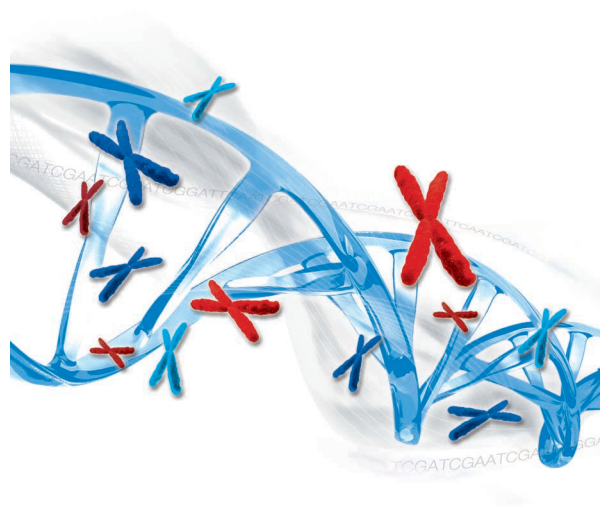


High-Throughput aCGH Analysis using Agilent HT Microarrays - Enzymatic Labeling of gDNA with the SureTag HT Kit For Blood, Cells, or Tissues

Protocol

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

Revision A0, November 2023



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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 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.

3 Sample Fragmentation

This chapter describes the Agilent recommended protocols for fragmenting gDNA for the labeling reaction.

4 Sample Labeling

This chapter describes the steps to fluorescently label the gDNA samples with cyanine 3 and cyanine 5.

5 Microarray Processing

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

6 Troubleshooting

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

7 Reference

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

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1 Before You Begin

Workflow Overview and Considerations **8**
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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.

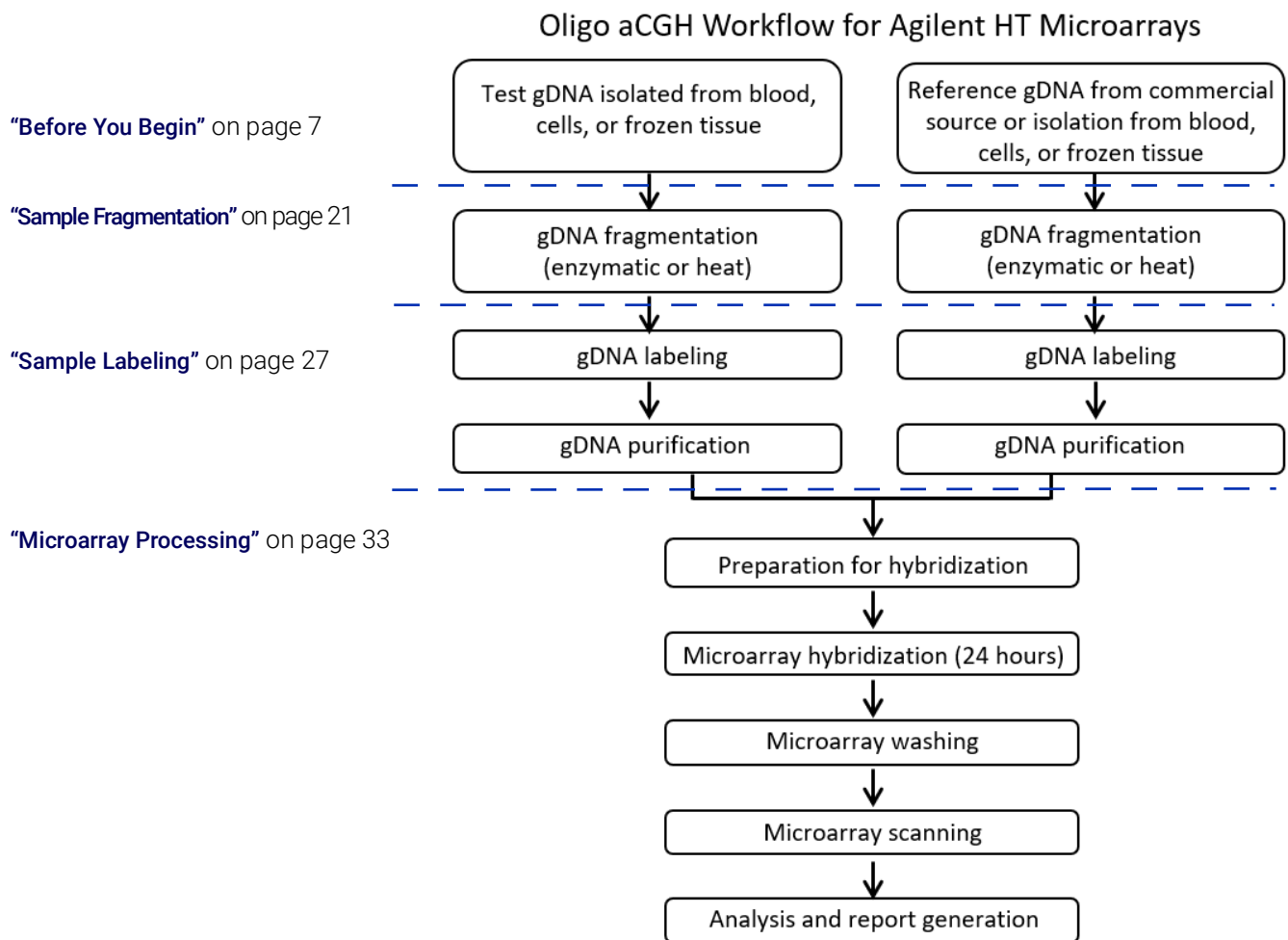


Figure 1 Workflow for sample preparation and HT microarray processing

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	40x	10x
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.

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.
- When preparing 96-well plates or 8x strip tubes for the thermal cycler, use domed or flat cap strips (8x strips) to cap the strip tubes or the wells of the plate. Use a compression mat if compatible with your thermal cycler.
- If using 96-well plates, use only semi-skirted plates (e.g., Agilent p/n 401334 or equivalent). Do not use unskirted plates.
- 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.

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.

Materials Required

gDNA Samples

The recommended input for the workflow is either 125 ng (for 16-pack format) or 162.5 ng (for 24-pack format) of high-quality gDNA from the test sample and reference.

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

Table 2 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 3 lists the Agilent equipment, reagent kits, and software required for the aCGH workflow for Agilent HT microarrays.

Table 3 Required Agilent Equipment, Reagents, and Software

Description	Part number
Hybridization gasket slides	<p>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)</p> <p>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)</p>
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 <i>Agilent G2545A Hybridization Calibration Procedure</i> (publication G2545-90002).	Agilent p/n G2545A
Hybridization oven rotator for Agilent Microarray Hybridization Chambers	Agilent p/n G2530-60029
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 (25) or p/n 5188-5380 (100)
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 64.

† Agilent Feature Extraction software, version 12.2 or higher, can also support extraction of HT microarrays. See **Table 23** on page 52 for the appropriate QC metrics.

Other Required Materials

Table 4 lists the remaining required materials (reagents, equipment, and plasticware) needed to complete the aCGH workflow.

Table 4 Other required reagents, equipment, and plasticware

Description	Vendor and part number
Thermal cycler with heated lid	Various suppliers
DNase-free tube strips, 200- μ L (strips of 8) and DNase-free tube cap strips, domed or flat (strips of 8) or DNase-free 96-well PCR plates, 200- μ L, semi-skirted or DNase-free PCR tubes, 200- μ L	Agilent p/n 401428 or equivalent Agilent p/n 401425 or Agilent p/n 410096 or equivalent Agilent p/n 401334 or equivalent semi-skirted plates Agilent p/n 410091 or equivalent
Nuclease-free distilled water	Thermo Fisher Scientific p/n 10977-015 or equivalent
Ethanol, 100% (molecular biology grade)	Sigma-Aldrich p/n E7023-6 \times 500ML or equivalent
1 \times TE buffer (pH 8.0), molecular grade	Promega p/n V6231 or equivalent
Microcentrifuge	Eppendorf p/n 5430 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
Magnetic separator device, low-elution	Permagen 96-well PCR Post Magnet Low Elution Plate, p/n LE400, or equivalent low-elution magnet
1.5 L glass dish	Pyrex p/n 213-R or equivalent
Magnetic stir plate with heating element ($\times 2$)	Corning p/n 6795-420D or equivalent
Magnetic stir bar, 7.9 \times 38.1 mm ($\times 2$)	VWR p/n 58948-150 or equivalent
250 mL capacity slide-staining dishes with lids ($\times 3$) 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
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

Table 4 Other required reagents, equipment, and plasticware (continued)

Description	Vendor and part number
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 5 lists the materials used only in the recommended assays for gDNA quantitation and quality analysis described in **Chapter 2**. These assays 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 5** may not be needed.

