- You can use the same processing plate that was used as the Labeling Master Mix source plate (if that protocol was performed the same day).

Table 41 Hybridization Master Mix source plate for 16-pack arrays - 1 to 6 columns of sample pairs

Master Mix Solution	Position on Source Plate	Volume per well Based on Number of Columns of Sample Paris in the Run (1 to 6 Columns)					
		1 Column	2 Columns	3 Columns	4 Columns	5 Columns	6 Columns
Hybridization Master Mix	Column 5 (A5-H5)	29.0 µL	46.0 µL	63.0 µL	85.0 µL	102.0 µL	119.0 µL

Table 42 Hybridization Master Mix source plate for 24-pack arrays - 1 to 6 columns of sample pairs

Master Mix Solution	Position on Source Plate	Volume per well Based on Number of Columns of Sample Paris in the Run (1 to 6 Columns)					
		1 Column	2 Columns	3 Columns	4 Columns	5 Columns	6 Columns
Hybridization Master Mix	Column 5 (A5-H5)	22.3 µL	32.6 µL	42.9 µL	58.2 µL	68.5 µL	78.8 µL

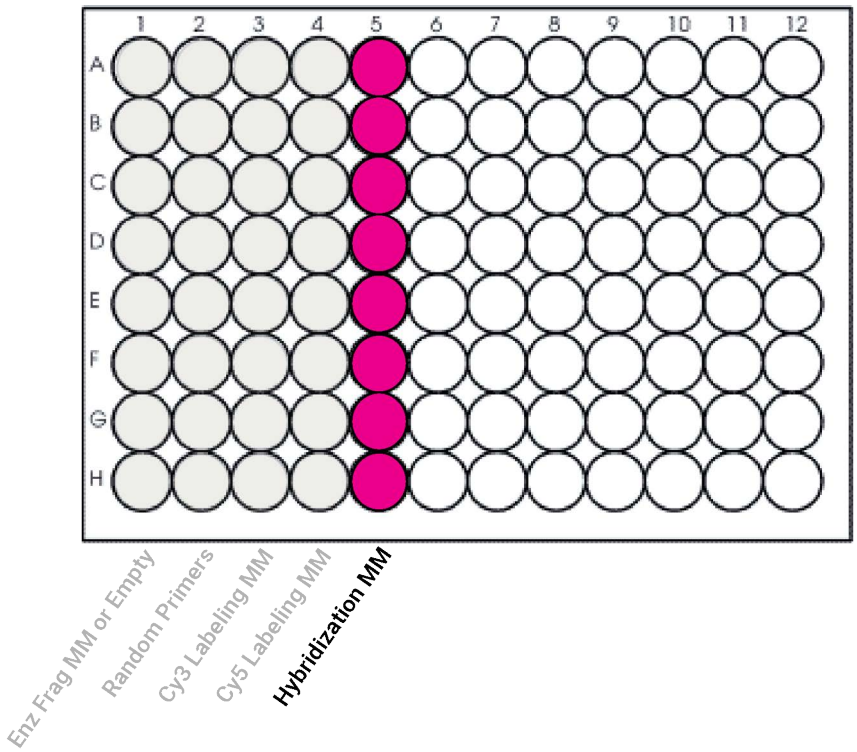


Figure 12 Configuration of the Hybridization Master Mix source plate for protocol **04 HybSetup_SureTagHT**. The wells of column 5 contain the Hybridization Master Mix. The solutions dispensed into columns 1–4 during previous steps are shown in light shading.

Load the NGS Bravo and run the protocol

7 On the VWorks form, under **Protocol Parameters**, make selections for steps 1 through 5. Refer to **Table 43** for details.

Table 43 Protocol parameters for Hybridization Setup protocol

Protocol Parameter Step	Selection
1	Select **04 HybSetup_SureTagHT.**
2	Select the type of plate for the thermal cycler.
3	Select the type of processing plate (Eppendorf twin.tec or Armadillo).
4	Select the pack size of the microarray (16 or 24).
5	Select the number of columns of sample pairs in the gDNA sample plate. A full plate (6 columns of test samples and 6 columns of reference samples) is 6 columns of sample pairs.

8 Click **Display Initial Bravo Deck Setup**.

The Bravo Deck Setup diagram displays the required Bravo deck configuration for the protocol.

9 Load the Bravo deck according to the Bravo Deck Setup diagram. The setup is also displayed in **Table 44**.

Table 44 Initial Bravo deck configuration for Hybridization Setup protocol

Bravo deck location	Content
2	New tip box
4	Processing plate containing purified, labeled gDNA samples
6	Empty thermal cycler plate seated in red aluminum insert (used for sample processing)
8	Empty tip box
9	Hybridization Master Mix source plate prepared on **page 54**

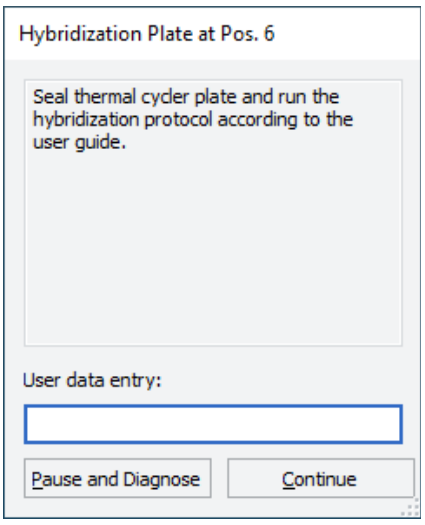
10 Update the **Current Tip State** indicator on the form to match the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively.

11 Click **Run Selected Protocol**.

The NGS Bravo executes the run, combining each test sample with its corresponding reference sample and adding the required volume of Hybridization Master Mix.

Running the protocol takes approximately 5 minutes.

12 When you see the prompt below, remove the thermal cycler plate from position 6 of the Bravo deck. Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing setting of 165°C and 1.0 seconds. Click **Continue** in the prompt.



13 Centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal and to eliminate air bubbles.

14 Immediately place the plate in the thermal cycler. Close the lid and run the thermal cycler program in **Table 38**.

15 While the thermal cycler program is running, confirm that the hybridization oven is set to 67°C.

CAUTION

The remainder of the workflow must be performed manually (i.e., without the use of NGS Bravo automation). As soon as the thermal cycler program reaches the 37°C hold step, begin **"Step 3. Prepare the hybridization assembly."** Process samples in batches of 16 (if using 16-pack arrays) or batches of 24 (if using 24-pack arrays). Keep the remaining samples in the 37° thermal cycler. Make sure that all hybridization reactions are processed within 1 hour of reaching the 37°C hold step.

Step 3. Prepare the hybridization assembly

Keep the hybridization samples in the 37°C thermal cycler until ready to load onto the microarray slide in **step 1** on **page 60**. Prepare the hybridization assembly for one microarray slide at a time. Then, if preparing multiple slides, repeat the hybridization assembly procedure for the remaining sets of hybridization samples.

Agilent recommends using the Agilent Hyb Station (p/n G5765A) to prepare the hybridization assembly. Use of the Hyb Station simplifies the preparation process, improves efficiency and throughput, and reduces the chance of sample leakage. Refer to the *Agilent Hyb Station Reference Card* (publication G5765-90000) for instructions.

