Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis

Enzymatic Labeling for Blood, Cells or Tissues (with a High Throughput option)

Protocol

Version 6.1, August 2009

Research Use Only. Not for Diagnostic Procedures.

Agilent Technologies

Notices

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In This Guide...

This guide describes Agilent's recommended operational procedures to analyze DNA copy number variations using Agilent 60-mer oligonucleotide microarrays for array-based comparative genomic hybridization (aCGH) analysis. This protocol is specifically developed and optimized to enzymatically label DNA from blood, cells or frozen tissues. For processing FFPE samples, follow the *Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis (ULS Labeling for Blood, Cells, Tissues or FFPE) Protocol v3.1* (p/n G4410-90020).

1 Before You Begin

This chapter contains information (such as procedural notes, safety information, required reagents and equipment) that you should read and understand before you start an experiment.

2 DNA Isolation

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

3 Sample Preparation

This chapter describes the two methods to process genomic DNA (gDNA) prior to labeling.

4 Sample Labeling

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

5 Microarray Processing and Feature Extraction

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

6 Troubleshooting

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

7 Reference

This chapter contains reference information related to the protocol.

What's New in Version 6.1

- The use of the Agilent Ozone-Barrier Slide Cover is described.
- Additional part numbers are added.
- Additional guidelines on yield and specific activity after labeling.
- Guidelines are expanded to enable skipping of the restriction digestion step.

What's New in Version 6.0

- Guidelines are expanded to enable processing in 96-well plates and to support the use of the new Genomic DNA Enzymatic Labeling Kit (p/n 5190-0449).
- Processing of 1x1M, 2x400K, 4x180K and 8x60K microarrays is supported.
- Wash Procedure B (with Stabilization and Drying Solution) is improved. This procedure is used to wash microarray slides in an environment in which the ozone level exceeds 5 ppb.
- Troubleshooting instructions are included.

Content

1 Before You Begin

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

Procedural Notes

- Follow the procedure described in this document to isolate gDNA from blood, cells, or frozen tissues, to increase the likelihood of a successful experiment. For processing FFPE samples, refer to the *Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis (ULS Labeling for Blood, Cells, Tissues or FFPE) Protocol v3.1* (p/n G4410-90020).
- If the DNA isolation procedure described in this document cannot be followed, make sure that the DNA is free of RNA and protein contamination.
- To prevent contamination of reagents by nucleases, always wear powder-free laboratory gloves, and use dedicated solutions and pipettors with nuclease-free aerosol-resistant tips.
- Maintain a clean work area.
- Do not mix stock solutions and reactions containing gDNA or enzymes on a vortex mixer. Instead, mix the solutions and reactions by gently tapping the tube with your finger.
- Avoid repeated freeze-thaw cycles of solutions containing gDNA or enzymes.
- When preparing frozen reagent stock solutions for use:
  1. Thaw the aliquot as quickly as possible without heating above room temperature.
  2. Mix briefly on a vortex mixer, then spin in a microcentrifuge for 5 to 10 seconds to drive the contents off the walls and lid.
  3. Store on ice or in a cold block until use.
- In general, follow Biosafety Level 1 (BL1) safety rules.

Safety Notes

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

WARNING

- **Cyanine 3-dUTP and Cyanine 5-dUTP are considered hazardous by the OSHA Hazard Communication Standard (29 CFR 1910.1200). Contains material that causes damage to the following organs: kidneys, liver, cardiovascular system, respiratory tract, skin, eye lens or cornea, stomach. May be harmful if swallowed. Avoid contact with eyes, skin and clothing.**
- **2X Hi-RPM Hybridization Buffer is considered hazardous by the OSHA Hazard Communication Standard (29 CFR 1910.1200). Contains material that causes damage to the following organs: skin, central nervous system. May be harmful if swallowed. Avoid contact with eyes, skin and clothing.**
- **Triton is harmful if swallowed. Risk of serious damage to eyes. Wear suitable PPE. Triton is a component of Agilent's 2X Hi-RPM Hybridization Buffer.**
- **Agilent Stabilization and Drying Solution is considered hazardous by the OSHA Hazard Communication Standard (29 CFR 1910.1200). Flammable liquid and vapor. Keep away from heat, sparks and flame. Keep container closed. Use only with adequate ventilation. This solution contains material which causes damage to the following organs: kidneys, liver, cardiovascular system, upper respiratory tract, skin, central nervous system (CNS), eye, lens or cornea.**

Agilent Oligo CGH Microarray Kit Contents

Store entire kit at room temperature. After the microarray foil pouch is opened, store the microarray slides at room temperature (in the dark) under a vacuum desiccator or N_2 purge box. Do not store microarray slides in open air after breaking foil.

Catalog CGH Microarray Kits

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

See Table 1 and Table 2 for available designs.

Table 1 Available Catalog SurePrint HD CGH Microarray Kits

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

Table 2 Available Catalog SurePrint G3 CGH Microarray Kits

Part Number	Description
G4447A	SurePrint G3 Human CGH Microarray Kit 1x1M
G4448A	SurePrint G3 Human CGH Microarray Kit 2x400K
G4449A	SurePrint G3 Human CGH Microarray Kit 4x180K
G4450A	SurePrint G3 Human CGH Microarray Kit 8x60K
G4506A	SurePrint G3 Human High-Resolution Discovery Microarray 1x1M
G4507A	SurePrint G3 Human CNV Microarray Kit 2x400K

Unrestricted SurePrint HD and G3 CGH Microarray Kits

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

See Table 3 and Table 4 for available designs.

Table 3 Unrestricted High-Definition CGH Microarrays

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

Table 4 Unrestricted SurePrint G3 CGH Microarrays

Part Number	Description
G4824A, AMADID 021529	Unrestricted SurePrint G3 CGH Microarray, 1x1M, Human (same design as G4447A)
G4824A, AMADID 023642	Unrestricted SurePrint G3 CGH Microarray, 1x1M, Human (same design as G4506A)
G4825A, AMADID 021850	Unrestricted SurePrint G3 CGH Microarray, 2x400K, Human (same design as G4448A)
G4825A, AMADID 021365	Unrestricted SurePrint G3 CGH Microarray, 2x400K, Human (same design as G4507A)
G4826A, AMADID 022060	Unrestricted SurePrint G3 CGH Microarray, 4x180K, Human (same design as G4449A)
G4827A, AMADID 021924	Unrestricted SurePrint G3 CGH Microarray, 8x60K, Human (same design as G4450A)

Custom SurePrint HD and G3 Microarray Kits

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

See Table 5 and Table 6 for available formats.

Table 5 Custom High-Definition CGH Microarrays

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

Table 6 Custom SurePrint G3 CGH Microarrays

Part Number	Description
G4123A	SurePrint G3 Custom CGH Microarray, 1x1M
G4124A	SurePrint G3 Custom CGH Microarray, 2x400K
G4125A	SurePrint G3 Custom CGH Microarray, 4x180K
G4126A	SurePrint G3 Custom CGH Microarray, 8x60K

Required Equipment

Table 7 Required equipment

Description	Company and part no.
Agilent Microarray Scanner Bundle for 1x244K, 2x105K, 4x44K or 8x15K, *or* for 1x1M, 2x400K, 4x180K or 8x60K	 Agilent p/n G2565BA or G2565CA Agilent p/n G2565CA
Hybridization Chamber, stainless	Agilent p/n G2534A
Hybridization Chamber gasket slides, 5-pack (alternative packaging sizes are available) for 1x microarrays *or* for 2x microarrays *or* for 4x microarrays *or* for 8x microarrays	 Agilent p/n G2534-60003 Agilent p/n G2534-60002 Agilent p/n G2534-60011 Agilent p/n G2534-60014
Hybridization oven; temperature set at 65°C	Agilent p/n G2545A
Hybridization oven rotator for Agilent Microarray Hybridization Chambers	Agilent p/n G2530-60029
Ozone-barrier slide cover*	Agilent p/n G2505-60550
UV-Transilluminator with SYBR photographic filter	Alpha Innotech p/n Alphalmager 2000 or equivalent
Nuclease-free 1.5 mL microfuge tubes (sustainable at 95°C)	Ambion p/n AM12400 or equivalent
Magnetic stir bar (×2 or ×4)†	Corning p/n 401435 or equivalent
Magnetic stir plate (×1 or ×3)†	Corning p/n 6795-410 or equivalent
Magnetic stir plate with heating element	Corning p/n 6795-420 or equivalent
Microcentrifuge	Eppendorf p/n 5430 or equivalent
E-Gel Opener‡	Invitrogen p/n G5300-01
E-Gel PowerBase v.4‡	Invitrogen p/n G6200-04
UV-VIS spectrophotometer	NanoDrop 8000 or 2000, or equivalent
P10, P20, P200 and P1000 pipettes	Pipetman P10, P20, P200, P1000 or equivalent
1.5 L glass dish	Pyrex p/n 213-R or equivalent

Table 7 Required equipment **(continued)**

Description	Company and part no.
Vacuum Concentrator **	Thermo Scientific p/n DNA120-115 or equivalent
250 mL capacity slide-staining dish, with slide rack (×3 or ×5)†	Wheaton p/n 900200 or equivalent
Circulating water baths or heat blocks set to 30°C, 37°C, 65°C, and 95°C	
Ice bucket	
Clean forceps	
Powder-free gloves	
Sterile, nuclease-free aerosol barrier pipette tips	
Timer	
Vacuum dessicator or N_2 purge box for slide storage	
Vortex mixer	

* Optional when processing arrays in high ozone environments.