Table 5 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 Alphamager 2000 or equivalent

* Kit contents and storage temperatures are listed in **“Reagent Kit Components”** on page 64.

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2 gDNA Quantitation and Quality Analysis

Assess gDNA Quantity and Quality	18
Fluorometry	18
UV-VIS spectrophotometry	19
Agarose gel electrophoresis	20

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/ μ L 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 64 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 6** together on a vortex mixer for 2 to 3 seconds.

Table 6 Qubit working solution

Component	Amount
Qubit dsDNA BR Reagent	1 μ L
Qubit dsDNA BR Buffer	199 μ L

- 3 Load 190 μ L of Qubit working solution into the two 0.5-mL PCR tubes labeled for the standards.
- 4 Load 180 to 199 μ L 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:

$$\text{Sample concentration} = QF \text{ value} \times (200/y)$$

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.

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

- Record the A_{260}/A_{280} and 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 2**. The ideal 260/230 ratio for pure DNA is >1.8. High-quality gDNA samples have an A_{260}/A_{280} ratio of 1.8 to 2.0, which indicates the absence of contaminating proteins.

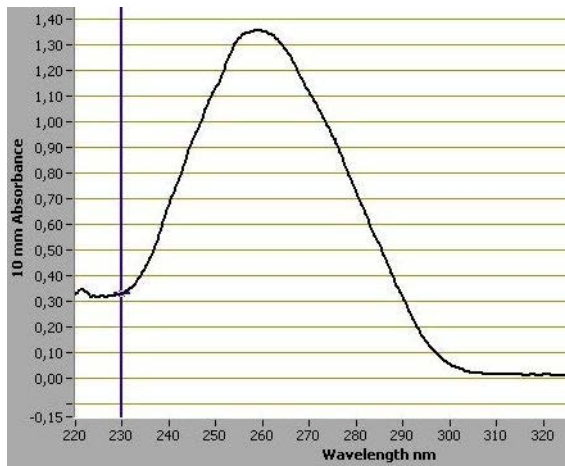


Figure 2 Typical spectrum of pure DNA

Agarose gel electrophoresis

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

- 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).
- 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.
- 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.
- Run the gel for 30 minutes as described in the manufacturer's instructions.
- Open the gel cassette with an E-Gel Opener as described in the manufacturer's instructions.
- 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.
- 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.

3 Sample Fragmentation

Enzymatic Fragmentation **22**

Step 1. Fragment the gDNA samples with digestion enzymes **22**

Step 2. Anneal the Random Primers **23**

Heat Fragmentation **25**

This chapter describes the Agilent recommended protocols for fragmenting gDNA for the labeling reaction.

Select between the following methods for DNA fragmentation.

- **“Enzymatic Fragmentation”** on page 22 – Fragments DNA using restriction digestion. This method is compatible with both CGH and CGH+SNP HT microarrays.
- **“Heat Fragmentation”** on page 25 – 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.

CAUTION

If using 96-well PCR plates, make sure to use semi-skirted plates (e.g., Agilent p/n 401334 or equivalent semi-skirted plates).

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 64 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

- 1 Pre-program the thermal cycler for the fragmentation reactions using the program in **Table 7**. If your thermal cycler requires entering a reaction volume setting, enter 6.5 μL . Start the program, then immediately pause the program as soon as *the thermal cycler reaches 37°C*.

Table 7 Thermal cycler program for enzymatic fragmentation (with heated lid)

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

- 2 If you have not already prepared a diluted working solution of 2 $\mu\text{g}/\mu\text{L}$ BSA, then prepare that solution as follows.
 - a Transfer 12.5 μL of the supplied 10 $\mu\text{g}/\mu\text{L}$ **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 $\mu\text{g}/\mu\text{L}$.
 - c After preparing the master mix in **step 5**, keep the unused portion of the diluted BSA working solution at -20°C for future assays.
- 3 For each test and reference gDNA sample, prepare a diluted stock of the appropriate concentration using **Nuclease Free Water**. See **Table 8** for the gDNA concentration required for your microarray format. Keep the diluted gDNA samples on ice.

Table 8 Concentration of diluted gDNA samples based on microarray format

Microarray format	Concentration of diluted gDNA
16-pack microarrays	35.7 ng/ μL
24-pack microarrays	46.4 ng/ μL

- 4 Add 3.5 μL of each diluted gDNA sample to a PCR tube or to the well of a 96-well PCR plate.

- Prepare the appropriate volume of Enzymatic Fragmentation Master Mix by combining the components in **Table 9**. Mix on a vortex mixer for 5 seconds, then spin briefly. Keep on ice.

Table 9 lists the volumes required per reaction as well as the required volumes (including excess) for 16 reactions, for 24 reactions, for 32 reactions (appropriate for one 16-pack microarray), and for 48 reactions (appropriate for one 24-pack microarray).

Table 9 Enzymatic Fragmentation Master Mix

Component	Volume per reaction	×16 reactions (including excess)	×24 reactions (including excess)	×32 reactions (including excess)	×48 reactions (including excess)
Nuclease Free Water	1.85 µL	33.3 µL	48.1 µL	64.8 µL	96.2 µL
10× Restriction Enzyme Buffer	0.65 µL	11.7 µL	16.9 µL	22.8 µL	33.8 µL
Diluted BSA (2 µg/µL) prepared in step 2	0.25 µL	4.5 µL	6.5 µL	8.75 µL	13.0 µL
Alu I	0.125 µL	2.25 µL	3.25 µL	4.4 µL	6.5 µL
Rsa I	0.125 µL	2.25 µL	3.25 µL	4.4 µL	6.5 µL
Total Volume	3.0 µL	54 µL	78.0 µL	105.15 µL	156.0 µL

- Add 3.0 µL of Enzymatic Fragmentation Master Mix to each tube or well containing a gDNA sample to make a total reaction volume of 6.5 µL. Cap the tubes or plate.
- Mix on a vortex mixer for 5 seconds, then spin briefly. Keep the reactions on ice.
- Place the tubes or plate in the thermal cycler. Resume the thermal cycler program in **Table 7**.
- Upon completion of the program, remove the tubes or plate, then spin briefly. Keep reactions on ice. Continue to **“Step 2. Anneal the Random Primers”** or store fragmented gDNA samples for later processing.

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

Step 2. Anneal the Random Primers

- Pre-program the thermal cycler for random primer annealing using the program in **Table 10**. If your thermal cycler requires entering a reaction volume setting, enter 7.75 µL. Start the program, then immediately pause the program as soon as the thermal cycler reaches 98°C.

Table 10 Thermal cycler program for random primer annealing (with heated lid)

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

- Add 1.25 µL of **Random Primers** to each gDNA reaction. Cap the tubes or plate.
- Mix the reactions on a vortex mixer at high speed for 10–15 seconds. Spin briefly.

- 4 Place the tubes or plate in the thermal cycler. Resume the thermal cycler program in **Table 10**.
- 5 Upon completion of the program, remove the tubes or plate, then spin briefly. Keep reactions on ice. Continue to **“Step 1. Label gDNA with Fluorescent Dyes (Cyanine 3 and Cyanine 5)”** on page 28.