Remove gasket slide from its packaging

NOTE

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

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

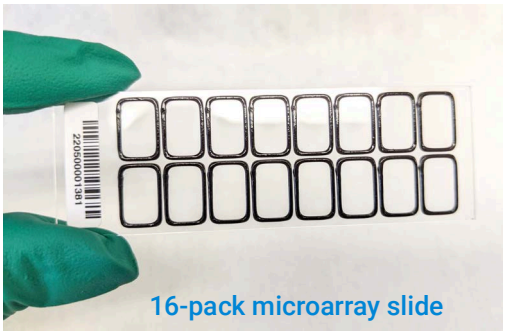
Figure 13 Removal of clear plastic covering

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

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

Insert the gasket slide into the chamber base

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



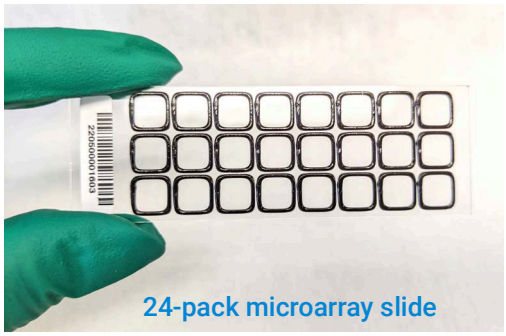


Figure 14 Gasket slide, gasket side

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

3 Position the gasket slide between the 4 chamber base guide posts (see **Figure 15**) with the barcode label resting over the base's rectangular barcode guide.

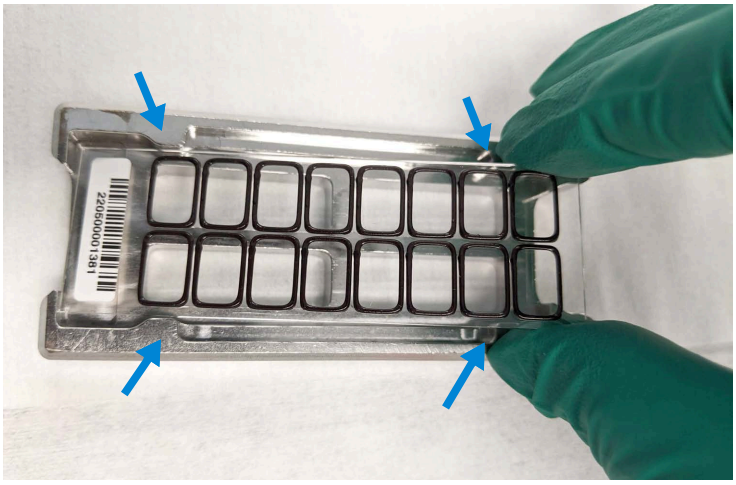


Figure 15 Chamber base, guide posts denoted with arrows

4 Gently place the gasket slide into the chamber base.

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

NOTE

If you are using the Agilent Hyb Station to set up the hybridization assembly, the chamber base is seated in the Hyb Station unit.

Slide and gasket are flush

Figure 16 Correct positioning of gasket slide in chamber base

Load the samples

NOTE

Agilent strongly recommends using a single channel pipette (e.g., Pipetman P10 or P20) to load the samples into the gasket wells. If you have significant experience processing Agilent HT microarrays, then you may consider using a 4-channel pipette to load the gasket wells horizontally or a 3-channel pipette to load the gasket wells vertically. Agilent does not recommend using an 8-channel pipette unless you are highly experienced with loading Agilent HT microarrays.

1 Slowly dispense the appropriate volume of hybridization sample mixture onto the gasket well so that the liquid forms a bead in the center of the well. Load all gasket wells before you add the microarray slide.

- 16-pack microarray: add 18 µL per gasket well
- 24-pack microarray: add 10 µL per gasket well

2 Visually inspect the gasket wells for proper loading of the hybridization sample mixtures.

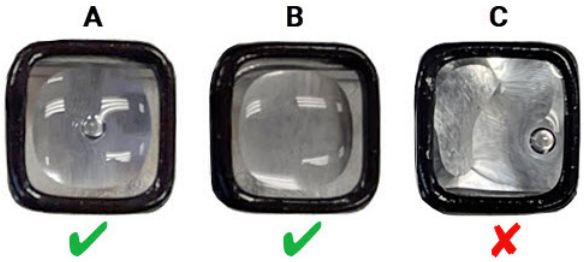


Figure 17 Gasket wells containing loaded sample mixtures. In panels A and B, the bead of liquid is intact. In panel C, the liquid is in contact with the gasket.

Each sample mixture should be sitting as a bead of liquid in the center of a gasket well (see panels A and B in **Figure 17**). The presence of a small air bubble is not a concern as long as the bead is intact (i.e., the sample mixture is not in contact with the gasket).

If the surface tension of the bead breaks, causing the sample mixture to come into contact with the edge of the gasket (e.g., panel C in **Figure 17**), use a clean, small-gauge pipette tip (e.g., a 10-µL tip) to spread the liquid evenly across the area of the gasket well. For detailed instructions, see the troubleshooting tip **"If a sample mixture comes into contact with the gasket during loading"** on page 80.

CAUTION Do not leave any of the gasket wells empty. If you do not have enough samples to load all wells, load the remaining wells with the equivalent volume of 1× HI-RPM Hybridization Buffer (diluted from 2× to 1× using nuclease-free water). Make sure that the gasket wells in the four corners of the slide contain hybridization sample mixture rather than 1× HI-RPM Hybridization Buffer.

Add the microarray slide

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

2 Use the four chamber base guideposts and rectangular end of the base to position the microarray slide as you lower it to within 3 mm (1/8") above the gasket slide, making sure the microarray slide is not tilted with respect to the gasket slide. Barcode ends of both the gasket slide and the microarray slide must line up at the corners of the chamber base. Once positioned, gently rest the microarray slide on the lower gasket slide. Refer to **Figure 18**.

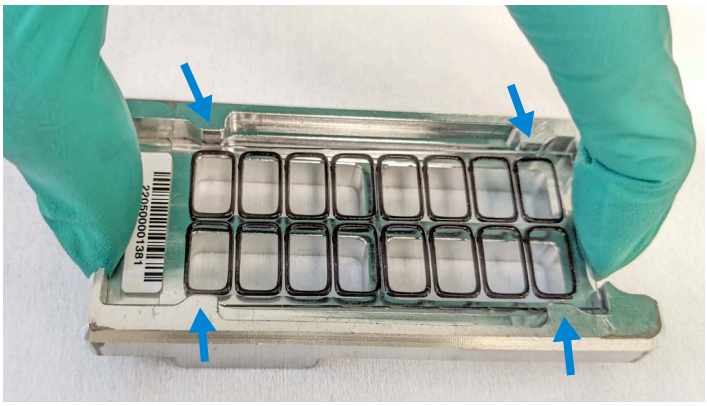


Figure 18 Chamber base with gasket and microarray slide applied, guide posts denoted with arrows

CAUTION

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

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

NOTE

If you are using the Agilent Hyb Station to set up the hybridization assembly, the microarray slide is added while the slide rests are engaged and then lowered onto the gasket slide by moving the lever to retract the slide rests. This avoids the need to manually drop the microarray slide directly onto the gasket slide, thereby minimizing the possibility of leakage.

Assemble the chamber

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

Figure 19 Chamber cover setting on chamber base in correct orientation

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

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

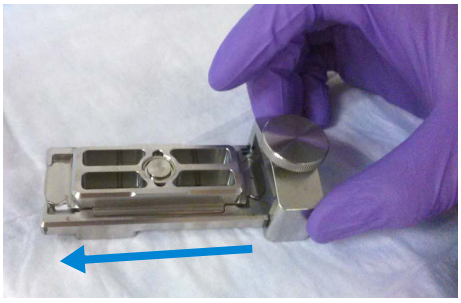


Figure 20 Slipping the clamp onto the chamber base

3 Firmly tighten the thumbscrew fully.

The slides will not be harmed by hand-tightening.

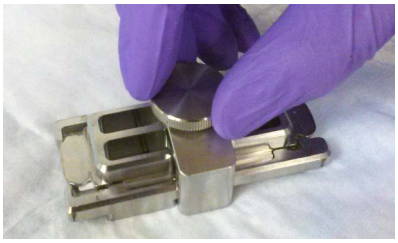


Figure 21 Tightening of the thumbscrew on the clamp

CAUTION

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

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

4 Rotate the final assembled chamber in a *vertical orientation*, clockwise, 2 to 3 times to wet the gaskets (see **Figure 22**).