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

‡ Optional when Invitrogen E-gels are used.

** Optional. Depends on array format and processing protocol used

Table 8 Optional, recommended when using the high throughput or amplification method for sample preparation.

Description	Company and part no.
Thermocycler with heated lid	Eppendorf p/n 950000015 or equivalent
96-well PCR plates	Eppendorf p/n 951020401 or equivalent
Centrifuge (for 96-well plate)	Eppendorf p/n 5810 or equivalent
Heat Sealer	Eppendorf p/n 951023078
Peel-it-lite Foil (removable)	Eppendorf p/n 951023205

Table 9 Optional, recommended when using high-throughput method on 2x microarrays.

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

Table 10 Optional equipment for DNA extraction from tissue or FFPE samples.

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

Required Reagents

Table 11 Required Reagents for gDNA Isolation

Description	Company and part no.
Phosphate Buffered Saline pH 7.4 (PBS)	Amresco p/n E504-500ML
Clear E-Gel 18-Pak (1.2% agarose, no stain)	Invitrogen p/n G5518-01
SYBR Gold Nucleic Acid Gel Stain	Invitrogen p/n S11494
TrackIt 1 Kb DNA Ladder	Invitrogen p/n 10488-072
Qiagen RNase A (100 mg/mL)	Qiagen p/n 19101
Qiagen DNeasy Blood & Tissue Kit	Qiagen p/n 69504
Qiagen Proteinase K (>600 mAU/mL, solution)	Qiagen p/n 19131
Ethanol (95% to 100% molecular biology grade)	Sigma p/n E7023-6x500ML

Table 12 Required Reagents for Enzymatic Sample Prep and Labeling

Description	Company and part no.
Agilent Genomic DNA Enzymatic Labeling Kit	Agilent p/n 5190-0449
DNase/RNase-free distilled water	Invitrogen p/n 10977-015
Microcon YM-30 filter units	Millipore p/n 42410
AutoScreen-96A Well plates*	GE Healthcare p/n 25-9005-98
For possible use as a reference sample: Human Genomic DNA *or* Mouse Genomic DNA *or* Rat Genomic DNA	 Promega p/n G1521 (female) or p/n G1471 (male) Jackson Labs p/n 000664 (female and male) Harlan Sprague Dawley (custom)
Alu I (10 U/µL)†	Promega p/n R6281
Rsa I (10 U/µL)†	Promega p/n R6371
1 × TE (pH 8.0), Molecular grade	Promega p/n V6231
GenElute PCR Clean-Up Kit‡	Sigma p/n NA1020
GenomePlex Complete Whole Genome Amplification Kit‡	Sigma p/n WGA2

* Optional component recommended if using the high-throughput protocol.

† Optional component recommended if doing a restriction digestion step.

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

Table 13 Required Reagents for Hybridization and Wash

Description	Company and part no.
Agilent Oligo aCGH Wash Buffer 1 and 2 set *or* Agilent Oligo aCGH Wash Buffer 1 (4 L) Agilent Oligo aCGH Wash Buffer 2 (4 L)	Agilent p/n 5188-5226 Agilent p/n 5188-5221 Agilent p/n 5188-5222
Stabilization and Drying Solution, 500 mL*	Agilent p/n 5185-5979
Agilent Oligo aCGH Hybridization Kit	Agilent p/n 5188-5220 (25) or p/n 5188-5380 (100)
Human Cot-1 DNA *or* Mouse Cot-1 DNA *or* Rat Hybloc	Invitrogen p/n 15279-011 or 15279-101 or Kreatech p/n EA-020 Invitrogen p/n 18440-016 Applied Genetics p/n RHB
Acetonitrile*	Sigma p/n 271004-1L

* Optional components recommended if wash procedure B is selected.

Required Hardware and Software

- Refer to the Agilent Scanner and Feature Extraction manuals for minimum memory requirements and other specifications. Go to http://www.agilent.com/chem.

2 DNA Isolation

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

Agilent's array-based Comparative Genomic Hybridization (aCGH) application uses a "two-color" process to measure DNA copy number changes in an experimental sample relative to a reference sample. The type of sample used as a reference is a matter of experimental choice; however, many experimenters use normal commercial gDNA as a reference sample.

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

For processing FFPE samples, follow the *Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis (ULS Labeling for Blood, Cells, Tissues or FFPE) Protocol v3.1* (p/n G4410-90020).

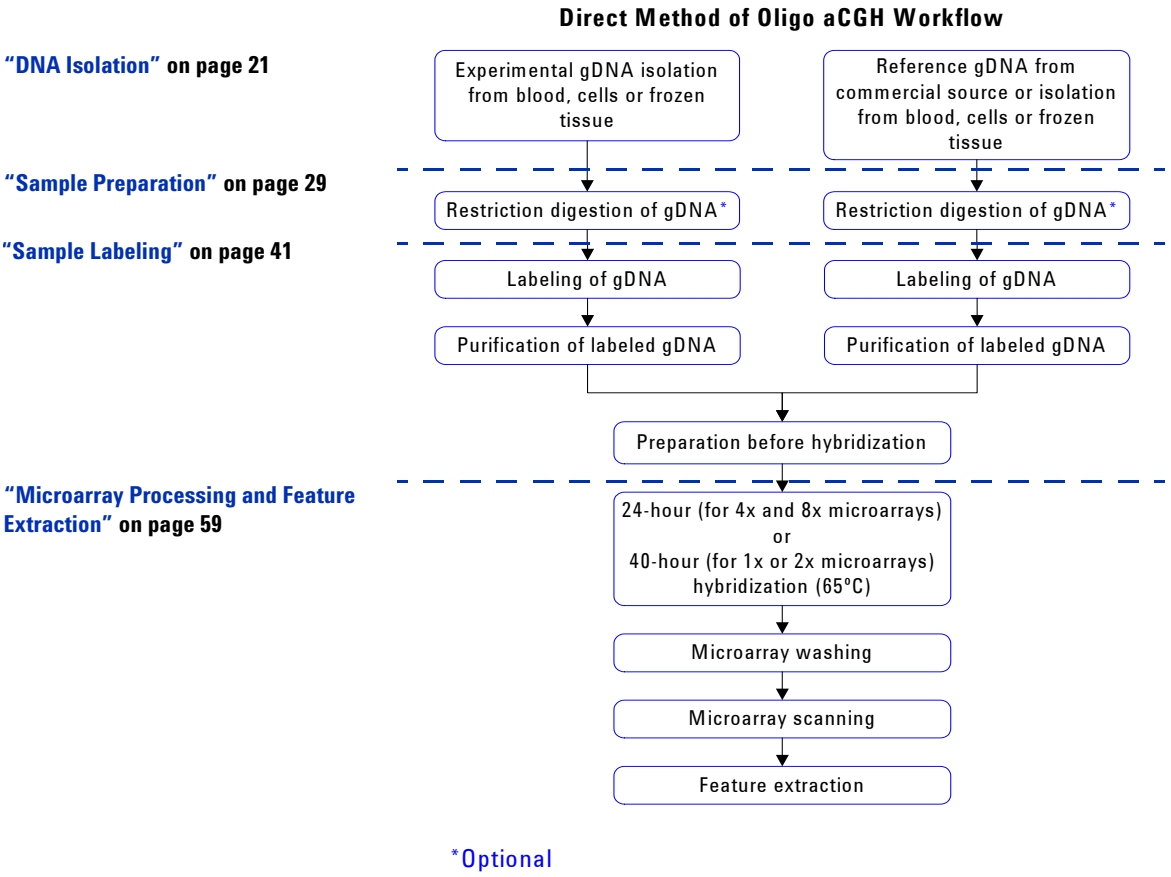

Figure 1 Direct workflow for sample preparation and microarray processing. Minimum of 0.5 µg (for 1x, 2x or 4x microarrays) or 0.2 µg (for 8x microarrays) starting gDNA per sample is required.