Heat Fragmentation

CAUTION

Do not use heat fragmentation if you are processing CGH+SNP HT microarrays. Use the enzymatic fragmentation procedure provided on [page 22](#) 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 64 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.

- 1 Pre-program the thermal cycler for the fragmentation reactions using the program in [Table 11](#). If your thermal cycler requires entering a reaction volume setting, enter 7.75 μL . Start the program, then immediately pause the program as soon as the thermal cycler reaches 98°C.

Table 11 Thermal cycler program for heat fragmentation (with heated lid)

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

- 2 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 for your microarray format. 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	19.2 ng/ μL
24-pack microarrays	25.0 ng/ μL

- 3 Add 6.5 μL of each diluted gDNA sample to a PCR tube or to the well of a 96-well PCR plate.
- 4 Add 1.25 μL of **Random Primers** to each tube or well containing a gDNA sample to make a total reaction volume of 7.75 μL . Cap the tubes or plate.
- 5 Mix on a vortex mixer for 5 seconds, then spin briefly.
- 6 Place the tubes or plate in the thermal cycler. Resume the thermal cycler program in [Table 11](#).
- 7 Upon completion of the program, remove the tubes or plate, then spin briefly. Keep reactions on ice. Continue to **“Step 1. Label gDNA with Fluorescent Dyes (Cyanine 3 and Cyanine 5)”** on page 28.

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4 Sample Labeling

Step 1. Label gDNA with Fluorescent Dyes (Cyanine 3 and Cyanine 5) **28**

Step 2. Purify Labeled gDNA **29**

Step 3. Determine Yield and Specific Activity **31**

This chapter describes the steps 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.

CAUTION

The magnetic separator device used for labeled gDNA purification must have a low-elution magnet. Agilent recommends the Permagen 96-well PCR Post Magnet Low Elution Plate, p/n LE400.

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

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 64 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.

CAUTION

The test/reference sample pairs must be treated identically during processing to avoid adversely affecting data quality. Using a thermal cycler for incubations helps ensure that the sample pairs are exposed to the same temperatures.

- 1 Pre-program the thermal cycler for the labeling reactions using the program in **Table 13**. If your thermal cycler requires entering a reaction volume setting, enter 12.5 μL . Start the program, then immediately pause the program as soon as the thermal cycler reaches 37°C.

Table 13 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 Prepare two Labeling Master Mixes (one with cyanine 3 and one with cyanine 5) by combining the components in **Table 14**. Mix on a vortex mixer for 5 seconds, then spin briefly. Keep on ice.

Table 14 lists the volumes required per reaction as well as the required volumes (including excess) for 8 reactions, for 16 reactions (appropriate for one 16-pack microarray), and for 24 reactions (appropriate for one 24-pack microarray).

NOTE

Make sure that **Cyanine 3-dUTP** and **Cyanine 5-dUTP** are fully thawed before preparing the Labeling Master Mixes.

Table 14 Labeling Master Mixes - one with cyanine 3 and one with cyanine 5

Component	Volume per reaction	×8 reactions (including excess)	×16 reactions (including excess)	×24 reactions (including excess)
5× Reaction Buffer	2.5 μL	22.5 μL	42.5 μL	65.0 μL
10× dNTPs	1.25 μL	11.3 μL	21.3 μL	32.5 μL
Cyanine 3-dUTP or Cyanine 5-dUTP	0.75 μL	6.75 μL	12.8 μL	19.5 μL
Exo (-) Klenow	0.25 μL	2.25 μL	4.25 μL	6.5 μL
Total volume	4.75 μL	42.8 μL	80.85 μL	123.5 μL

- 3 Add 4.75 μL of appropriate Labeling Master Mix to each tube or well containing fragmented gDNA stored on ice. Final volume of each reaction is 12.5 μL . Cap the tubes or plate after addition of the Labeling Master Mix.
 - Add the cyanine 3 Labeling Master Mix to fragmented gDNA samples that are to be labeled with cyanine 3.
 - Add the cyanine 5 Labeling Master Mix to fragmented gDNA samples that are to be labeled with cyanine 5.
- 4 Mix on a vortex mixer for 5 seconds, then spin briefly.
- 5 Place the tubes or plate in the thermal cycler. Resume the thermal cycler program in **Table 13**.
- 6 Upon completion of the program, remove the tubes or plate, then spin briefly. Keep reactions on ice.

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 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 64 for a list of kit components.

- 1 Prepare enough 80% ethanol to wash all of the reactions (including excess). The required volume of 80% ethanol is 200 μL per reaction.

The 80% ethanol is used in **step 9** below.

- 2 Prepare the SurePure Beads Master Mix by combining the components in **Table 15**.

Table 15 lists the volumes required per reaction as well as the required volumes (including excess) for 16, 24, 32, and 48 reactions.

NOTE

Because the stock vials of SurePure Beads and SureTag HT Buffer include volume overage, the SureTag HT and SureTag HT Complete Kits provide sufficient quantities of these components to prepare the master mix with the excess volumes indicated in the table.

Table 15 SurePure Beads Master Mix

Component	Volume per reaction	×16 reactions (including excess)	×24 reactions (including excess)	×32 reactions (including excess)	×48 reactions (including excess)
SureTag HT Buffer	30 μL	552 μL	828 μL	1.1 mL	1.66 mL
100% ethanol	120 μL	2.21 mL	3.31 mL	4.42 mL	6.62 mL
SurePure Beads	5 μL	92 μL	138 μL	184 μL	276 μL
Total volume	155 μL	2.854 mL	4.276 mL	5.704 mL	8.556 mL

- 3 Make sure that the SurePure Beads Master Mix is well mixed. Then add 155 μL of SurePure Beads Master Mix to each tube or well containing labeled gDNA.

Final volume of each reaction is 167.5 μL .
- 4 Mix the reactions on a vortex mixer until the SurePure Beads are fully suspended.

- 5 Briefly spin the reactions in a centrifuge. Make sure that no liquid remains on the walls and lid and that the beads are still suspended.
- 6 Incubate the reactions at room temperature for 5 minutes to allow the labeled gDNA to bind to the SurePure Beads.
- 7 Put the plate or tubes onto a magnetic separator device and incubate at room temperature for 5 minutes to pellet the SurePure Beads.

NOTE

If you are using the Permagen magnetic separator device, or another device that collects beads on the side of the tube bottom, use the following technique for best performance:

When initially putting the plate or tubes onto the device, position the plate/tubes such that the magnets are directly beneath the bottom of the wells/tubes. Once the beads have settled to the bottom, reposition the plate/tubes to the proper position on the device. This technique helps ensure that the beads are properly aggregated prior to washing.

- 8 With a pipette, carefully remove and discard the supernatant in each reaction without disturbing the pellet.
- 9 Add 200 μ L of 80% ethanol to each reaction to wash the beads. Do not remove the reactions from the magnetic separator device and do not mix. Incubate at room temperature for 1 minute.
- 10 With a pipette, carefully remove and discard the supernatant in each reaction without disturbing the pellet.
- 11 Remove the plate or tubes from the magnetic separator device. Spin briefly.
- 12 Without capping the tubes or plate, incubate the reactions in an open thermal cycler set to 37°C until beads are dry (typically <5 minutes). *Do not close the lid of the thermal cycler.* Check the samples every 30 seconds to monitor dryness of the pellets. Do not allow the beads to dry longer than necessary.
- 13 Remove the plate or tubes from the thermal cycler as soon as the pellets are completely dry. Keep at room temperature.
- 14 Add 5.5 μ L of 1 \times TE buffer (pH 8.0) to each reaction. Mix well by vortexing or by pipetting up and down.

Beads do not need to be resuspended at this step. Allowing the 1 \times TE buffer to wash over the bead pellet is sufficient for proper elution.
- 15 Spin the reactions in a centrifuge for 10–15 seconds, or long enough to drive the contents off the walls and lid.
- 16 Incubate the reactions at room temperature for 5 minutes to elute the labeled gDNA from the SurePure Beads.
- 17 Put the plate or tubes onto a magnetic separator device and incubate at room temperature for 5 minutes to pellet the SurePure Beads.