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

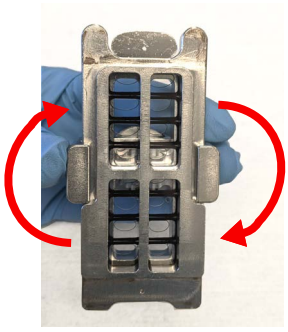


Figure 22 Rotation of the final assembled chamber

5 Inspect for good bubble formation.

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

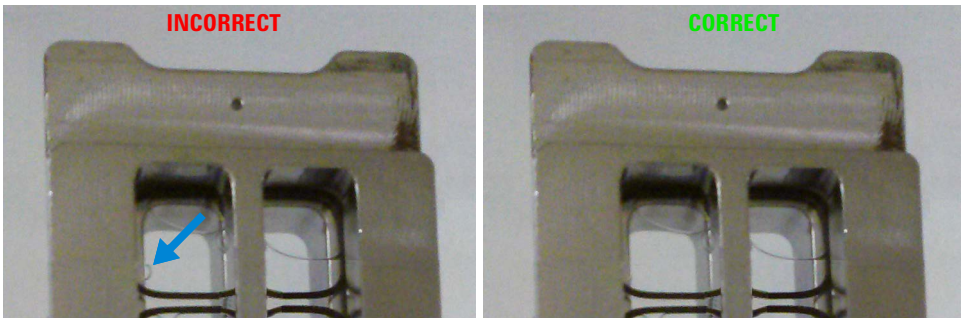


Figure 23 The slide on the left shows a stray, stationary bubble (denoted with arrow), which must be removed before hybridization. The slide on the right shows only large mixing bubbles, which move freely around the chamber when rotated. Bubbles are acceptable, as long as they move freely when you rotate the chamber.

6 Load the assembled chamber into the hybridization oven rotator rack, starting from the center of the rack (position 3 or 4 when counting from left to right). Refer to the figure below for correct and incorrect orientations. Close the oven door and set the rotator speed to 20 rpm.

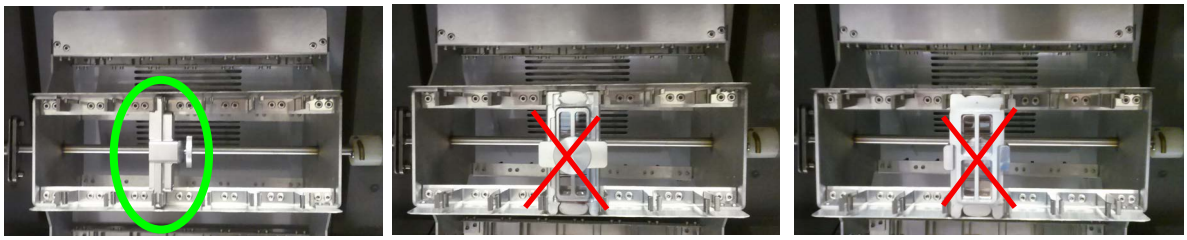


Figure 24 Assembled chambers in correct (left) and incorrect (middle and right) orientations

7 Repeat **"Step 3. Prepare the hybridization assembly"** (starting on **page 58**) for the remaining microarray slides.

Step 4. Hybridize

1 With all assembled chambers loaded into the rotator rack, hybridize at 67°C, with a rotator speed of 20 rpm, for 24 hours.

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

Step 5. Prewarm Oligo aCGH/ChIP-on-Chip Wash Buffer 2

Oligo aCGH/ChIP-on-Chip Wash Buffer 2 is included in the Oligo aCGH/ChIP-on-chip Wash Buffer Kit. See **"Agilent Oligo aCGH/ChIP-on-chip Wash Buffer Kit"** on page 84 for a list of kit components.

The temperature of Oligo aCGH/ChIP-on-Chip Wash Buffer 2 must be at 37°C ±1°C for optimal wash performance. You can either initiate the prewarming of the Oligo aCGH/ChIP-on-Chip Wash Buffer 2 on the same day that you load the assembled chambers into the hybridization oven, allowing the buffer to prewarm overnight, or you can initiate the prewarming approximately 2–3 hours before starting the wash procedure on **page 66**.

Agilent recommends washing no more than 5 microarray slides per group. Prewarm enough Oligo aCGH/ChIP-on-Chip Wash Buffer 2 for all groups (approximately 250 mL per group).

To prewarm the buffer overnight

1. Add the volume of buffer required to a new or well-cleaned storage bottle and place in an incubator or circulating water bath set to 37°C.
2. Allow the buffer to warm overnight.
3. Proceed to **"Wash the Microarray Slides"** on page 66.

To prewarm the buffer the day of the wash procedure

1. Add the volume of buffer required to a new or well-cleaned storage bottle and place the bottle in a water bath sitting on a hot plate.
2. Monitor the temperature of the water bath until the temperature is stabilized to 37°C.
3. Allow the buffer to warm for approximately 2 to 3 hours.
4. Proceed to **"Wash the Microarray Slides"** on page 66.

Wash the Microarray Slides

The wash procedure uses high-quality, ultra-pure, deionized water, such as Milli-Q ultrapure water or similar.

Always wear clean laboratory gloves throughout the slide washing procedure. Change gloves as needed to make sure they are clean.

NOTE

The microarray wash procedure must be done in environments where ozone levels are 5 ppb or less. You can 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.

Step 1. Wash equipment with ultra-pure water

Rinse slide-staining dishes, slide racks and stir bars thoroughly with ultra-pure water before use and in between washing groups.

1. Run copious amounts of ultra-pure water through the slide-staining dishes, slide racks and stir bars.
2. Empty out the water collected in the dishes at least five times.
3. Repeat **step 1** and **step 2** until all traces of contaminating material are removed.

NOTE

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

Step 2. Wash microarrays

This procedure uses reagents included in the Oligo aCGH/ChIP-on-chip Wash Buffer Kit. See **"Agilent Oligo aCGH/ChIP-on-chip Wash Buffer Kit"** on page 84 for a list of kit components.

Prior to starting the washing procedure, turn on the SureScan Microarray Scanner and the associated computer equipment and launch the Agilent Microarray Scan Control software.

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

Table 45 summarizes the wash conditions for the wash procedure. Detailed instructions follow.

Table 45 Wash conditions

Step	Dish	Wash buffer	Temperature	Incubation
Disassembly	#1	**Oligo aCGH/ChIP-on-Chip Wash Buffer 1**	Room temperature	—
1st wash	#2	**Oligo aCGH/ChIP-on-Chip Wash Buffer 1**	Room temperature	5 minutes with stirring
2nd wash	#3	**Oligo aCGH/ChIP-on-Chip Wash Buffer 2** (prewarmed to 37°C overnight)	37°C	5 minutes with stirring

1. Completely fill slide-staining dish #1 with **Oligo aCGH/ChIP-on-Chip Wash Buffer 1** at room temperature.
2. Prepare dish #2:
   a. Put a slide rack into slide-staining dish #2.
   b. Add a magnetic stir bar. Fill slide-staining dish #2 with enough **Oligo aCGH/ChIP-on-Chip Wash Buffer 1** at room temperature to cover the slide rack.
   c. Put this dish on a magnetic stir plate.
3. Prepare dish #3:
   a. Put a 1.5 L glass dish on a magnetic stir plate with heating element.
   b. Put the slide-staining dish #3 into the 1.5 L glass dish.
   c. Fill the 1.5 L glass dish with ultra-pure water.
   d. Fill the slide-staining dish #3 approximately three-fourths full with **Oligo aCGH/ChIP-on-Chip Wash Buffer 2** (warmed to 37°C).
   e. Add a magnetic stir bar.
   f. Turn on the heating element and maintain temperature of **Oligo aCGH/ChIP-on-Chip Wash Buffer 2** at 37°C. Monitor with a thermometer.
4. Remove one hybridization chamber from the incubator and resume rotation of the others. Record whether bubbles formed during hybridization and if all bubbles are rotating freely. Visually check for volume loss.
5. Prepare the hybridization chamber disassembly.
   a. Put the hybridization chamber assembly on a flat surface and loosen the thumbscrew, turning counter-clockwise.