Step 1. gDNA Extraction

1. Equilibrate a thermomixer to 55°C and heat block or water bath to 70°C.
2. For blood with nonnucleated erythrocytes (mammals):
   a. Put 20 µL proteinase K (supplied with Qiagen DNeasy Blood & Tissue Kit) into the bottom of a 1.5 mL microfuge tube.
   b. Add 50 to 100 µL anticoagulated blood.
   c. Add enough PBS to make a total volume of 220 µL.
   d. Go to step 7.
3. For blood with nucleated erythrocytes (such as chicken):
   a. Put 20 µL proteinase K (supplied with Qiagen DNeasy Blood & Tissue Kit) into the bottom of a 1.5 mL microfuge tube.
   b. Add 5 to 10 µL anticoagulated blood.
   c. Add enough PBS to make a total volume of 220 µL.
   d. Go to step 7.
4. For cells:
   a. Spin a maximum of $5x10^6$ cells in a centrifuge for 5 minutes at 300 x g. Resuspend the pellet in 200 µL PBS.
   b. Add 20 µL proteinase K (supplied with Qiagen DNeasy Blood & Tissue Kit).
   c. Go to step 7.
5. For frozen tissue:
   a. Cut up to 25 mg frozen tissue (up to 10 mg for spleen tissue) into small pieces and put into a 1.5 mL microfuge tube.
   b. Add 180 µL Buffer ATL (supplied with Qiagen DNeasy Blood & Tissue Kit).
   c. Add 20 µL proteinase K (supplied).
   d. Mix well on a vortex mixer.
   e. Incubate in a thermomixer at 55°C shaking at 450 rpm until the tissue is completely lysed.

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

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

g Go to step 7.

6 For further purification of extracted DNA:

a Take a maximum 25 µg of DNA.

b Add enough PBS to make a total volume of 220 µL.

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

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

8 Add 200 µL Buffer AL (supplied) to each sample, mix thoroughly on a vortex mixer, and incubate at 70°C for 10 minutes in a heat block or water bath. Spin in a microcentrifuge for 30 seconds at 6,000 x g to drive the contents off the walls and lid.

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

10 Transfer the sample mixture onto a DNeasy Mini spin column in a 2 mL collection tube (supplied). Spin in a centrifuge at 6,000 x g for 1 minute. Discard the flow-through and collection tube. Put the DNeasy Mini spin column in a new 2 mL collection tube (supplied).

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

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

13 Before using for the first time, prepare Buffer AW2 by adding 100% ethanol to the Buffer AW2 bottle (supplied; see bottle label for volume).

Mark the appropriate check box to indicate that ethanol was added to the bottle.

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

15 Put the DNeasy Mini spin column in a clean 1.5 mL microcentrifuge tube, and pipette 200 µL of Buffer AE (supplied) directly onto the center of the DNeasy column membrane.

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

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

Step 2. gDNA Quantitation and Quality Analysis

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

See "FFPE Tissues" in the *Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis (ULS Labeling for Blood, Cells, Tissues or FFPE) Protocol v3.1* (p/n G4410-90020) for details on how to isolate gDNA from FFPE tissues.

Use the NanoDrop ND-1000 UV-VIS Spectrophotometer (or equivalent) to assess gDNA concentration and purity. Use the agarose gel electrophoresis to assess gDNA intactness and the average molecular weight for each sample.

UV-VIS Spectrophotometry

1. In the Nanodrop program menu, select **Nucleic Acid Measurement**, then select **Sample Type** to be **DNA-50**.
2. Use 1.5 µL of elution buffer to blank the instrument.
3. Use 1.5 µL of each gDNA sample to measure DNA concentration. Record the gDNA concentration (ng/µL) for each sample. Calculate the yield as

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

4. Record the $\mathbf{A_{260}/A_{280}}$ and $\mathbf{A_{260}/A_{230}}$ ratios. High-quality gDNA samples should have an $\mathbf{A_{260}/A_{280}}$ ratio of 1.8 to 2.0, indicating the absence of contaminating proteins, and an $\mathbf{A_{260}/A_{230}}$ ratio of >2.0, indicating the absence of other organic compounds such as guanidinium isothiocyanate, alcohol and phenol as well as cellular contaminants such as carbohydrates.

Agarose Gel Electrophoresis

1. Load 20 ng gDNA for each sample in a volume of 10 µL nuclease-free water in the well of a single-comb 1.2% Clear E-Gel. (No need to add loading buffer in this system).
2. As a control, load 20 ng of commercial Human Genomic DNA in a volume of 10 µL nuclease free water in one of the wells of the E-Gel.
3. Mix 5 µL TrackIt 1 Kb DNA Ladder with 95 µL deionized water and load 10 µL of the diluted ladder in one of the wells of the E-Gel.
4. Run the gel for 30 minutes as described in Invitrogen's instructions.
5. Open the gel cassette with E-Gel Opener as described in Invitrogen's instructions.
6. Stain the gel with SYBR Gold Nucleic Acid Gel Stain (diluted 1:10,000 by adding 10 µL of SYBR Gold Nucleic Acid Gel Stain to 100 mL of nuclease-free water) in a plastic tray for 15 minutes.
7. Visualize the gel on the UV-transilluminator using a SYBR Gold photographic filter.

3 Sample Preparation

This chapter describes Agilent's two recommended options to process genomic DNA (gDNA) prior to labeling.

You can choose between two methods for sample preparation prior to labeling: "Direct Method" on page 30 and "Amplification Method" on page 34. Figure 1 on page 22 and Figure 2 on page 35 show the respective workflows.

Direct Method

CAUTION

For optimal performance, use high quality, intact template genomic DNA. If the DNA isolation procedure described in this protocol cannot be followed, make sure that the DNA is free of RNA and protein contamination. If needed, repurify already isolated DNA and start from step 6 on page 24 in the previous chapter.

Make sure that the gDNA is completely in solution by pipetting up and down. If needed, incubate at 37°C for 30 minutes. If the gDNA concentration > 350 ng/µL, dilute 1:2 in water and requantitate to make sure quantitation is accurate.

Use the Direct Method if you have at least 0.5 µg (for 1x, 2x or 4x microarrays) or 0.2 µg (for 8x microarrays) of starting genomic DNA. You must use equal amounts of genomic DNA for both the experimental and reference channels. The required gDNA input amount depends on the microarray format used (see Table 14) and whether a restriction digestion is done before the labeling reaction.

NOTE

For a wide variety of samples, high quality micorarray data is achieved when a restriction digestion step is used before the labeling step. But you can also achieve high quality data if you replace the restriction digestion step by a longer incubation at 95°C after you add the random primers in the labeling reaction step. See "Step 1. Fluorescent Labeling of Genomic DNA" on page 42.

If you have 50 ng to <0.5 µg (for 1x, 2x or 4x microarrays) or <0.2 µg (for 8x microarrays) genomic DNA, see "Amplification Method" on page 34.

Table 14 Requirement of gDNA Input Amount and Volume per Microarray

Microarray format	gDNA input amount requirement (µg)	Volume of gDNA with restriction digestion (µL)	Volume of gDNA without restriction digestion (µL)
1x microarray	0.5 to 3.0	20.2	26
2x microarray or 4x microarray	0.5 to 1.5	20.2	26
8x microarray	0.2 to 0.5	10.1	13

Restriction Digestion

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

1. Equilibrate heat blocks or water baths to 37°C and 65°C or use a thermocycler.
2. Thaw 10X Buffer C and Acetylated BSA (supplied with Rsa I). Briefly mix on a vortex mixer and spin in a microcentrifuge.

   Store all reagents on ice while in use and return promptly to -20°C.
3. For each reaction, add the amount of genomic DNA to the appropriate nuclease-free tube or well in the PCR plate and add enough nuclease-free water to bring to the final volume listed in Table 14 on page 30.
4. Prepare the Digestion Master Mix by mixing the components in Table 15 or Table 16, based on the microarray format used, on ice in the order indicated.

Table 15 Preparation of Digestion Master Mix (for 1x, 2x and 4x microarrays)

Component	Per reaction (µL)	× 16 rxns (µL) (including excess)	× 48 rxns (µL) (including excess)	x 96 rxns (µL) (including excess)
Nuclease-free water	2.0	34	100	200
10X Buffer C*	2.6	44.2	130	260
Acetylated BSA (10 µg/µL)*	0.2	3.4	10	20
Alu I (10 U/µL)	0.5	8.5	25	50
Rsa I (10 U/µL)	0.5	8.5	25	50
Final volume of Digestion Master Mix	**5.8**	**98.6**	**290**	**580**

* Supplied with the restriction enzyme Rsa I.