The labeled gDNA is in the supernatant.
- 18 With a pipette, carefully transfer the entire volume of supernatant from each reaction to the well of a fresh 96-well plate or a fresh tube. Keep the purified labeled gDNA samples on ice.

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 \times TE buffer (pH 8.0) to blank the instrument.
- 3 Use 1 μL of purified labeled gDNA for quantitation. Measure the absorbance at $A_{260\text{nm}}$ (DNA), $A_{550\text{nm}}$ (cyanine 3), and $A_{650\text{nm}}$ (cyanine 5).
- 4 Calculate the Specific Activity of the labeled gDNA:

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

*pmol dyes per μg gDNA

- 5 Record the gDNA concentration ($\text{ng}/\mu\text{L}$) for each sample. Calculate the yield as

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

Refer to **Table 16** for expected yield of labeled gDNA and specific activity after labeling and purification, when starting with high quality gDNA.

Table 16 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 55.

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5 Microarray Processing

Hybridize Labeled gDNA to HT Microarray **34**

Wash the Microarray Slides **44**

Microarray Scanning and Analysis **50**

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

Hybridize Labeled gDNA to HT Microarray

This procedure uses reagents included in the Oligo aCGH/ChIP-on-chip Hybridization Kit. See **“Agilent Oligo aCGH/ChIP-on-chip Hybridization Kit”** on page 64 for a list of kit components.

To practice setting up the hybridization assembly, prepare a 1:1 **2× HI-RPM Hybridization Buffer** and water mix and use a microscope slide or used microarray slide, and a gasket slide. You can use the same slide to practice wash and placement of slide in the slide holder. Refer to the *Agilent Microarray Hybridization Chamber User Guide* (publication G2534-90004) for in-depth instructions on how to load samples, assemble and disassemble chambers, as well as other helpful tips.

Before you begin, make sure you read and understand **“Secure Fit” Slide Box Opening Instructions** on page 65 and **“Microarray Handling Tips”** on page 67.

Step 1. Reconstitute the aCGH Blocking Agent

CAUTION

The aCGH Blocking Agent is supplied as a lyophilized reagent that can be reconstituted to either a 10× concentration or a 40× concentration depending on which Agilent microarrays you are using. The Agilent HT microarrays require the 40× concentration. Make sure to reconstitute the aCGH Blocking Agent to a 40× concentration following the instructions below. After reconstitution, mark the “40X Blocking Agent” check box on the reagent vial.

- 1 Add 337.5 µL of nuclease-free distilled water to the vial containing lyophilized **aCGH Blocking Agent**. The reconstituted aCGH Blocking Agent is at a concentration of 40×.
- 2 Leave at room temperature for 60 minutes and mix on a vortex mixer to reconstitute sample before use or storage.

NOTE

Reconstituted 40× aCGH Blocking Agent is stable for 120 days when stored at –20°C and can be frozen and thawed up to 16 times.

Step 2. Prepare labeled gDNA for hybridization

- 1 In a fresh PCR tube or the well of a fresh 96-well plate, combine the purified labeled gDNA from the test sample with the purified labeled gDNA from the corresponding reference sample. Use the entire volume of both samples. Keep the mixture on ice and protected from light.

The total volume of the mixture is approximately 9 µL.

NOTE

Each test sample should be paired with a reference sample that has the same yield (as measured in **“Step 3. Determine Yield and Specific Activity”** on page 31). If you prepared multiple samples of labeled gDNA for use as a reference, select the one with a yield that most closely matches that of the test sample. Do not pair test samples with a reference sample that has a significantly different yield or specific activity. Refer to **Chapter 6, “Troubleshooting”** for further guidance.

- Pre-program the thermal cycler for the hybridization reactions using the program in **Table 17**. 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). Start the program, then immediately pause the program *as soon as the thermal cycler reaches 98°C*.

Table 17 Thermal cycling program for hybridization

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 36)

- Prepare the appropriate volume of Hybridization Master Mix by combining the components in either **Table 18** (if using 16-pack arrays) or **Table 19** (if using 24-pack arrays). Mix well by pipetting up and down. Spin briefly and keep on ice.

The tables list the volumes required per reaction as well as the required volumes (including excess) for 16 or 24 reactions.

Table 18 Hybridization Master Mix for 16-pack arrays

Component	Volume per hybridization reaction	×16 reactions (including excess)
Nuclease-free water	2.51 μL	45.2 μL
Cot-1 DNA (1.0 mg/mL)	1.11 μL	20.0 μL
aCGH Blocking Agent (40×)	0.61 μL	11.0 μL
2× HI-RPM Hybridization Buffer	12.2 μL	220.0 μL
Total Volume	16.43 μL	296.2 μL

Table 19 Hybridization Master Mix for 24-pack arrays

Component	Volume per hybridization reaction	×24 reactions (including excess)
Cot-1 DNA (1.0 mg/mL)	0.78 μL	20.3 μL
aCGH Blocking Agent (40×)	0.43 μL	11.2 μL
2× HI-RPM Hybridization Buffer	8.59 μL	223.3 μL
Total Volume	9.8 μL	254.8 μL

- In a fresh PCR tube or the well of a fresh 96-well plate, add the appropriate volume of Hybridization Master Mix and labeled gDNA mixture. **Table 20** lists the required volumes per hybridization reaction for each array format, as well as the final total volume of each reaction and the volume that will be loaded onto the gasket slide on **page 38**.

Table 20 Volumes per hybridization reaction

Component	Volume per hybridization for 16-pack arrays	Volume per hybridization for 24-pack arrays
Hybridization Master Mix	16.5 μL	9.8 μL
Labeled gDNA mixture*	8.0 μL	7.38 μL
Total Volume	24.5 μL	17.18 μL
Volume to be added to gasket well	18.0 μL	10.0 μL

* If you do not have enough labeled gDNA mixture to add the volume listed in the table, consult the section of the Troubleshooting chapter entitled **“If the volume of the labeled DNA mixture is less than that needed for hybridization.”**.

- Mix the hybridization reactions on a vortex mixer for 2–3 seconds or until thoroughly mixed, then briefly spin in a centrifuge.

NOTE

The hybridization reactions will contain bubbles after mixing on the vortex mixer. The bubbles dissipate early in the thermal cycling program and do not interfere with hybridization.

- Cap the tubes or plate and place in the thermal cycler. Resume the thermal cycler program in **Table 17**.
- While the thermal cycler program is running, confirm that the hybridization oven is set to 67°C.

CAUTION

As soon as the thermal cycler program reaches the 37°C hold step, begin **“Step 3. Prepare the hybridization assembly”** on page 36, processing 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 38**. 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 3 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.