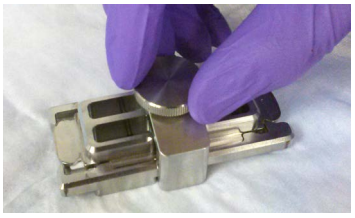


Figure 25 Loosening of the thumbscrew

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

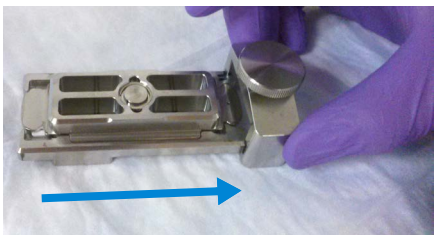


Figure 26 Removal of the clamp

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

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

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

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

b Gently twist the forceps to separate the slides.

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

d While grasping the microarray slide with thumb and forefinger (as shown in **Figure 27**) remove the microarray slide from the dish #1 and quickly put into the slide rack in the slide-staining dish #2 containing **Oligo aCGH/ChIP-on-Chip Wash Buffer 1** at room temperature. Minimize exposure of the slide to air. *Touch only the barcode portion of the microarray slide or its edges!*

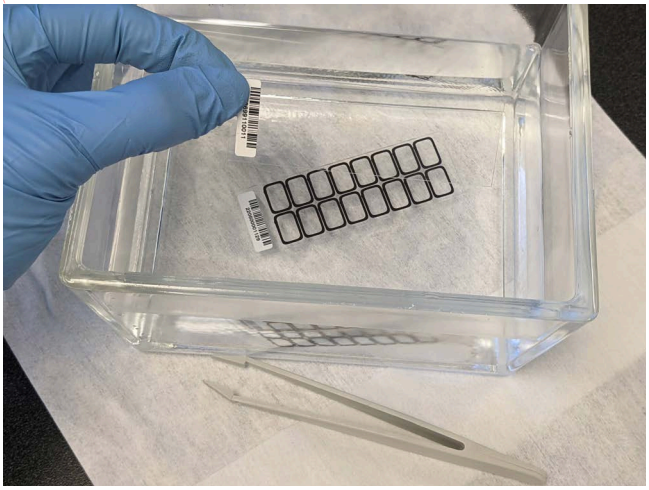


Figure 27 Removal of the microarray slide from dish #1

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

8 When all slides in the group are put into the slide rack in slide-staining dish #2, stir at approximately 550 rpm for 5 minutes.

Adjust the setting to get good but not vigorous mixing. The buffer is being well mixed when it is just forming a dimple at the surface.

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

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

   a Activate the magnetic stirrer.

   b Wash microarray slides for 5 minutes.

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

10 Slowly remove the slide rack trying to minimize droplets on the slides. It should take 5 to 10 seconds to remove the slide rack. Place the slide rack on a clean stack of lint-free, disposable laboratory wipes.

11 Remove the slides from the slide rack one at a time, tapping the edge of each slide on the stack of laboratory wipes to remove any remaining droplets, and then putting it in a slide holder.

   See **"Step 3. Put slides in a slide holder"** on page 69 for detailed instructions on putting slides into slide holders.

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

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

Step 3. Put slides in a slide holder

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

An Agilent SureScan Microarray Scanner is required for HT microarrays.

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

1 Before you insert the slide into a slide holder, inspect the slide holder for any dust or fingerprints. If found, remove the dust or fingerprints with compressed air or a soft, dust-free cloth. If the slide holder is scratched, worn, or damaged, has a lid that does not close tightly, or has a hinge that does not move freely, discard the slide holder and select a different one.

2 Place the slide holder on a flat surface, with the clear cover facing up, and the tab on the right. This helps to ensure that you have the slide aligned properly when you insert it into the slide holder.

3 Gently push in and pull up on the tabbed end of the clear plastic cover to open the slide holder.

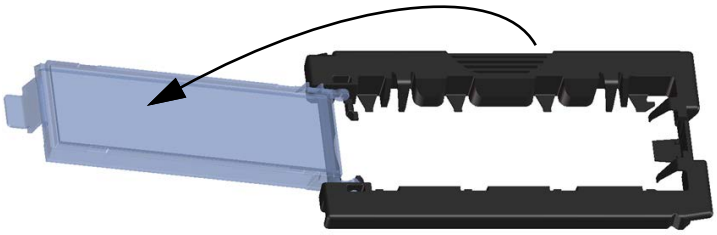


Figure 28 Opening the slide holder

4 Insert the slide into the holder.

a Hold the slide at the barcode end and position the slide over the open slide holder. Make sure that the active microarray surface faces up with the barcode on the left, as shown in **Figure 29**.

b Carefully place the end of the slide without the barcode label onto the slide ledge. See **Figure 29**.

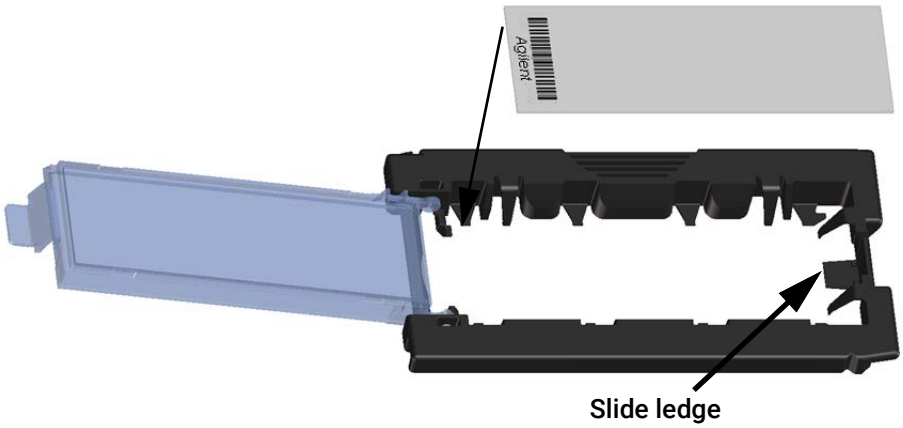


Figure 29 Inserting slide into the slide holder

c Gently lower the slide into the slide holder. See **Figure 30**.

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

CAUTION An improperly inserted slide can damage the scanner.

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

f Close the plastic slide cover, gently pushing on the tab end until you hear it “click”. See **Figure 31**. Make sure that the slide holder is completely closed.