Table 16 Preparation of Digestion Master Mix (for 8x microarrays)

Component	Per reaction (µL)	× 16 rxns (µL) (including excess)	× 48 rxns (µL) (including excess)	x 96 rxns (µL) (including excess)
Nuclease-free water	1	17	50	100
10X Buffer C*	1.3	22.1	65	130
Acetylated BSA (10 µg/µL)*	0.1	1.7	5	10
Alu I (10 U/µL)	0.25	4.25	12.5	25
Rsa I (10 U/µL)	0.25	4.25	12.5	25
Final volume of Digestion Master Mix	**2.9**	**49.3**	**145**	**290**

* Supplied with the restriction enzyme Rsa I.

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

6 Incubate the samples:

a Transfer sample tubes to a circulating water bath or heat block at 37°C. Incubate at 37°C for 2 hours.

b Transfer sample tubes to a circulating water bath or heat block at 65°C. Incubate at 65°C for 20 minutes to inactivate the enzymes.

c Move the sample tubes to ice.

or

Transfer sample tubes or plates to a thermocycler. Program the thermocycler according to the following table and run the program:

Table 17

Step	Temperature	Time
Step I	37 °C	2 hours
Step II	65 °C	20 minutes
Step III	4° C	hold

7 *Optional.* Take 2 µL of the digested genomic DNA and run on a 0.8% agarose gel stained with SYBR Gold (Invitrogen p/n S-11494) to assess the completeness of the digestion. The majority of the digested products should be between 200 bp and 500 bp in length.

8 Proceed directly to "Sample Labeling" on page 41, or store digested genomic DNA for up to a month at -20°C.

Amplification Method

CAUTION

For optimal performance, use high quality, intact template genomic DNA. If the DNA isolation procedure described in this protocol cannot be followed, make sure that the DNA is free of RNA and protein contamination. If needed, repurify already isolated DNA and start from step 6 on page 24 in the previous chapter.

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

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

Use the Amplification Method if you have limited amounts of genomic DNA. If you have 0.5 µg (for 1x, 2x or 4x microarrays) or 0.2 µg (for 8x microarrays) or more genomic DNA, see "Direct Method" on page 30.

Reference

GenomePlex Whole Genome Amplification (WGA) Kit. Product Code WGA2. Technical Bulletin. Sigma-Aldrich. 2006. TR/PHC 06/05-1

Genomic Amplification

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

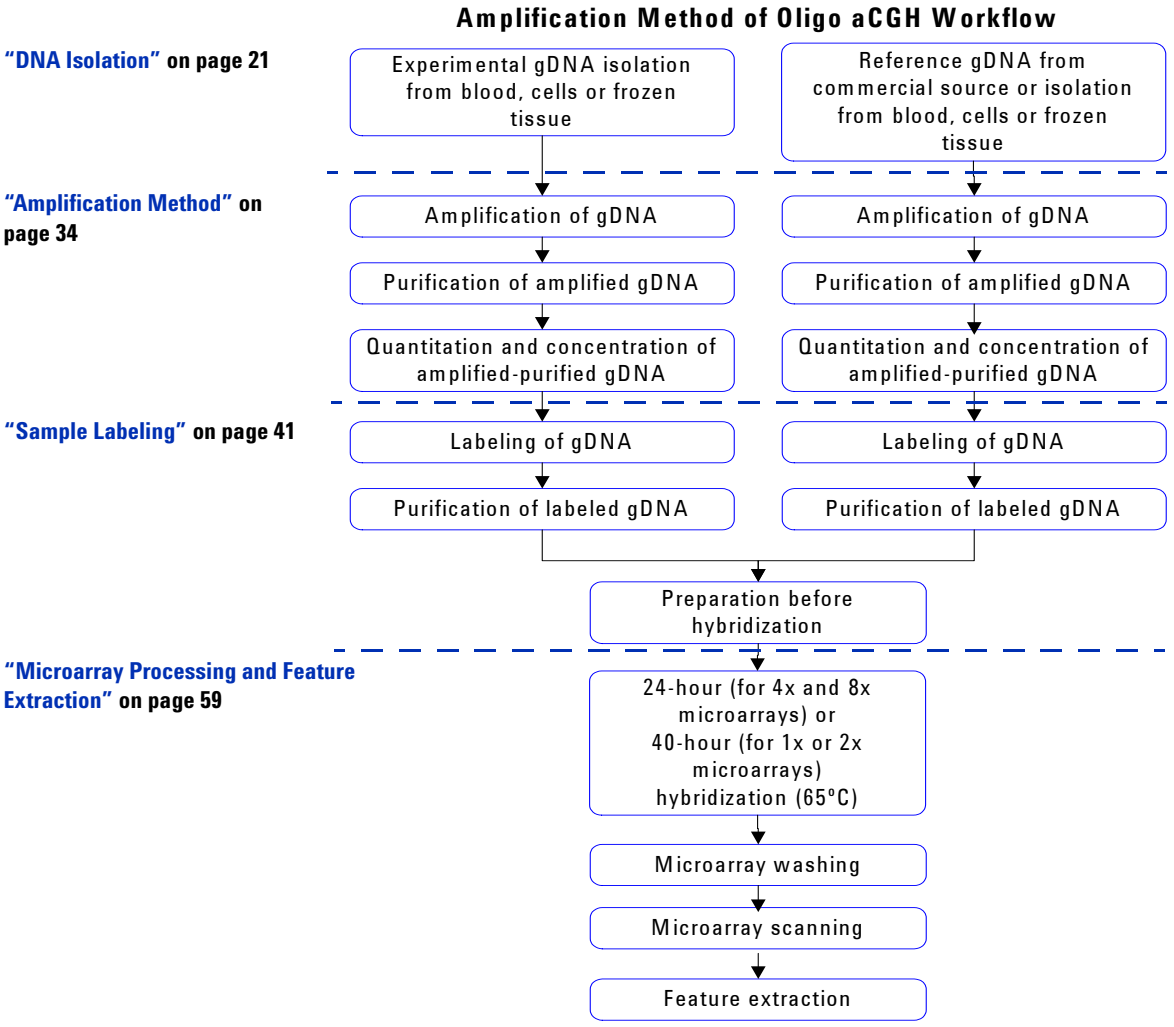

Figure 2 Amplification workflow for sample preparation and microarray processing. Minimum of 50 ng starting gDNA per sample is required.

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

Step 1. Fragmentation

1 Add 50 ng genomic DNA to a 0.2 mL nuclease-free PCR tube or plate. Add nuclease-free water to bring to a final volume of 10 µL.

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

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

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

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

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

Step 2. Library Preparation

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

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

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

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

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

6 Place sample in a thermocycler and incubate as shown in Table 18.

Table 18 Library Preparation Isothermal Reaction using thermocycler (total time approximately 1 hour)

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

7 Remove samples from the thermocycler and spin briefly in a centrifuge to drive the contents off the walls and lid. Samples may be amplified immediately or stored at -20°C for up to three days.

Step 3. Amplification

1 Prepare the Amplification Master Mix by mixing the components in Table 19 on ice.

Table 19 Preparation of Amplification Master Mix

	Volume (µL)	x16 rxns (µL) including excess	x48 rxns (µL) including excess	x96 rxns (µL) including excess
10X Amplification Master Mix	7.5	127.5	375	750
Nuclease-free water	47.5	807.5	2,375	4,750
WGA DNA Polymerase	5	85	250	500
Final volume of Amplification Master Mix	**60**	**1,020**	**3,000**	**6,000**

2 Add 60 µL of Amplification Master Mix to each 15 µL reaction from the previous step to make a total volume of 75 µL.

3 Mix thoroughly, spin briefly in a centrifuge to drive the contents of the walls and lid, and place the samples in a thermocycler with heated lid. Run the program below:

Table 20 PCR Amplification (total time approximately 2 hours)

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

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

Step 4. Purification of PCR products

Use Sigma's GenElute PCR Clean-Up Kit for the purification of amplified gDNA.

1. Before using for the first time, dilute the Wash Solution Concentrate with 48 ml of 100% ethanol.
2. Insert a GenElute Miniprep Binding Column (with a blue O-ring) into a provided collection tube, if not already assembled. Add 0.5 mL of the Column Preparation Solution to each miniprep column and spin in a centrifuge at 12,000 g for 30 seconds to 1 minute. Discard the eluate, but keep the collection tube.

   The Column Preparation Solution maximizes binding of the DNA to the membrane resulting in more consistent yields.
3. Add 375 µL of Binding Solution to each 75 µL sample. Transfer the solution into the binding column. Spin the column in a centrifuge at maximum speed (12,000 to 16,000 x g) for 1 minute. Discard the eluate, but keep the collection tube.
4. Place the binding column into the same collection tube. Apply 0.5 mL of diluted Wash Solution to the column and spin in a centrifuge at maximum speed for 1 minute. Discard the eluate, but retain the collection tube.
5. Place the column into the same collection tube. Spin the column in a centrifuge at maximum speed for 2 minutes, without any additional wash solution, to remove excess ethanol. Discard any residual eluate as well as the collection tube.
6. Transfer the column to a fresh 2 mL collection tube. Apply 50 µL of Elution Solution (10 mM Tris, pH 8.0) to the center of each column. Incubate at room temperature for 1 minute.
7. To elute the DNA, spin the column in a centrifuge at maximum speed for 1 minute.