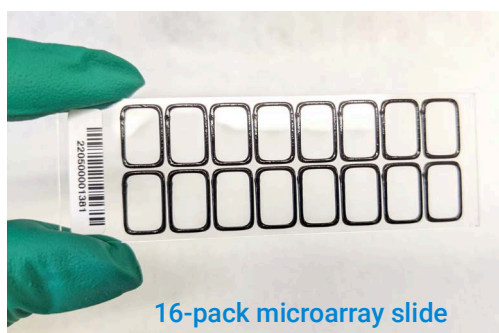


Figure 4 Gasket slide, gasket side

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

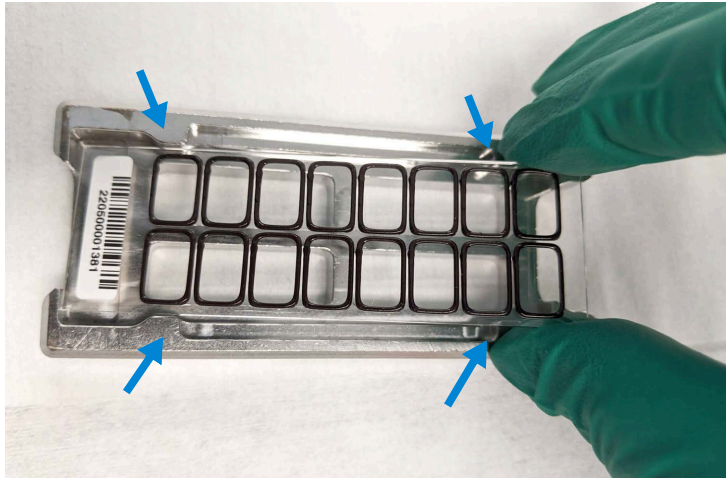


Figure 5 Chamber base, guide posts denoted with arrows

- 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 6 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 μ L per gasket well
 - 24-pack microarray: add 10 μ L per gasket well

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

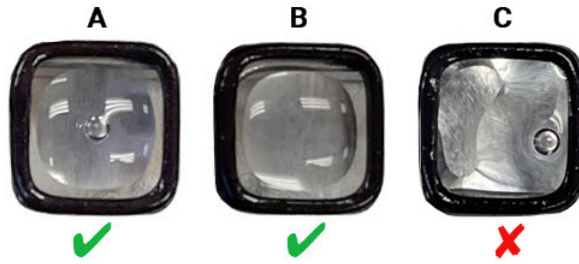


Figure 7 Gasket wells containing loaded sample mixtures. In panels A and B, the bead of liquid is intact. In panel C, the liquid is in contact with the gasket.

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 7**). 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 7**), use a clean, small-gauge pipette tip (e.g., a 10- μ L 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 59.

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 \times HI-RPM Hybridization Buffer (diluted from 2 \times to 1 \times using nuclease-free water). Make sure that the gasket wells in the four corners of the slide contain hybridization sample mixture rather than 1 \times 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 8**.

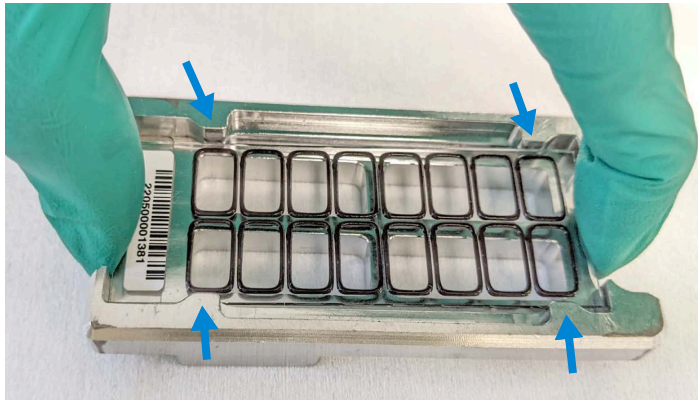


Figure 8 Chamber base with gasket and microarray slide applied, guide posts denoted with arrows

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.

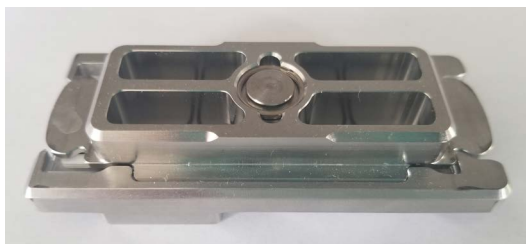


Figure 9 Chamber cover setting on chamber base in correct orientation

- 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.

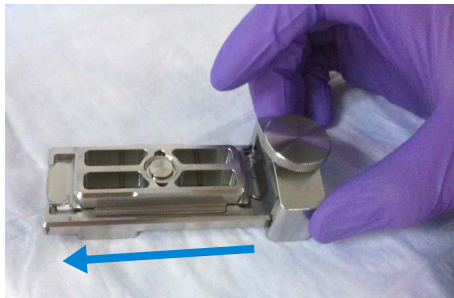


Figure 10 Slipping the clamp onto the chamber base

- 3 Firmly tighten the thumbscrew fully.
The slides will not be harmed by hand-tightening.

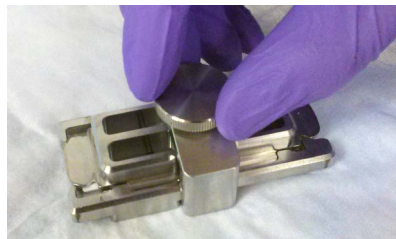


Figure 11 Tightening of the thumbscrew on the clamp

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 12**).

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

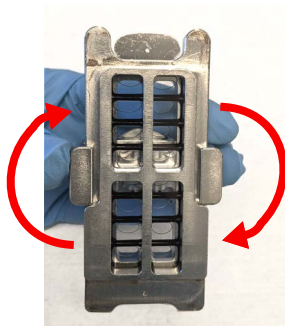


Figure 12 Rotation of the final assembled chamber

- 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.

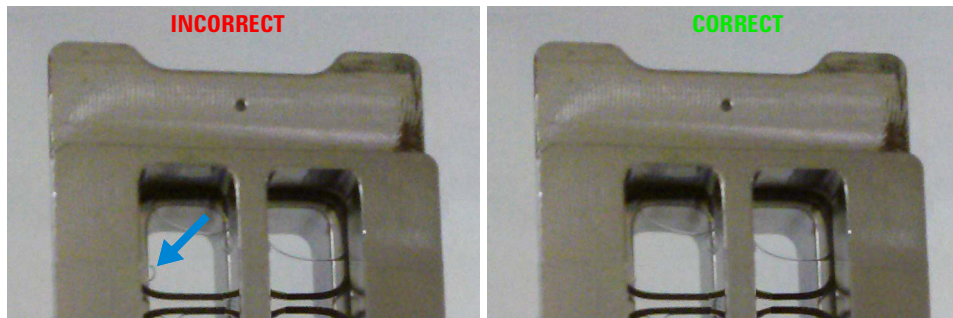


Figure 13 The slide on the left shows a stray, stationary bubble (denoted with arrow), which must be removed before hybridization. The slide on the right shows only large mixing bubbles, which move freely around the chamber when rotated. Bubbles are acceptable, as long as they move freely when you rotate 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 14 Assembled chambers in correct (left) and incorrect (middle and right) orientations

- 7 Repeat “**Step 3. Prepare the hybridization assembly**” (starting on [page 36](#)) 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 64 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 44**.

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 44.

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 44.

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 64 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 21 summarizes the wash conditions for the wash procedure. Detailed instructions follow.

Table 21 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 15** Loosening of the thumbscrew

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

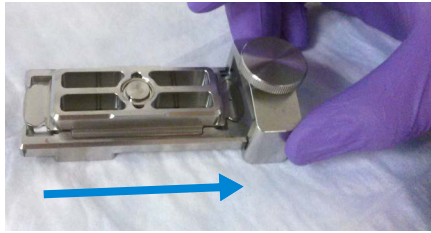


Figure 16 Removal of the clamp

- 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 17**) 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!*

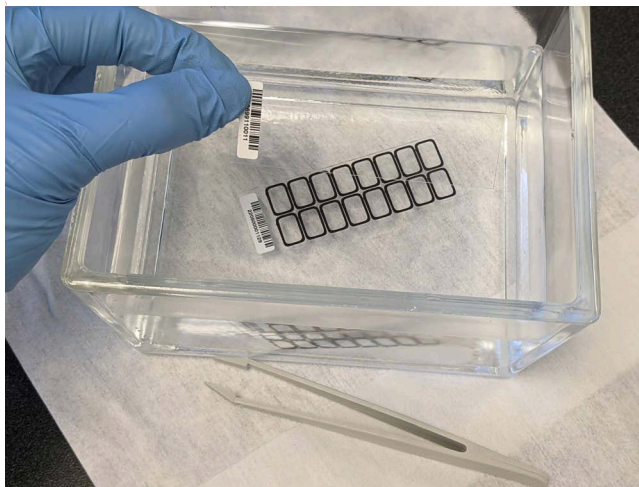


Figure 17 Removal of the microarray slide from dish #1

- 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 47 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.