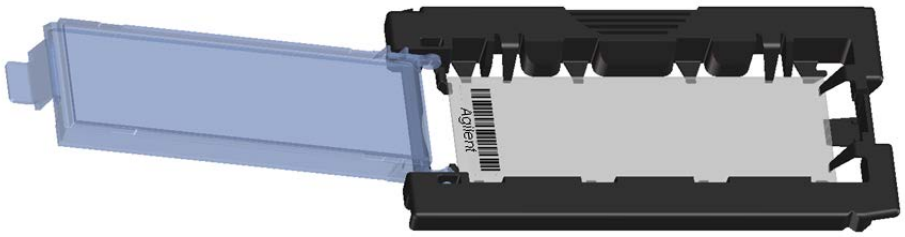


Figure 30 Slide inserted in slide holder – cover open

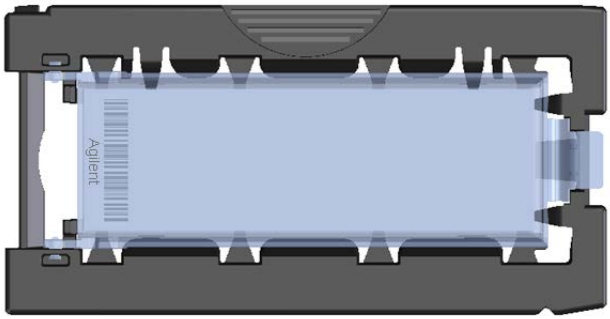


Figure 31 Slide inserted in slide holder – cover closed

Microarray Scanning and Analysis

Step 1. Scan the microarray slides

1 Put assembled slide holders into the scanner cassette. Refer to **Figure 32** and **Figure 33**.

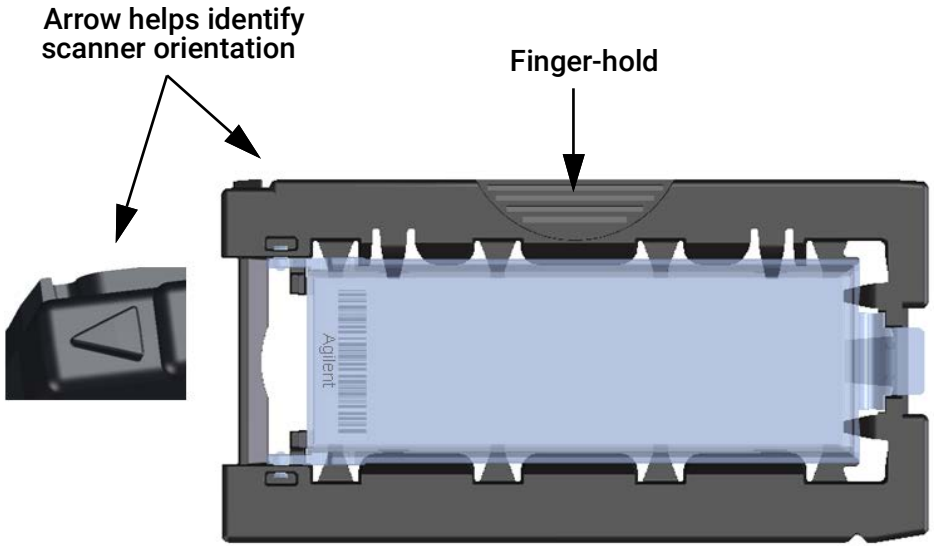


Figure 32 Slide holder helps you to insert slides correctly

Figure 33 Inserting slide holder into cassette

2 Select **Protocol AgilentG3_CGH**.

Table 46 lists the scan settings for this protocol

Table 46 Scan Settings for protocol AgilentG3_CGH

Dye Channels	Red+Green
Scan Region	FullAgilentSlide
Resolution	3 µm
Tiff Dynamic Range	16 bit
Red PMT Sensitivity	100%
Green PMT Sensitivity	100%
XDR Ratio	<No XDR>

3 Verify that the Scanner status in the main window says **Scanner Ready**.

4 Click **Start Scan**.

Step 2. Set up on Analysis Workflow in CytoGenomics

Feature extraction is the process by which data is extracted from the scanned microarray TIFF image (.tif) and translated into log ratios, allowing researchers to identify aberrations in their samples. The Agilent CytoGenomics software (version 5.3 and higher) for Microsoft Windows supports feature extraction of the 16-pack and 24-pack HT microarrays for analysis of human gDNA samples. During the extraction and analysis process, Agilent CytoGenomics generates feature extraction files, QC results, and aberration reports.

Set up an Analysis Workflow in Agilent CytoGenomics to analyze your data.

NOTE

If you are processing CGH+SNP HT microarrays, make sure that the appropriate reference sample is selected during the analysis workflow setup.

Microarray QC Metrics

NOTE

If the automatic gridding of the TIFF image fails during the Analysis Workflow, then the CytoGenomics QC report includes an "Evaluate Grid" warning and/or the *IsGoodGrid* QC metric is rated as "Evaluate." See **"If CytoGenomics reports a gridding error"** on page 82 of the **Troubleshooting** chapter for guidance.

The metric thresholds in **Table 47** are only appropriate for high-quality DNA samples analyzed with Agilent HT microarrays by following the standard operational procedures provided in this user guide. These metrics are reported in the Feature Extraction QC report generated by the Agilent CytoGenomics software. They can be used to assess the relative data quality from a set of microarrays in an experiment. In some cases, they can indicate potential processing errors that have occurred or suggest that the data from particular microarrays might be compromised. Many factors can influence the range of these metrics, including the microarray format (16-pack or 24-pack), biological sample source, quality of starting gDNA, experimental processing, 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 CGH+SNP HT microarrays, make sure the DerivativeLR_Spread (DLRSD) is <0.2.

Table 47 QC metric thresholds for Enzymatic labeling

Metric name	Excellent	Good	Evaluate
g_BGNoise and r_BGNoise	< 15	15 to 25	> 25
g_Signal Intensity	> 400	200 to 400	< 200
r_Signal Intensity	> 350	150 to 350	< 150
g_Signal2Noise and r_Signal2Noise	> 60	25 to 60	< 25
gRepro and rRepro	0 to 0.1	0.1 to 0.2	<0 or > 0.2
DerivativeLR_Spread	< 0.2	0.2 to 0.3	> 0.3