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

Step 5. Quantitation of Amplified-Purified gDNA

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

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

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

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

4 Calculate the amplification yield (µg) as

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

Step 6. Preparation of Amplified-Purified gDNA before Labeling

1 Add 2 µg of amplified-purified gDNA to a 1.5 mL nuclease-free tube or well in the PCR plate and bring to a final volume of 26 µL (1x, 2x, 4x microarrays) or 13 µL (8x microarrays) with nuclease-free water.

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

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

You can concentrate the gDNA to dryness and resuspend in water. Do not excessively dry the gDNA because the pellets will become difficult to resuspend.

Proceed directly to "Sample Labeling" on page 41 or store amplified-purified DNA at -20°C.

4 Sample Labeling

The Agilent Genomic DNA Enzymatic Labeling Kit (Agilent p/n 5190-0449) uses random primers and the exo-Klenow fragment to differentially label genomic DNA samples with fluorescent-labeled nucleotides. For Agilent's Oligo aCGH application, the experimental sample is labeled with one dye while the reference sample is labeled with the other dye. The “polarity” of the sample labeling is a matter of experimental choice.

Step 1. Fluorescent Labeling of Genomic DNA

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.

1 Equilibrate heat blocks or water baths to 95°C, 37°C and 65°C, or use a thermocycler.

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

3 Add Random Primers (supplied with the Agilent Genomic DNA Enzymatic Labeling Kit):

- For 1x, 2x and 4x microarrays, add 5 µL of Random Primers to each reaction tube containing 26 µL of gDNA to make a total volume of 31 µL (or 24 µL of gDNA to make a total volume of 29 µL if the optional agarose gel step at step 7 on page 33 was done). Mix well by pipetting up and down gently.
- For 8x microarrays, add 2.5 µL of Random Primers to each PCR tube that contains 13 µL of gDNA to make a total volume of 15.5 µL (or 11 µL of gDNA to make a total volume of 13.5 µL if the optional agarose gel step on page 33 was done). Mix well by pipetting up and down gently.

4 Transfer sample tubes to a circulating water bath or heat block at 95°C. Incubate at 95°C for 3 minutes (with restriction digestion) or 10 minutes (without restriction digestion), then move to ice and incubate on ice for 5 minutes.

or

Transfer sample tubes to a thermocycler. Program the thermocycler according to Table 21 and run the program.

Table 21 DNA denaturation and fragmentation using a thermocycler

Step	Temperature	Time (with restriction digestion)	Time (without restriction digestion)
Step 1	95°C	3 minutes	10 minutes
Step 2	4°C	hold	hold

NOTE

For a wide variety of samples, high quality micorarray data is achieved when a restriction digestion step is used before the labeling step. But you can also achieve high quality data if you replace the restriction digestion step by a longer incubation at 95°C after you add the random primers in the labeling reaction step. See "Step 1. Fluorescent Labeling of Genomic DNA" on page 42.

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

6 For 1x, 2x and 4x microarrays:

a Prepare one Cy3 and one Cy5 Labeling Master Mix by mixing the components in Table 22 on ice in the order indicated.

Table 22 Preparation of Labeling Master Mix (for 1x, 2x and 4x microarrays)

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

* Do *not* add nuclease-free water if you skipped the optional agarose gel step (step 7 on page 33).

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

7 For 8x microarrays:

a Prepare one Cy3 and one Cy5 Labeling Master Mix by mixing the components in Table 23 on ice in the order indicated.

Table 23 Preparation of Labeling Master Mix (for 8x microarrays)

Component	Per reaction (µL)	x 8 rxns (µL) (including excess)	x 24 rxns (µL) (including excess)	x 48 rxns (µL) (including excess)
Nuclease-free water*	2.0*	17*	50*	100*
5X Buffer	5.0	42.5	125	250
10X dNTP	2.5	21.25	62.5	125
Cyanine 3-dUTP (1.0 mM) or Cyanine 5-dUTP (1.0 mM)	1.5	12.75	37.5	75
Exo-Klenow fragment	0.5	4.25	12.5	25
Final volume of Labeling Master Mix	**9.5 or 11.5***	**80.75 or 97.75***	**237.5 or 287.5***	**475 or 575***

* Do *not* add nuclease-free water if you skipped the optional agarose gel step (step 7 on page 33).

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

8 Incubate the samples:

a Transfer sample tubes to a circulating water bath or heat block at 37°C. Incubate at 37°C for 2 hours.

b Transfer sample tubes to a circulating water bath or heat block at 65°C. Incubate at 65°C for 10 minutes to inactivate the enzyme.

c Move the sample tubes to ice.

or

Transfer sample tubes to a thermocycler. Program the thermocycler according to Table 24 and run the program.

Table 24 DNA labeling using a thermocycler

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

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

Step 2. Clean-up of Labeled Genomic DNA

Labeled genomic DNA can be purified using individual Microcon YM-30 filters or GE Healthcare 96-well plates.

NOTE Keep Cyanine-3 and Cyanine-5 labeled genomic DNA samples separated throughout this clean-up step.

Microcon YM-30 filters *without* vacuum concentration

1. Spin the labeled genomic DNA samples in a centrifuge for 1 minute at 6,000 x g to drive the contents off the walls and lid.
2. Add 430 µL of 1X TE (pH 8.0) to each reaction tube.
3. Place a Microcon YM-30 filter into a 1.5-mL microfuge tube (supplied) and load each labeled gDNA into the filter. Spin 10 minutes at 8,000 × g in a microcentrifuge at room temperature. Discard the flow-through.
4. Add 480 µL of 1X TE (pH 8.0) to each filter. Spin for 10 minutes at 8,000 × g in a microcentrifuge at room temperature. Discard the flow-through.
5. Invert the filter into a fresh 1.5-mL microfuge tube (supplied). Spin for 1 minute at 8,000 × g in a microcentrifuge at room temperature to collect purified sample.
6. Measure and record volume (µL) of each eluate. If sample volume exceeds the Sample Volume listed in Table 25, return sample to its filter and spin 1 minute at 8,000 × g in a microcentrifuge at room temperature. Discard the flow-through. Repeat step 5.
7. Repeat step 6 until each sample volume is less than or equal to the Sample Volume listed in Table 25.
8. Bring total sample volume to the Sample Volume listed in Table 25 with 1X TE (pH 8.0).
9. Take 1.5 µL of each sample to determine yield and specific activity. See "To determine yield , degree of labeling or specific activity" on page 53. Refer to Table 27 on page 54 for expected yield of labeled genomic DNA and specific activity after labeling and clean-up, when starting with high quality genomic DNA.

10 Combine test and reference sample using the appropriate cyanine 5-labeled sample and cyanine 3-labeled sample for a Total Mixture Volume listed in Table 25. Use the appropriate container listed in Table 25.

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

Microcon YM-30 filters with vacuum concentration (such as SpeedVac)

1. Spin the labeled genomic DNA samples in a centrifuge for 1 minute at 6,000 x g to drive the contents off the walls and lid.
2. Add 430 µL of 1X TE (pH 8.0) to each reaction tube.
3. Place a Microcon YM-30 filter into a 1.5-mL microfuge tube (supplied) and load each labeled gDNA into the filter. Spin 10 minutes at 8,000 × g in a microcentrifuge at room temperature. Discard the flow-through.
4. Add 480 µL of 1X TE (pH 8.0) to each filter. Spin for 10 minutes at 8,000 × g in a microcentrifuge at room temperature. Discard the flow-through.
5. Invert the filter into a fresh 1.5-mL microfuge tube (supplied). Spin for 1 minute at 8,000 x g in a microcentrifuge at room temperature to collect purified sample.
6. Place sample tubes in a concentrator with tops open and evaporate to dryness. This may take 20 to 40 minutes depending on sample volume.

   Do not excessively dry the gDNA because the pellets will become difficult to resuspend.
7. Reconstitute each sample with water to a final volume equal to the Sample Volume listed in Table 25.
8. Take 1.5 µL of each sample to determine yield and specific activity. See "To determine yield , degree of labeling or specific activity" on page 53. Refer to Table 27 on page 54 for expected yield of labeled genomic DNA and specific activity after labeling and clean-up, when starting with high quality genomic DNA.
9. Combine test and reference sample using the appropriate cyanine 5-labeled sample and cyanine 3-labeled sample for a Total Mixture Volume listed in Table 25 on page 49. Use the appropriate container as listed in Table 25.