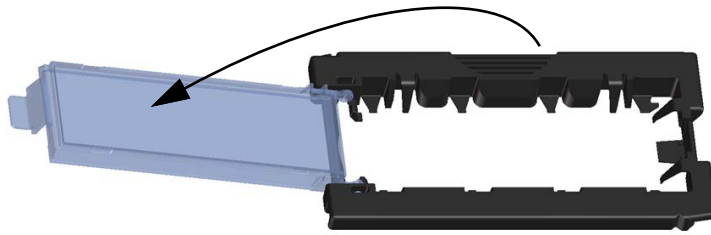


Figure 18 Opening the slide holder

- 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 19**.
 - b Carefully place the end of the slide without the barcode label onto the slide ledge. See **Figure 19**.

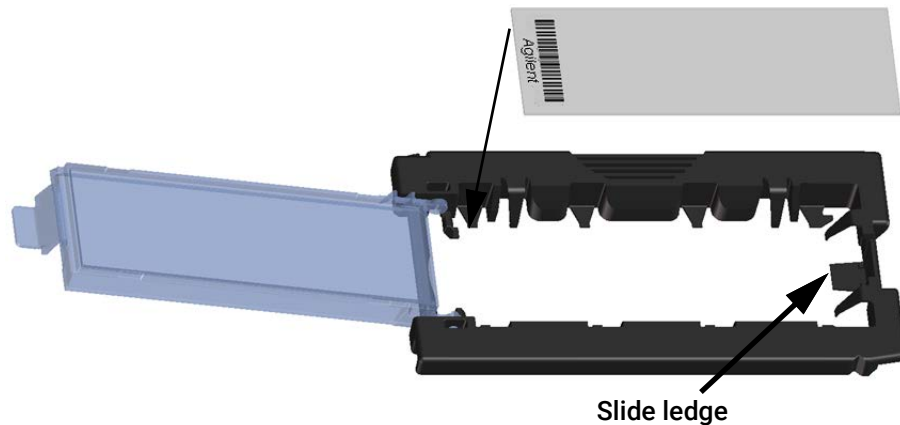


Figure 19 Inserting slide into the slide holder

- c Gently lower the slide into the slide holder. See **Figure 20**.
 - 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 21**. Make sure that the slide holder is completely closed.

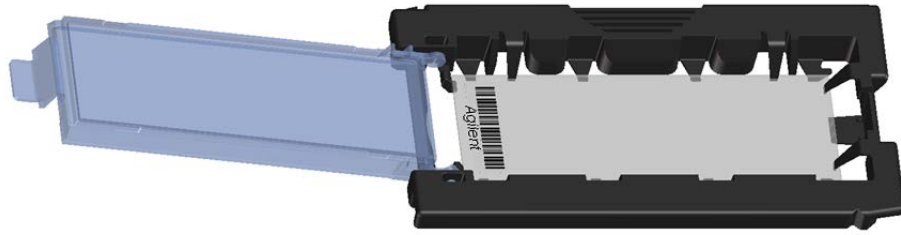


Figure 20 Slide inserted in slide holder – cover open

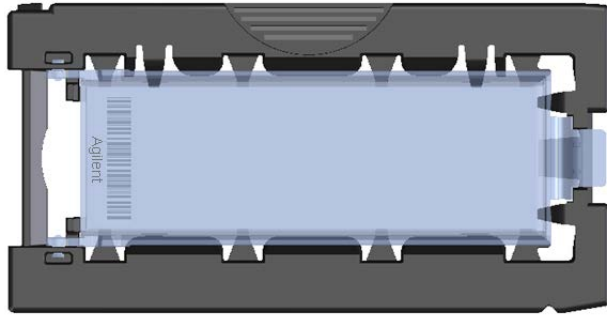


Figure 21 Slide inserted in slide holder – cover closed

Microarray Scanning and Analysis

Step 1. Scan the microarray slides

- 1 Put assembled slide holders into the scanner cassette. Refer to [Figure 22](#) and [Figure 23](#).

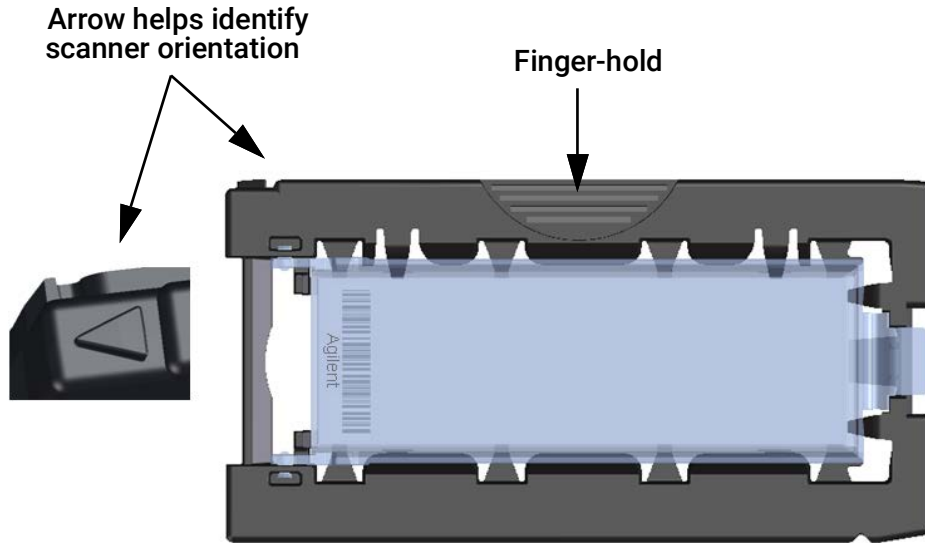


Figure 22 Slide holder helps you to insert slides correctly

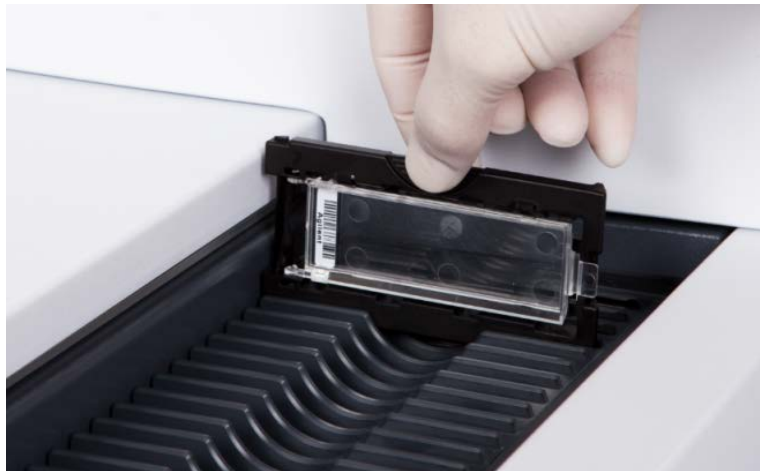


Figure 23 Inserting slide holder into cassette

- 2 Select **Protocol AgilentG3_CGH**.

[Table 22](#) lists the scan settings for this protocol

Table 22 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 61 of the **Troubleshooting** chapter for guidance.

The metric thresholds in **Table 23** 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.