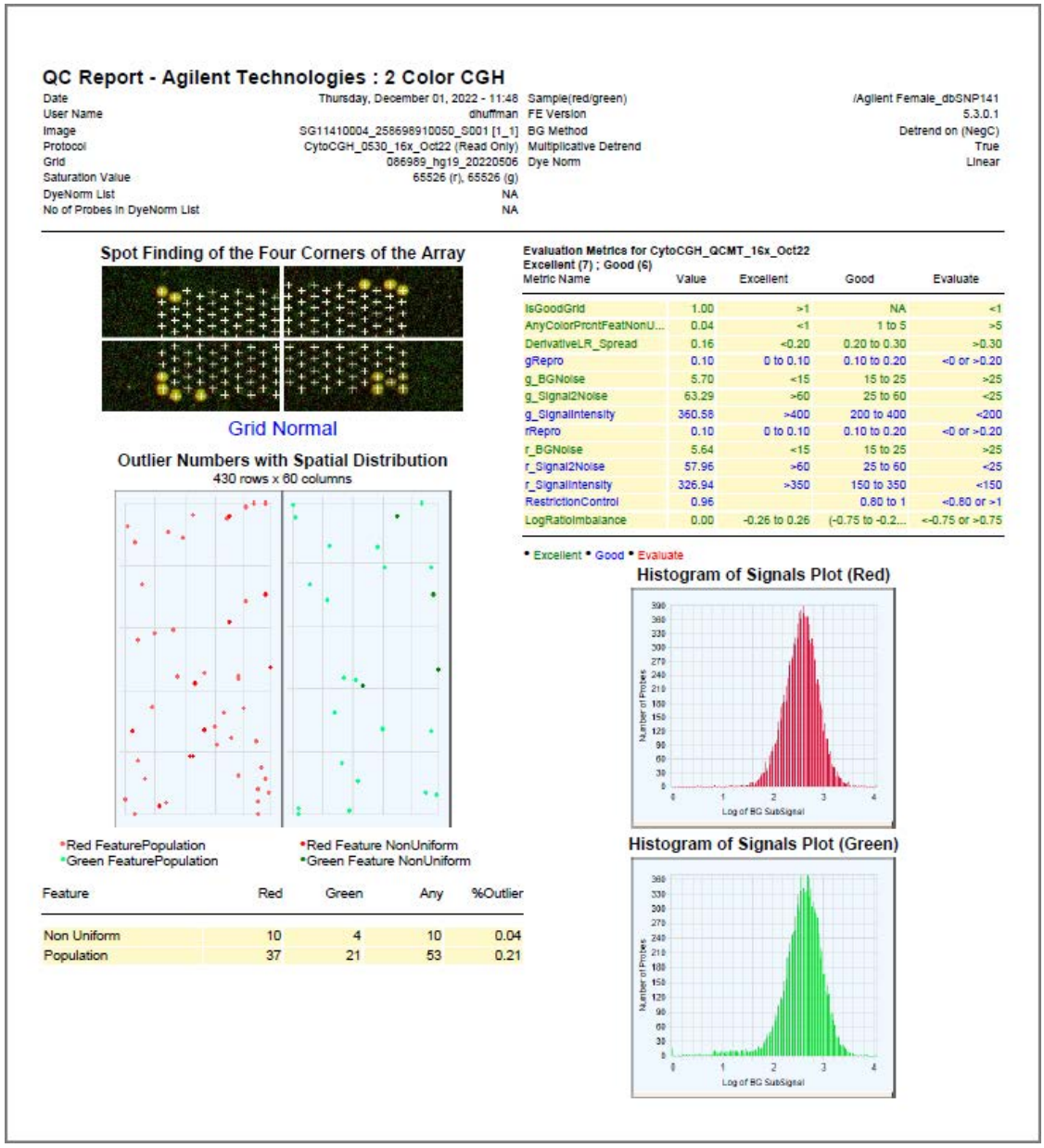


Figure 34 Feature Extraction QC Report, page 1

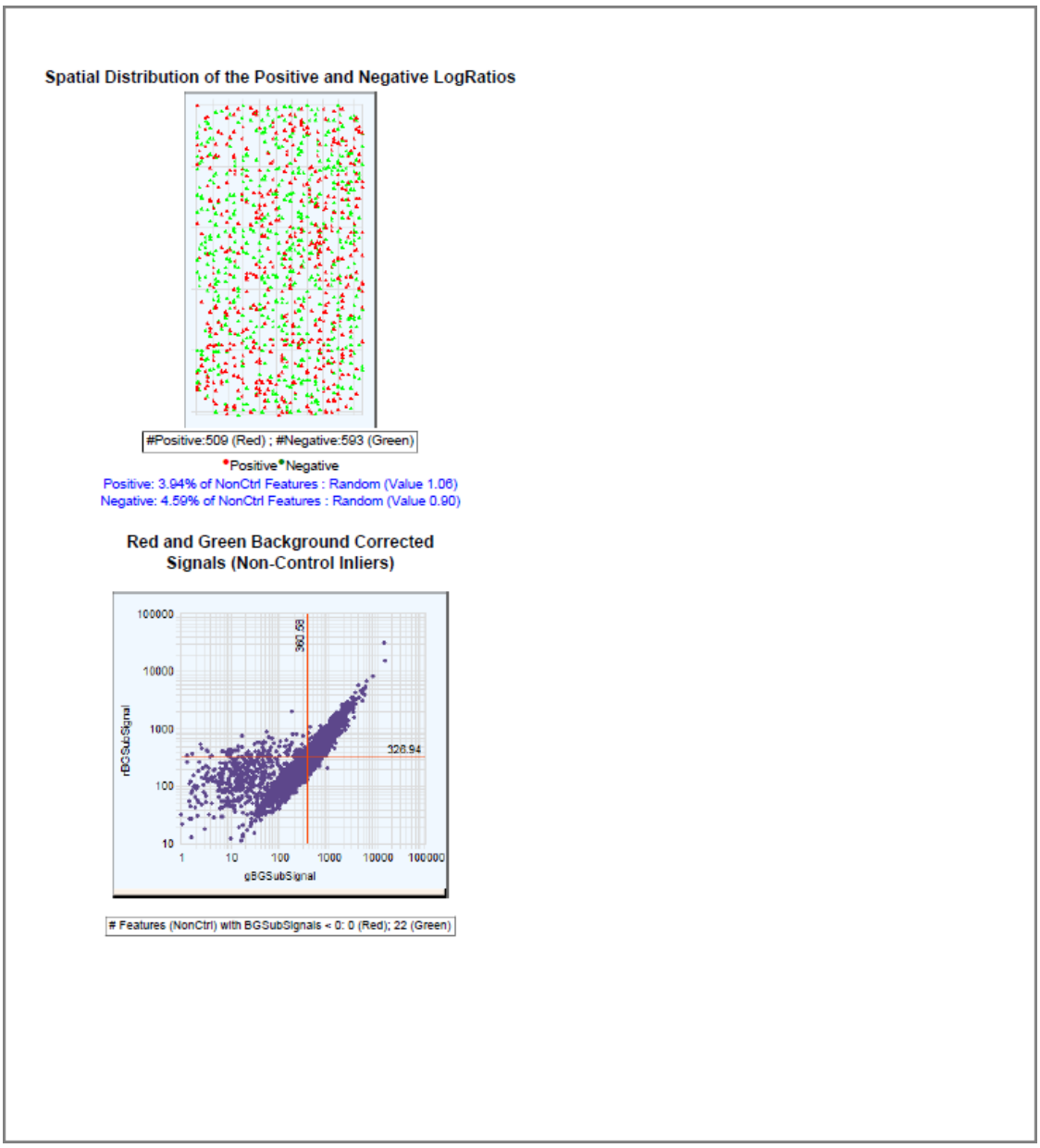


Figure 35 Feature Extraction QC Report, page 2

7 Troubleshooting

This chapter contains tips for troubleshooting potential issues with the HT aCGH protocol.

If the gDNA sample has a low A_{260}/A_{230} or A_{260}/A_{280} value

A low $\mathbf{A_{260}/A_{230}}$ value can indicate contaminants in the gDNA, such as residual salt or organic solvents. A low A_{260}/A_{280} value indicates residual protein. Either condition can result in low specific activity (pmol dye/µg DNA). See **"Step 3. Determine Yield and Specific Activity"** on page 50.

- Repurify the DNA. Agilent recommends using the QIAGEN DNeasy Blood & Tissue Kit, which 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 A_{260} value.
- Make sure to calibrate the spectrophotometer with the appropriate buffer.

If the gDNA sample has poor quality due to residual RNA

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

- Repurify the DNA. Agilent recommends using the QIAGEN DNeasy Blood & Tissue Kit. Include treatment with RNase A in the purification procedure.

If the gDNA sample has poor quality due to degradation

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

- Check DNA on a 1 to 1.5% agarose gel. If DNA that was isolated from cells, blood or frozen tissue is degraded, then repurify the DNA using the QIAGEN DNeasy Blood & Tissue Kit.
- If you use the heat fragmentation method to fragment the gDNA, make sure that the DNA is not over-fragmented prior to labeling. Possible causes are incorrect temperature or length of incubation at 98°C, or evaporation (use a thermal cycler with heated lid). Make sure most of the heat fragmented products are between 1000 and 3000 bases in length.
- Use only DNA isolated from cells, blood, or frozen tissue. FFPE samples are not supported for use on HT microarrays.

If the estimated concentration of gDNA is too high or low

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

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

- Make sure that the gDNA is completely in solution by pipetting up and down. If needed, incubate at 37°C for 30 minutes. If the gDNA concentration is > 350 ng/µL, dilute 1:2 in an appropriate buffer (e.g., 1×TE buffer, pH 8.0). Quantitate again to make sure quantitation is accurate.
- Different DNA isolation methods can create different quantitation artifacts, the risk of assay noise is higher when the test and reference DNA samples are isolated from different sources. If you used a spectrophotometer (such as the NanoDrop) for the initial measurement, also use a double-stranded DNA-based fluorometer (such as the Qubit) to verify.
- If needed, repurify the DNA using the QIAGEN DNeasy Blood & Tissue Kit.

If the labeled DNA has low specific activity not due to poor sample quality

Low specific activity 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 3-dUTP and Cyanine 5-dUTP at –20°C. Keep enzymes on ice and return to –20°C as quickly as possible.
- Double check incubation times and temperatures (use a calibrated thermometer), and use a thermal cycler with heated lid.
- Evaporation can be a problem when you process samples at high temperatures. Make sure that sample tubes are well closed to avoid evaporation.
- Make sure that the pipettes 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.
- If you are using a multichannel pipette to dispense master mixes, be aware that some of the master mixes are viscous. Check if the pipette tips contain any residual liquid after dispensing and, if needed, dispense the master mixes more slowly. Reaction volumes are very low; failure to fully dispense the master mixes into the reactions could impact performance.