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

Table 25 Sample volume and total mixture volumes

Microarray	Cy3 or Cy5 sample volume after purification	Total mixture volume after Nanodrop and combining	Container
1x	80.5 µL	158 µL	microfuge tube
2x	41 µL	79 µL	microfuge tube or tall chimney plate
4x	21 µL	39 µL	microfuge tube, tall chimney plate, or PCR plate
8x	9.5 µL	16 µL	microfuge tube, tall chimney plate, or PCR plate

GE Healthcare 96-well plates

NOTE

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

For 1x, 2x, 4x you can use two wells of the AutoScreen-96A Well plates (GE Healthcare p/n 25-9005-98) per sample or concentrate the samples down to 25 µL with a vacuum concentrator (such as a Speed Vac). You need to supply two 96-well PCR plates (Eppendorf p/n 951020401 or equivalent). Label one plate "wash plate" and the other plate "collection plate". The wash plate can be re-used in next experiments.

1 Remove the purification plates from the foil storage pouch.

   If the purification plates were stored at 4°C, allow them to equilibrate to ambient temperature before use (approximately 2 hours).

2 Carefully remove the top and bottom seal of the purification plates.

   Once the bottom seal is removed, keep the purification plates on top of a wash plate. Do not allow the bottom surface to come in contact with laboratory benchtop liners, wipes, or other materials.

3 Place the purification plates in re-usable wash plates.

4 Pre-spin the purification plates in a centrifuge for 5 minutes at 910 x g.

5 Discard the flow-through from the wash plates, and place the purification plates back to the same wash plates.

6 Add the 150 µL nuclease free water to the purification plates.

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

8 Discard the flow-through.

9 Transfer the purification plates to the sample collection plate.

10 Add labeled genomic DNA to the purification plates:

   - For 1x, 2x, 4x microarray samples that were not concentrated to 25 µL, add 2x25 µL labeled genomic DNA to two separate wells.
   - For 1x, 2x, 4x microarray samples that were concentrated to 25 µL with concentrator and for 8x microarray samples, add 1x25 µL labeled genomic DNA to one well.

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

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

13 Take 1.5 µL of each sample to determine the yield and specific activity. See "To determine yield , degree of labeling or specific activity" on page 53.

Refer to Table 27 on page 54 for expected yield of labeled genomic DNA and specific activity after labeling and clean-up, when starting with high quality genomic DNA.

14 Combine the test and reference sample using the appropriate cyanine 5-labeled sample and cyanine 3-labeled sample. Use the appropriate container listed in Table 26. Add 1X TE or use a concentrator to bring to the Total Mixture Volume in Table 26.

If needed, you can concentrate the combined Cy5- and Cy3-labeled gDNA mixture to dryness and resuspend in water to the final volume listed in Table 26. Do not excessively dry the samples because the pellets will become difficult to resuspend.

Table 26 Total mixture volumes

Micro array	Treatment prior to purification	Cy3 or Cy5 sample volume after purification	Volume after Nanodrop and combining	1X TE volume	Total mixture volume	Container
1x	without vacuum concentration*	40 µL	77 µL	81 µL	158 µL	microfuge tube
	with vacuum concentration	20 µL	37 µL	121 µL	158 µL	microfuge tube
2x	without vacuum concentration*	40 µL	77 µL	2 µL	79 µL	microfuge tube or tall chimney plate
	with vacuum concentration	20 µL	37 µL	42 µL	79 µL	microfuge tube or tall chimney plate
4x	without vacuum concentration*	40 µL	77 µL	0 µL†	39 µL	microfuge tube, tall chimney plate or PCR plate
	with vacuum concentration	20 µL	37 µL	2 µL	39 µL	microfuge tube, tall chimney plate or PCR plate
8x	without vacuum concentration	20 µL	37 µL	0 µL†	16 µL	microfuge tube, tall chimney plate or PCR plate

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

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

To determine yield , degree of labeling or specific activity

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

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

2 Use 1.5 µL of TE to blank the instrument.

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

4 Calculate the Degree or Labeling or Specific Activity of the labeled genomic DNA:

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

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

*pmol dyes per µg genomic DNA

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

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

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

Refer to Table 27 for expected yield of labeled genomic DNA and specific activity after labeling and clean-up, when starting with high quality genomic DNA.

Table 27 Expected Yield and Specific Activity after Labeling and Clean-up

Input gDNA (µg)	Yield (µg)	Specific Activity of Cyanine-3 Labeled Sample (pmol/µg)	Specific Activity of Cyanine-5 Labeled Sample (pmol/µg)
0.2*	2.5 to 3	15 to 25	15 to 20
0.5	5 to 7	25 to 40	20 to 35
3.0	7 to 10	35 to 55	25 to 40

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

The Cy3 and Cy5 yield after labeling should be the same. If not, refer to "Troubleshooting" on page 83.

Step 3. Preparation of Labeled Genomic DNA for Hybridization

1 Prepare the **10X Blocking Agent**:

a Add 1,350 µL of nuclease-free water to the vial containing lyophilized 10X Blocking Agent (supplied with Agilent Oligo aCGH Hybridization Kit).

b Leave at room temperature for 60 minutes and mix on a vortex mixer to reconstitute sample before use or storage.

NOTE The 10X Blocking Agent can be prepared in advance and stored at -20°C.

2 Prepare the samples for hybridization:

a Equilibrate water baths or heat blocks to 95°C and 37°C or use a thermocycler.

b Mix the components according to the microarray format in Table 28 through Table 31 below to prepare the Hybridization Master Mix.

Table 28 Preparation of Hybridization Master Mix for 1x microarray

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

* Use Cot-1 DNA from the appropriate species.

† Supplied with Agilent Oligo aCGH Hybridization Kit

Table 29 Preparation of Hybridization Master Mix for 2x microarray

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

* Use Cot-1 DNA from the appropriate species.

† Supplied with Agilent Oligo aCGH Hybridization Kit

Table 30 Preparation of Hybridization Master Mix for 4x microarray

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

* Use Cot-1 DNA from the appropriate species.

† Supplied with Agilent Oligo aCGH Hybridization Kit

Table 31 Preparation of Hybridization Master Mix for 8x microarray

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

* Use Cot-1 DNA from the appropriate species.

† Supplied with Agilent Oligo aCGH Hybridization Kit

c Add the appropriate volume of the Hybridization Master Mix to the 1.5 mL microfuge tube, tall chimney plate well or PCR plate well containing the labeled gDNA to make the total volume listed in Table 32.

Table 32 Volume of Hybridization Master Mix per hybridization

Microarray format	Volume of Hybridization Master Mix	Total volume
1x microarray	362 µL	520 µL
2x microarray	181 µL	260 µL
4x microarray	71 µL	110 µL
8x microarray	29 µL	45 µL

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

e Transfer sample tubes to a circulating water bath or heat block at 95°C. Incubate at 95°C for 3 minutes, then immediately transfer sample tubes to a circulating water bath or heat block at 37°C. Incubate at 37°C for 30 minutes.

or

Transfer sample tubes to a thermocycler. Program the thermocycler according to the following table and run the program:

Table 33

Step	Temperature	Time
Step I	95 °C	3 minutes
Step II	37 °C	30 minutes

f Remove sample tubes from the water bath, heat block or thermocycler. Spin 1 minute at 6000 × g in a centrifuge to collect the sample at the bottom of the tube.

The samples are ready to be hybridized.

5 Microarray Processing and Feature Extraction

Microarray processing consists of hybridization, washing, and scanning.

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

Step 1. Microarray Hybridization

NOTE

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

Microarray Handling Tips

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

The hybridization sample mixture is applied directly to the gasket slide and not to the microarray slide. Then the active side of the microarray slide is put on top of the gasket slide to form a "sandwich slide pair".

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

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

Hybridization Assembly

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

CAUTION

Keep the temperature of hybridization sample mixtures as close to 37°C as possible. To do this, process them in small batches and/or put them on a heat block, thermocycler or in an oven.

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

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

5 Hand-tighten the clamp onto the chamber.

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

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

8 Hybridize at 65°C for 40 hours (for 1x or 2x microarrays) or for 24 hours (for 4x or 8x microarrays).

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

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

Step 2. Wash Preparation

NOTE

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

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

Before you begin, determine which wash procedure to use:

Table 34 Wash procedure to follow

Ozone level in your lab	Wash Procedure	Ozone-Barrier Slide Cover
< 5 ppb	"Wash Procedure A (without Stabilization and Drying Solution)" on page 66	No
> 5 ppb < 10 ppb	"Wash Procedure A (without Stabilization and Drying Solution)" on page 66	Yes
> 10 ppb	"Wash Procedure B (with Stabilization and Drying Solution)" on page 69	Yes

Equipment Preparation

CAUTION

Do not use detergent to wash the staining dishes as some detergents may leave fluorescent residue on the dishes. If you do, you must ensure that all traces are removed by thoroughly rinsing with Milli-Q water.