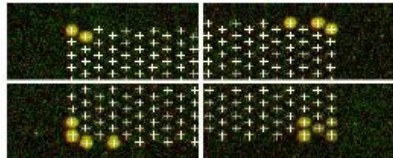
Table 23 QC metric thresholds for Enzymatic labeling

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

QC Report - Agilent Technologies : 2 Color CGH

Date	Thursday, December 01, 2022 - 11:48	Sample(red/green)	/Agilent Female_dbSNP141
User Name	dhuffman	FE Version	5.3.0.1
Image	SG11410004_258698910050_S001 [1_1]	BG Method	Detrend on (NegC)
Protocol	CytoCGH_0530_16x_Oct22 (Read Only)	Multiplicative Detrend	True
Grid	086989_hg19_20220506	Dye Norm	Linear
Saturation Value	65526 (r), 65526 (g)		
DyeNorm List	NA		
No of Probes in DyeNorm List	NA		

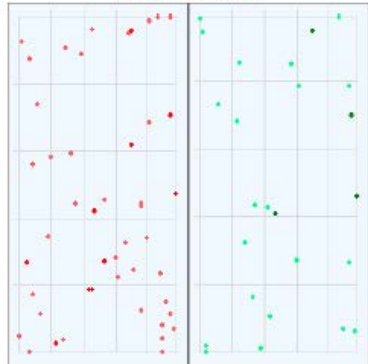
Spot Finding of the Four Corners of the Array



Grid Normal

Outlier Numbers with Spatial Distribution

430 rows x 60 columns



• Red FeaturePopulation • Red Feature NonUniform
• Green FeaturePopulation • Green Feature NonUniform

Feature	Red	Green	Any	%Outlier
Non Uniform	10	4	10	0.04
Population	37	21	53	0.21

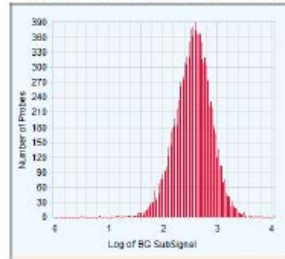
Evaluation Metrics for CytoCGH_QCMT_16x_Oct22

Excellent (7) ; Good (6)

Metric Name	Value	Excellent	Good	Evaluate
IsGoodGrid	1.00	>1	NA	<1
AnyColorPrintFeatNonU...	0.04	<1	1 to 5	>5
DerivativeLR_Spread	0.16	<0.20	0.20 to 0.30	>0.30
gRepro	0.10	0 to 0.10	0.10 to 0.20	<0 or >0.20
g_BGNoise	5.70	<15	15 to 25	>25
g_Signal2Noise	63.29	>60	25 to 60	<25
g_SignalIntensity	360.58	>400	200 to 400	<200
rRepro	0.10	0 to 0.10	0.10 to 0.20	<0 or >0.20
r_BGNoise	5.64	<15	15 to 25	>25
r_Signal2Noise	57.96	>60	25 to 60	<25
r_SignalIntensity	326.94	>350	150 to 350	<150
RestrictionControl	0.96		0.80 to 1	<0.80 or >1
LogRatioImbalance	0.00	-0.26 to 0.26	(-0.75 to -0.2...	<-0.75 or >0.75

• Excellent • Good • Evaluate

Histogram of Signals Plot (Red)



Histogram of Signals Plot (Green)

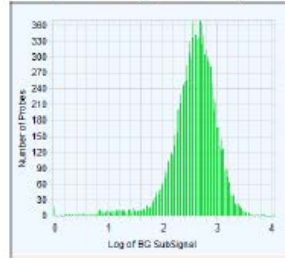


Figure 24 Feature Extraction QC Report, page 1

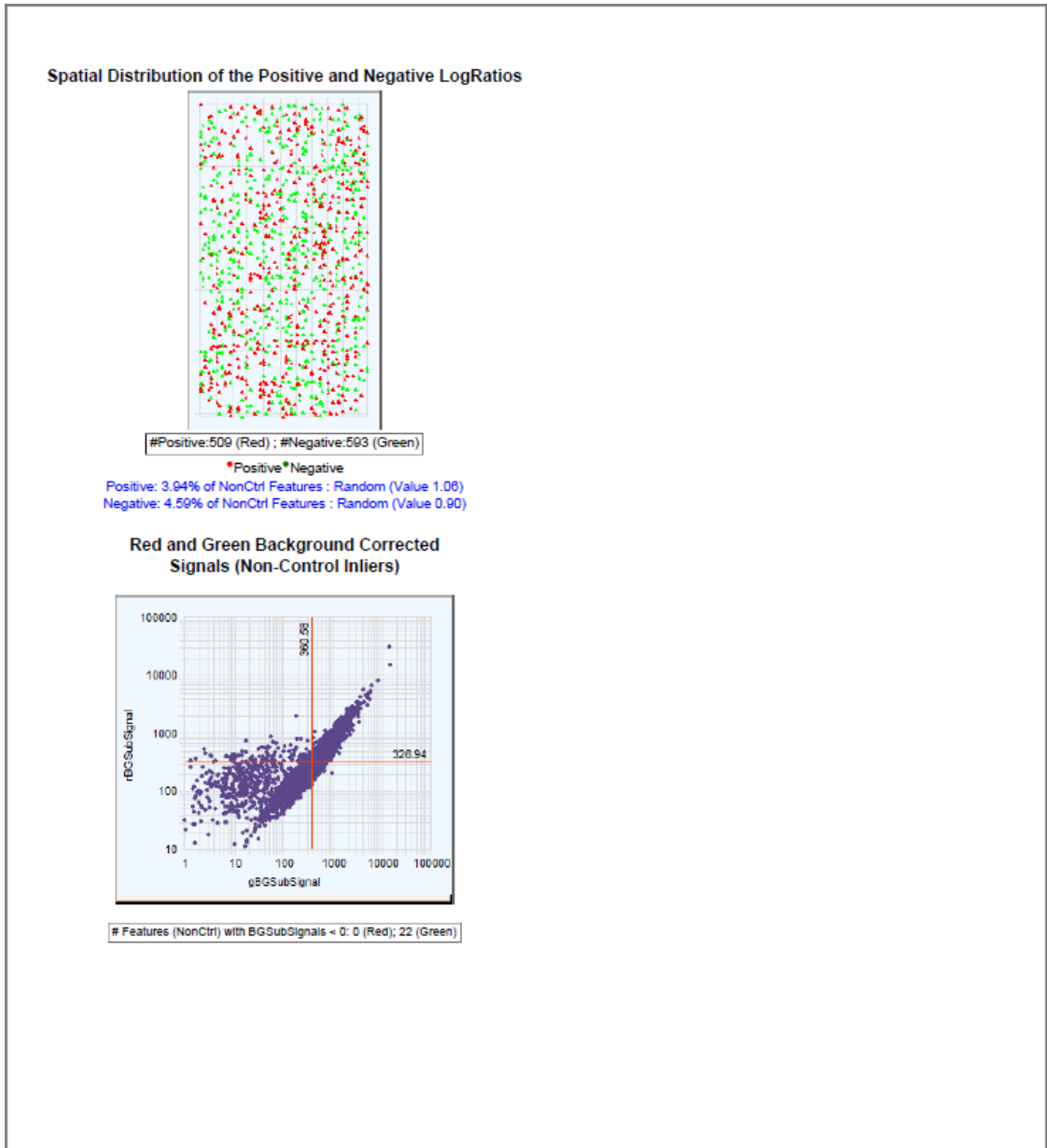


Figure 25 Feature Extraction QC Report, page 2

6 Troubleshooting

- If the gDNA sample has a low A260/A230 or A260/A280 value **56**
- If the gDNA sample has poor quality due to residual RNA **56**
- If the gDNA sample has poor quality due to degradation **56**
- If the estimated concentration of gDNA is too high or low **57**
- If the labeled DNA has low specific activity not due to poor sample quality **57**
- If the labeled DNA has low yield not due to poor sample quality **58**
- If a sample mixture comes into contact with the gasket during loading **59**
- If you observe post-labeling signal loss **59**
- If the feature extraction QC metrics indicate a high BGNoise value **60**
- If the feature extraction QC metrics indicate poor reproducibility **60**
- If CytoGenomics reports a gridding error **61**

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 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/ μ g DNA). See **“Step 3. Determine Yield and Specific Activity”** on page 31.