If the labeled DNA has low yield not due to poor sample quality

Possible sample loss during clean-up after labeling.

- Use the procedures in **"Step 2. Purify Labeled gDNA"** on page 46 to remove unreacted dye. Other purification techniques result in the loss of shorter fragments.
- Make sure to store the SurePure Beads at 4°C (do not freeze). You do not need to allow the beads to equilibrate to room temperature before adding them to the SurePure Beads Master Mix. If the SurePure Beads did freeze, then those beads are unusable. Obtain a new stock of SurePure Beads before proceeding.

If a sample mixture comes into contact with the gasket during loading

When dispensing the hybridization sample mixtures into the gasket wells of the gasket slide, the optimal outcome is for the mixture to form a bead of liquid that sits in the center of the well. The presence of a small air bubble is not a concern as long as the surface tension of the bead is intact and the sample mixture is not in contact with the gasket.

- If the surface tension of the liquid bead breaks, causing the sample mixture to come into contact with the edge of the gasket (**Figure 36**, panel **A**), use the sample loading rescue procedure described below.
  - First, pop any air bubbles that may be present in the sample mixture (**Figure 36**, panel **B**). Use a clean, small-gauge pipette tip (e.g., a 10-µL tip) to gently pop the bubble. Keep the pipette tip nearly parallel with the gasket slide and gently tap the bubble with the edge of the pipette tip opening. Use a fresh pipette tip for each sample. Never attempt to pop a bubble using a wet or previously used pipette tip as the capillary action will aspirate the sample mixture.
  - Then, use a NEW pipette tip to spread the sample mixture evenly across the area of the gasket well (**Figure 36**, panel **C**). Make sure that the entire surface inside the gasket is covered with sample mixture (**Figure 36**, panel **D**).

Figure 36 Sample loading rescue procedure

If you observe post-labeling signal loss

Signal loss can be due to hybridization or wash conditions that are too stringent.

- Check that the oven temperature is 67°C. If needed, recalibrate the hybridization oven. Follow the steps in *Agilent G2545A Hybridization Calibration Procedure* (publication number G2545-90002).
- Check that the temperature of Wash 2 is 37°C.
- Check that Wash 2 was not accidentally used instead of Wash 1.

Because cyanine 5 is more prone to degradation by ozone than cyanine 3, if you see signal loss predominantly in the red (cyanine 5) channel, then the loss may be due to exposure of the slide to atmospheric ozone or oxidants from pollution and/or lab equipment (e.g., compressors and centrifuges). A common indication of ozone degradation is a gradient of red signal loss across the slide that is most prominent on the edges of the slide (where exposure to air is greatest) than in the center of the slide.

- 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.

If the feature extraction QC metrics indicate a high BGNoise value

High BGNoise can cause lower signal-to-noise values (see **Table 23** for thresholds) and higher DLRSD values. BGNoise is defined as the standard deviation of the signals on the negative controls. If the BGNoise is high, examine the microarray image for visible non-uniformities. High BGNoise is often introduced during hybridization or washes.

- Make sure that the oven is calibrated. Follow the steps in *Agilent G2545A Hybridization Calibration Procedure* (publication number G2545-90002).
- Sample hybridization at incorrect temperatures affects the stringency of the hybridization.
- Make sure that wash dishes, racks and stir bars are clean. Do not use tap water or detergents to clean wash equipment. If needed, rinse wash equipment with acetonitrile followed by rinses with Milli-Q ultrapure water.
- If you observe patterns of bright fluorescence or swirls across the image of the scanned slide, add an additional wash step for the slides using acetonitrile following the procedure below.
  1. In the fume hood, fill a slide-staining dish approximately three-fourths full with acetonitrile.
  2. Put the slides in a slide rack and transfer the slide rack to the slide-staining dish containing acetonitrile. Leave the rack in the dish for 20 seconds.
  3. Slowly remove the slide rack and scan the slides immediately.

If the feature extraction QC metrics for a 24-pack array indicate high BGNoise values and low Signal2Noise values

On the 24-pack arrays, a combination of high background noise (both r_BGNoise and g_BGNoise) and low signal-to-noise ratios (both r_Signal2Noise and g_Signal2Noise) may be an indication that the hybridization chamber is too loose.

- When setting up the hybridization chamber, firmly tighten the thumbscrew as fully as you can by hand tightening.
- Never use tools to tighten the thumbscrew.

If the feature extraction QC metrics indicate poor reproducibility

Poor reproducibility (see **Table 47** 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 in the center of the gasket wells to prevent spills.
- Use the Agilent Hyb Station to prepare the hybridization assembly. Use of the Hyb Station reduces the chance of sample leakage, which can negatively impact reproducibility. Refer to the Agilent Hyb Station Reference Card (publication G5765-90000) for instructions.
- Check that the oven is rotating.

If CytoGenomics reports a gridding error

During the feature extraction process of the CytoGenomics Analysis Workflow, the software performs automatic gridding of the TIFF image. If automatic gridding is unsuccessful, then the CytoGenomics QC report includes an “Evaluate Grid” warning and/or the IsGoodGrid QC metric is rated as “Evaluate.”

- Make sure that the four arrays positioned in the corners of the microarray slide contain a hybridization sample. The Feature Extraction for CytoGenomics software uses the corners of the slide for placing and optimizing the grid fit during TIFF image extraction.
- Perform manual gridding of the array. See the *Feature Extraction for CytoGenomics User Guide* for instructions. The guide available on the Agilent website or from within the Feature Extraction for CytoGenomics software module (go to **Help > User Guide**). For more information on the array extraction algorithms, see the *Feature Extraction for CytoGenomics Reference Guide*.

8 Reference

This chapter contains reference information and other helpful tips that pertain to this protocol.

Reagent Kit Components

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

Qubit dsDNA BR Assay Kit p/n Q32850

Component	Recommended Storage Temperature
Qubit dsDNA BR Reagent	2°C to 8°C
Qubit dsDNA BR Buffer	≤ 30°C
Qubit dsDNA BR Standard #1	2°C to 8°C
Qubit dsDNA BR Standard #2	2°C to 8°C

Agilent SureTag HT Complete Kit and SureTag HT Kit p/n G9978A or G9978B

Component	Recommended Storage Temperature
Human Reference DNA (Male and Female)*	4°C
10× Restriction Enzyme Buffer	–20°C
BSA	–20°C
Alu I	–20°C
Rsa I	–20°C
Nuclease Free Water	–20°C
Exo (-) Klenow	–20°C
5× Reaction Buffer	–20°C
Cyanine 5-dUTP	–20°C
Cyanine 3-dUTP	–20°C
10× dNTPs	–20°C
Random Primers	–20°C
SureTag HT Buffer	Room temperature
SurePure Beads	4°C

* Included in the SureTag HT Complete Kit only.