- Always use clean equipment when conducting the wash procedures.

- Use only dishes that are designated and dedicated for use in Agilent oligo aCGH experiments.

Cleaning with Milli-Q Water Wash

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

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

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

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

Cleaning with Acetonitrile Wash (Wash Procedure B Only)

Acetonitrile wash removes any remaining residue of Agilent Stabilization and Drying Solution from slide-staining dishes, slide racks and stir bars that were used in previous experiments with "Wash Procedure B (with Stabilization and Drying Solution)" on page 69.

WARNING **Do acetonitrile washes in a vented fume hood. Acetonitrile is highly flammable and toxic.**

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

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

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

d Wash for 5 minutes at room temperature.

e Discard the acetonitrile as is appropriate for your site.

f Repeat step a through step e.

g Air dry everything in the vented fume hood.

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

Prewarming Oligo aCGH Wash Buffer 2 (Overnight)

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

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

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

Prewarming Stabilization and Drying Solution (Wash Procedure B Only)

The Agilent Stabilization and Drying Solution contains an ozone scavenging compound dissolved in acetonitrile. The compound in solution is present in saturating amounts and may precipitate from the solution under normal storage conditions. If the solution shows visible precipitation, warming of the solution will be necessary to redissolve the compound. Washing slides using Stabilization and Drying Solution showing visible precipitation will have profound adverse affects on array performance.

WARNING **The Agilent Stabilization and Drying Solution is a flammable liquid. Warming the solution will increase the generation of ignitable vapors. Use gloves and eye/face protection in every step of the warming procedures.**

WARNING **Do not use a hot plate, oven, an open flame or a microwave. Do not increase temperature rapidly. Warm and mix the material away from ignition sources.**

WARNING **Failure to follow the outlined process will increase the potential for fire, explosion, and possible personal injury.**

1 Put a clean magnetic stir bar into the Stabilization and Drying Solution bottle and recap.

2 Partially fill a plastic bucket with hot water at approximately 40°C to 45°C (for example from a hot water tap).

3 Put the Stabilization and Drying Solution bottle into the hot water in the plastic bucket.

4 Put the plastic bucket on a magnetic stirrer (*not a hot-plate*) and stir.

5 The hot water cools to room temperature. If the precipitate has not all dissolved replenish the cold water with hot water.

6 Repeat step 5 until the solution is clear.

7 After the precipitate is completely dissolved, allow the solution to equilibrate to room temperature prior to use.

CAUTION Do not filter the Stabilization and Drying solution, or the concentration of the ozone scavenger may vary.

Step 3. Microarray Washing

Before you begin, determine which wash procedure to use:

Table 35 Wash procedure to follow

Ozone level in your lab	Wash Procedure	Ozone-Barrier Slide Cover
< 5 ppb	"Wash Procedure A (without Stabilization and Drying Solution)" on page 66	No
> 5 ppb < 10 ppb	"Wash Procedure A (without Stabilization and Drying Solution)" on page 66	Yes
> 10 ppb	"Wash Procedure B (with Stabilization and Drying Solution)" on page 69	Yes

Wash Procedure A (without Stabilization and Drying Solution)

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

Table 36 lists the wash conditions for the Wash Procedure A without Stabilization and Drying Solution.

Table 36 Wash conditions

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

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

3 Put the prewarmed 1.5 L glass dish filled with water and containing slide-staining dish #3 on a magnetic stir plate with heating element. Fill the slide-staining dish #3 approximately three-fourths full with Oligo aCGH Wash Buffer 2 (warmed to 37°C). Add a magnetic stir bar. Turn on the heating element and maintain temperature of Oligo aCGH Wash Buffer 2 at 37°C; monitor using a thermometer.

4 Remove one hybridization chamber from incubator and record time. Record whether bubbles formed during hybridization and if all bubbles are rotating freely.

5 Prepare the hybridization chamber disassembly.

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

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

   c With gloved fingers, remove the array-gasket sandwich from the chamber base by grabbing the slides from their ends. Keep the microarray slide numeric barcode facing up as you quickly transfer the sandwich to slide-staining dish #1.

   d Without letting go of the slides, submerge the array-gasket sandwich into slide-staining dish #1 containing Oligo aCGH Wash Buffer 1.

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

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

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

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

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

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

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

13 Put the slides in a slide holder:

- In environments in which the ozone level exceeds 5 ppb, immediately put the slides with Agilent barcode facing up in a slide holder with an ozone-barrier slide cover on top of the array as shown in Figure 3.

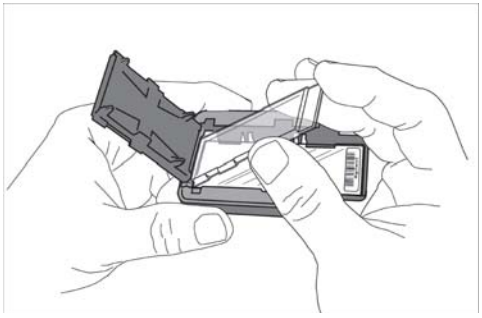

Figure 3 Inserting the ozone-barrier slide cover

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

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

Wash Procedure B (with Stabilization and Drying Solution)

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

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

The acetonitrile (dish #4) and Stabilization and Drying Solution (dish #5) below may be reused for washing up to 4 batches of 5 slides (total 20 slides) in one experiment. Do not pour the Stabilization and Drying Solution back in the bottle.

WARNING **The Stabilization and Drying Solution must be set-up in a fume hood. Put the Wash Buffer 1 and Wash Buffer 2 set-up areas close to, or preferably in, the same fume hood. Use gloves and eye/face protection in every step of the washing procedure.**

Table 37 lists the wash conditions for the Wash Procedure B with Stabilization and Drying Solution.

Table 37 Wash conditions

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

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

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

3 Put the prewarmed 1.5 L glass dish filled with water and containing slide-staining dish #3 on a magnetic stir plate with heating element. Fill the slide-staining dish #3 approximately three-fourths full with Oligo aCGH Wash Buffer 2 (warmed to 37°C). Add a magnetic stir bar. Turn on the heating element and maintain temperature of Oligo aCGH Wash Buffer 2 at 37°C; monitor using a thermometer.

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

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

6 Remove one hybridization chamber from incubator and record time. Record whether bubbles formed during hybridization, and if all bubbles are rotating freely.

7 Prepare the hybridization chamber disassembly.

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

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

   c With gloved fingers, remove the array-gasket sandwich from the chamber base by grabbing the slides from their ends. Keep the microarray slide numeric barcode facing up as you quickly transfer the sandwich to slide-staining dish #1.

   d Without letting go of the slides, submerge the array-gasket sandwich into slide-staining dish #1 containing Oligo aCGH Wash Buffer 1.

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

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

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

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

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

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

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

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

NOTE

The acetonitrile and the Stabilization and Drying Solution may be reused for washing of up to four batches of five slides (that is, total 20 microarray slides) in one experiment. Do not pour the Stabilization and Drying Solution back in the bottle. After each use, rinse the slide rack and the slide-staining dish that were in contact with the Stabilization and Drying Solution with acetonitrile followed by a rinse in Milli-Q water.

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

17 Immediately put the slides with Agilent barcode facing up in a slide holder with an ozone-barrier slide cover on top of the array as shown in Figure 3 on page 68.

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

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

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

Agilent C Scanner Settings

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

1. Put assembled slide holders with or without the ozone-barrier slide cover into scanner carousel.
2. Select Start Slot *m* End Slot *n* where the letter *m* represents the Start slot where the first slide is located and the letter *n* represents the End slot where the last slide is located.
3. Select **Profile Agilent G3_CGH** for 1x1M, 2x400K, 4x180K and 8x60K microarrays. Select **Profile Agilent HD_CGH** for 1x244K, 2x105K, 4x44K and 8x15K microarrays.
4. Verify that
   - **Slide ID** is set to **<Auto Detect>.**
   - **Channels** is set to **R+G**
   - **Scan region** is set to **Agilent HD (61 × 21.6 mm)**.
   - **Resolution (µm)** is set to **3 µm** for 1x1M, 2x400K, 4x180K and 8x60K microarrays, and **5 µm** for 1x244K, 2x105K, 4x44K and 8x15K microarrays.
   - **Tiff** is set to **16 bit**
   - **R PMT** is set to **100%**.
   - **G PMT** is set to **100%**.
   - **XDR** is set to **<No XDR>**.
   - **Output Path Browse** is set for desired location.
5. Verify that the Scanner status in the main window says Scanner Ready.
6. Click **Scan Slot *m*-*n*** on the Scan Control main window where the letter *m* represents the Start slot where the first slide is located and the letter *n* represents the End slot where the last slide is located.