- 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 29 to remove unreacted dye. Other purification techniques result in the loss of shorter fragments.
- In the purification procedure, Agilent recommends the Permagen 96-well PCR Post Magnet Low Elution Plate (or equivalent low-elution magnet) to pellet the SurePure Beads.
- 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 the volume of the labeled DNA mixture is less than that needed for hybridization

The hybridization reaction requires either 8.0 μL (for 16-pack arrays) or 7.38 μL (for 24-pack arrays) of the purified, labeled DNA mixture, which consists of the combined test and reference samples. Consult the tips below if you have an insufficient volume.

- If evaporation is suspected as the cause of the low volume, and the volume of labeled DNA mixture is within 1 μL of the target volume, then add 1 μL of nuclease-free water to the DNA mixture.
- If evaporation is not suspected as the cause of the low volume and the volume of labeled DNA mixture is within 10% of the target volume — i.e., 7.2 μL for 16-pack arrays or 6.6 μL for 24-pack arrays — then proceed with the protocol, adding additional nuclease-free water to the hybridization reaction to make up for the missing volume (see **Table 20** on page 36 for reaction volumes).
- If the yields of the individual test and reference gDNA samples (as measured in **“Step 3. Determine Yield and Specific Activity”** on page 31) were on the high end of the expected range (e.g., >3.0 ng), then proceed with the protocol, adding nuclease-free water to the hybridization reaction to make up for the missing volume (see **Table 20** on page 36 for reaction volumes).

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 26, panel A**), use the sample loading rescue procedure described below.
 - First, pop any air bubbles that may be present in the sample mixture (**Figure 26, panel B**). Use a clean, small-gauge pipette tip (e.g., a 10- μ L 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 26, panel C**). Make sure that the entire surface inside the gasket is covered with sample mixture (**Figure 26, panel D**).



Figure 26 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 23](#) 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*.

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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.

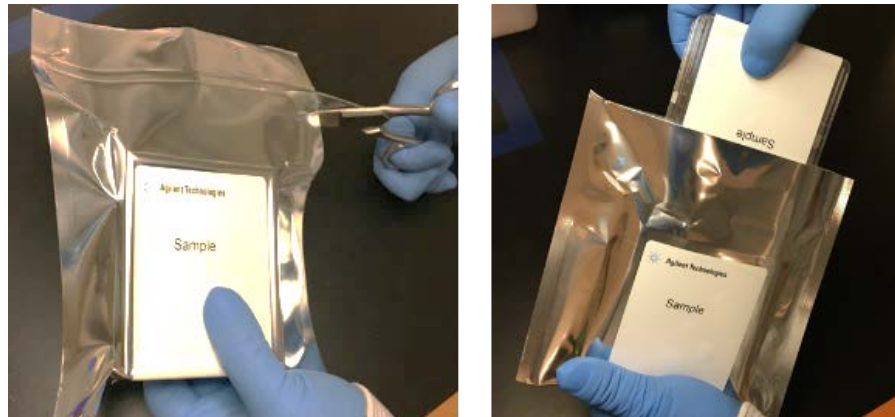


Figure 27 Opening foil pouch (left) and removing the slide box (right)

- 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.

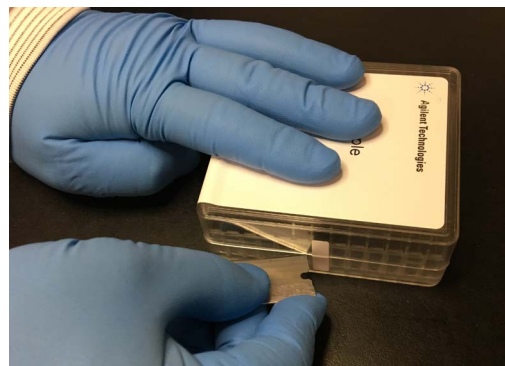


Figure 28 Cutting the sealing tape

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

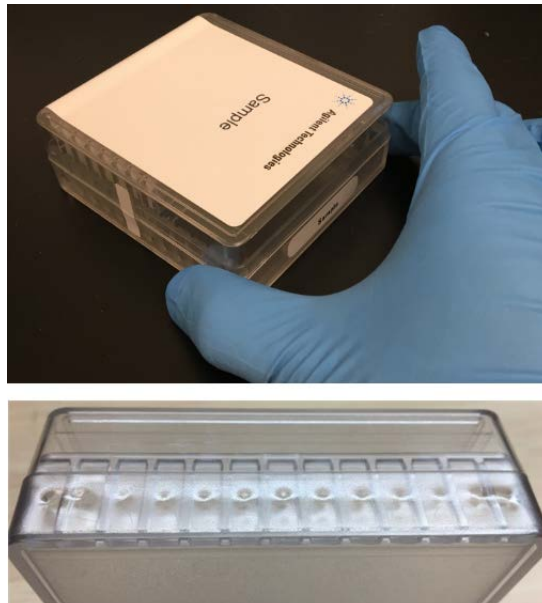


Figure 29 Gripping the base at the indentations (top) and close-up of the indentations (bottom)

- 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.

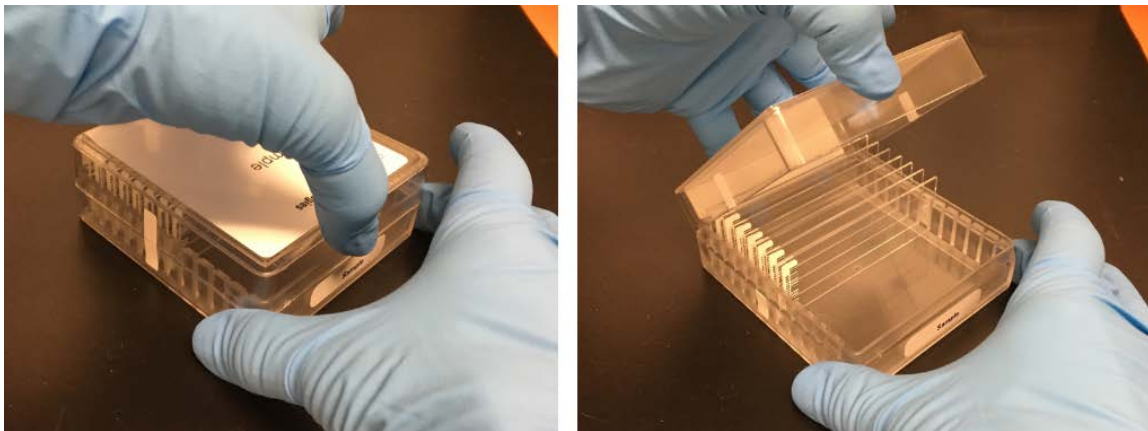


Figure 30 Grasping the lid (left) and lifting the lid from the base (right)

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

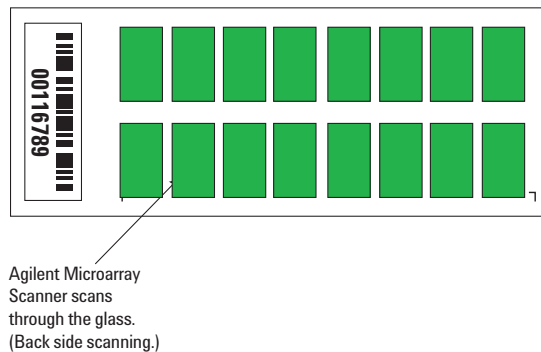
You must familiarize yourself with the assembly and disassembly instructions for use with the Agilent Microarray Hybridization Chamber (G2534A) and gasket slides. Practice slide kits are available.

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 31 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

	Array 1_1	Array 1_2	Array 1_3	Array 1_4	Array 1_5	Array 1_6	Array 1_7	Array 1_8	
B A R C O D E	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>
	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>
	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 32 16-pack microarray slide

Arrays

B A R C O D E	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>
	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: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>
	Array 2_1	Array 2_2	Array 2_3	Array 2_4	Array 2_5	Array 2_6	Array 2_7	Array 2_8
	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>
	Array 3_1	Array 3_2	Array 3_3	Array 3_4	Array 3_5	Array 3_6	Array 3_7	Array 3_8

Barcode Number _____

Figure 33 24-pack microarray slide

In This Book

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