Agilent Oligo aCGH/ChIP-on-chip Hybridization Kit p/n 5188-5220 or 5188-5380

Component	Recommended Storage Temperature
2× HI-RPM Hybridization Buffer	Room temperature
aCGH Blocking Agent	Lyophilized: Room temperature Reconstituted: –20°C

Agilent Oligo aCGH/ChIP-on-chip Wash Buffer Kit p/n 5188-5226

Component	Recommended Storage Temperature
Oligo aCGH/ChIP-on-Chip Wash Buffer 1	Room temperature
Oligo aCGH/ChIP-on-Chip Wash Buffer 2	Room temperature

“Secure Fit” Slide Box Opening Instructions

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

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

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

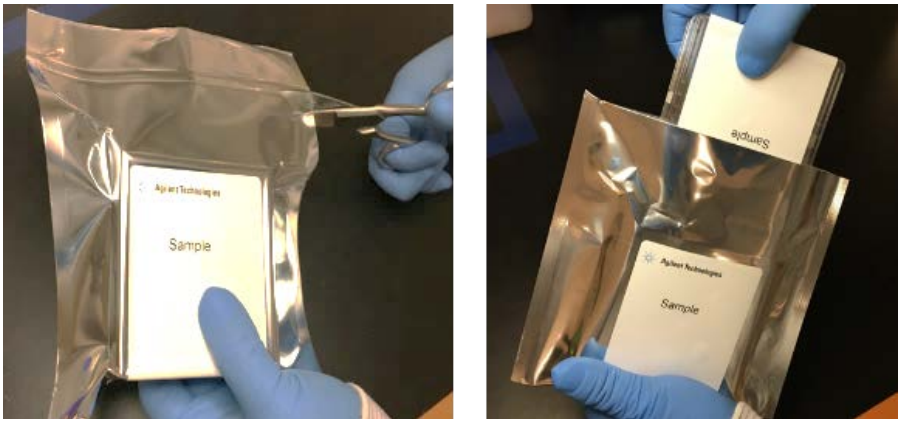


Figure 37 Opening foil pouch (left) and removing the slide box (right)

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

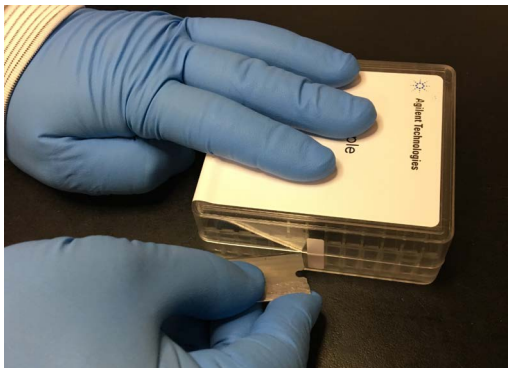


Figure 38 Cutting the sealing tape

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

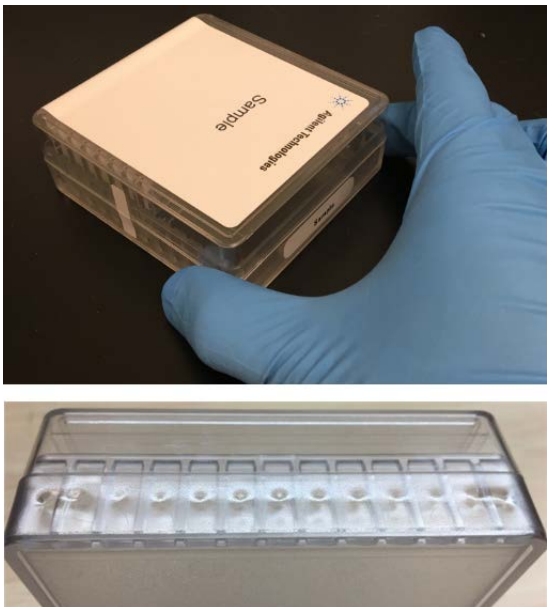


Figure 39 Gripping the base at the indentations (top) and close-up of the indentations (bottom)

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

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

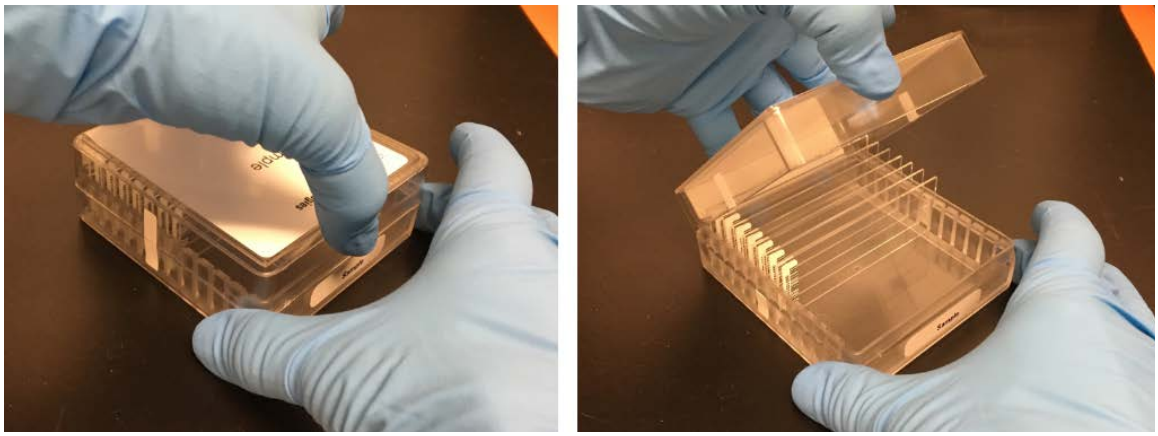


Figure 40 Grasping the lid (left) and lifting the lid from the base (right)

Microarray Handling Tips

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

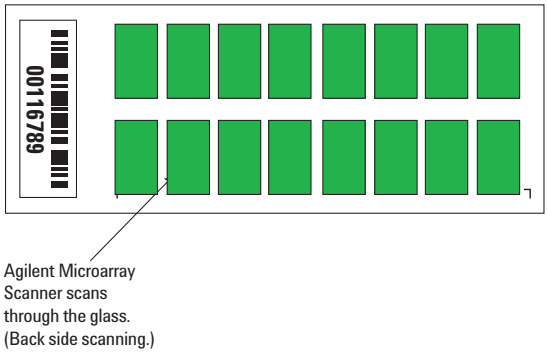
CAUTION You must familiarize yourself with the assembly and disassembly instructions for use with the Agilent Microarray Hybridization Chamber (G2534A) and gasket slides. Practice slide kits are available.

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

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

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

Agilent Microarray Layout and Orientation



Microarrays are printed on the side of the glass with the “Agilent”-labeled barcode (also referred to as the “active side” or “front side”).

The side of the glass with the numeric barcode (as shown on the left) is the “inactive side” or “back side.” The scanner scans through this side of the glass.

Figure 41 Layout of an Agilent HT microarray slide (16-pack format)

Agilent oligo microarrays formats and the resulting “microarray design files” are based on how the Agilent microarray scanner images 1-inch x 3-inch glass slides. Agilent designed its microarray scanner to scan through the glass slide (back side scanning). The glass slide is securely placed in an Agilent microarray slide holder with the “Agilent” labeled barcode facing the opening of the slide holder. In this orientation, the “active side” containing the microarrays is protected from potential damage by fingerprints and other elements. Once securely placed, the numeric barcode, non-active side of the slide, is visible from the outside of the slide holder.

Array/Sample tracking on microarray slides

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

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

Arrays

BARCODE	Array 1_1	Array 1_2	Array 1_3	Array 1_4	Array 1_5	Array 1_6	Array 1_7	Array 1_8
	Sample:	Sample:	Sample:	Sample:	Sample:	Sample:	Sample:	Sample:
	Sample:	Sample:	Sample:	Sample:	Sample:	Sample:	Sample:	Sample:
	Array 2_1	Array 2_2	Array 2_3	Array 2_4	Array 2_5	Array 2_6	Array 2_7	Array 2_8

Barcode Number _____

Figure 42 16-pack microarray slide

Arrays

BARCODE								
	Sample: Array 1_1	Sample: Array 1_2	Sample: Array 1_3	Sample: Array 1_4	Sample: Array 1_5	Sample: Array 1_6	Sample: Array 1_7	Sample: Array 1_8
	Sample: Array 2_1	Sample: Array 2_2	Sample: Array 2_3	Sample: Array 2_4	Sample: Array 2_5	Sample: Array 2_6	Sample: Array 2_7	Sample: Array 2_8
	Sample: Array 3_1	Sample: Array 3_2	Sample: Array 3_3	Sample: Array 3_4	Sample: Array 3_5	Sample: Array 3_6	Sample: Array 3_7	Sample: Array 3_8

Barcode Number _____

Figure 43 24-pack microarray slide

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In This Book

This guide contains information to run the protocol for High-Throughput aCGH Analysis using NGS Bravo Automation and the Agilent SureTag HT Reagents.

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