Agilent A and B Scanner Settings

Agilent Scanner Control software v7.0 is recommended for 5 µm scans of 1x, 2x, 4x and 8x density microarrays.

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

2 Put assembled slide holders into scanner carousel.

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

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

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

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

7 Click **Scan Slot *m*-*n*** on the Scan Control main window where the letter *m* represents the Start slot where the first slide is located and the letter *n* represents the End slot where the last slide is located.

GenePix Scanner Settings

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

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

- Refer to the manufacturer's user guide for appropriate scanner settings.
- Refer to "Agilent Microarray Layout and Orientation" on page 91 for appropriate slide layout and orientation in GenePix scanner.

Step 5. Data Extraction using Feature Extraction Software

The Feature Extraction (FE) software v10.5 or higher supports extraction of microarray TIFF images (.tif) of Agilent CGH microarrays scanned on the Agilent C Scanner but does *not* support extraction of Agilent CGH microarrays on the GenePix 4000B scanner. The Feature Extraction (FE) software v9.5 supports extraction of microarray TIFF images (.tif) of Agilent CGH microarrays scanned on the Agilent Scanner B and GenePix 4000B Scanner.

Figure 4 shows an example of Agilent 1M CGH microarray image opened in Feature Extraction software v10.5 in both full and zoomed view.

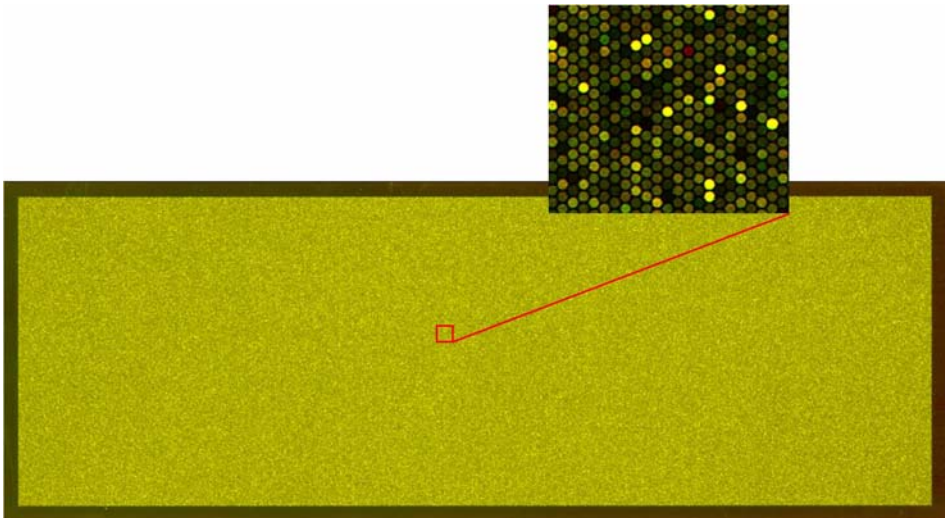

Figure 4 Agilent SurePrint G3 1x1M CGH microarray shown in red and green channels: full and zoomed view

1 Open the Agilent Feature Extraction (FE) program.

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

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

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

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

- For auto assignment of the grid template, the image must be generated from an Agilent scanner and have an Agilent barcode.
- For auto assignment of the CGH FE protocol, the **default CGH protocol** must be specified in the FE Grid Template properties.

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

3 Set FE Project Properties.

a Select the **Project Properties** tab.

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

c In all other sections, verify that at least the following default settings as shown in Figure 5 below are selected.

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

For FE 10.5, the metric sets are part of the protocol, and there is no need to set them.

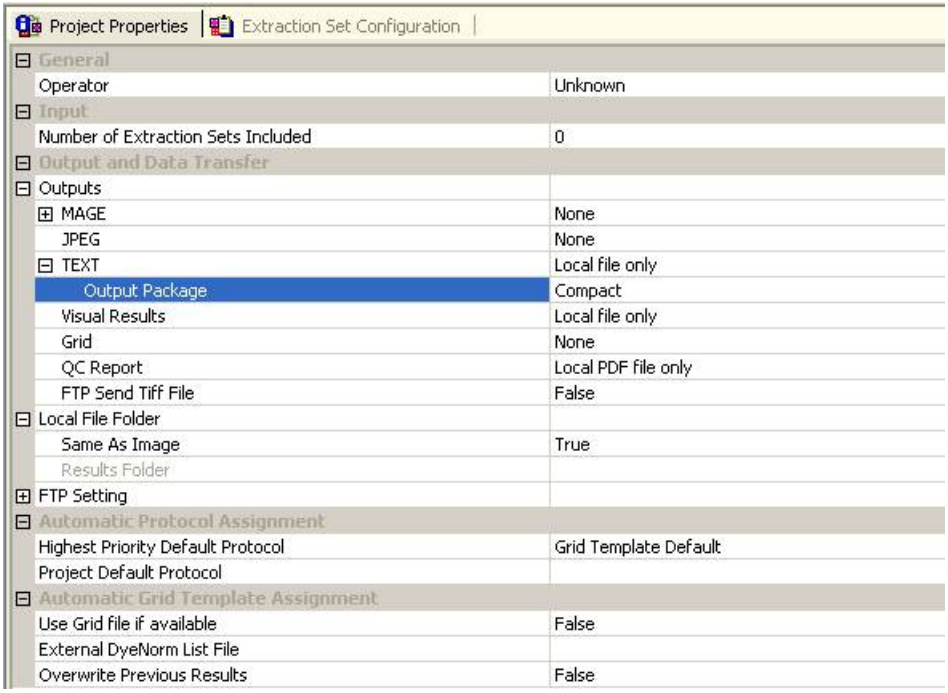

Figure 5 Default settings in FE 10.5

4 Check the Extraction Set Configuration.

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

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

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

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

After a new grid template is added to the Grid Template Browser, remember to specify the default protocol for the new grid template if you want the Feature Extraction program to automatically assign an FE protocol to an extraction set.

c Verify that the CGH-v4_95_Feb07 (in FE 9.5) or CGH_105_Dec08 (in FE 10.5) protocol is assigned to each extraction set in the **Protocol Name** column.

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

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

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

7 Select **Project > Start Extracting**.

8 After the extraction is completed successfully, view the QC report for each extraction set by double-clicking the QC Report link in the **Summary Report** tab. Determine whether the grid has been properly placed by inspecting **Spot Finding of the Four Corners of the Array**. See Figure 6 and Figure 7.

Microarray QC Metrics for high DNA quality samples

These metrics are only appropriate for high-quality DNA samples analyzed with Agilent CGH microarrays by following the standard operational procedures provided in this user guide. These metrics are exported to a table in the Feature Extraction QC report (in FE 9.5, select **CGH_QCMT_Feb08** in Project Properties before extraction) and in Genomic Workbench. They can be used to assess the relative data quality from a set of microarrays in an experiment. In some cases, they can indicate potential processing errors that have occurred or suggest that the data from particular microarrays might be compromised. Many factors can influence the range of these metrics including the microarray format (1x, 2x, 4x or 8x) biological sample source, quality of starting gDNA, experimental processing, scanner sensitivity and image processing. The value guidelines presented below represent the thresholds that Agilent has observed when analyzing samples using this protocol.

Table 38 QC metric thresholds for Enzymatic labeling

Metric	Excellent	Good	Poor
BGNoise	< 5	5 to 10	> 10
Signal Intensity	> 150	50 to 150	< 50
Signal to Noise	> 100	30 to 100	< 30
Reproducibility	< 0.05	0.05 to 0.2	> 0.2
DLRSpread	< 0.2	0.2 to 0.3	> 0.3

QC Chart Tool

At times the Feature Extraction program is used in a production environment, where the biological samples are similar, microarray processing protocols are identical and monitoring run-to-run consistency is an important goal. The Feature Extraction program can help monitor this consistency with the optional QC Chart Tool. The QC Chart Tool extracts summary statistics from a set of Feature Extraction output text files and can be used to generate metric sets that can be imported into the Feature Extraction program for analysis of each batch of microarrays processed. Only one metric set can be assigned to a Feature Extraction project. When that project is run, the Feature Extraction program summarizes the metric statistics on each microarray's QC report and shows if the thresholds (if any) were exceeded. In addition, at the end of the project, a summary chart can be opened to display graphically what the results are for each metric for each microarray. The QC Chart Tool can be downloaded at www.agilent.com/chem/FEQCMetrics.

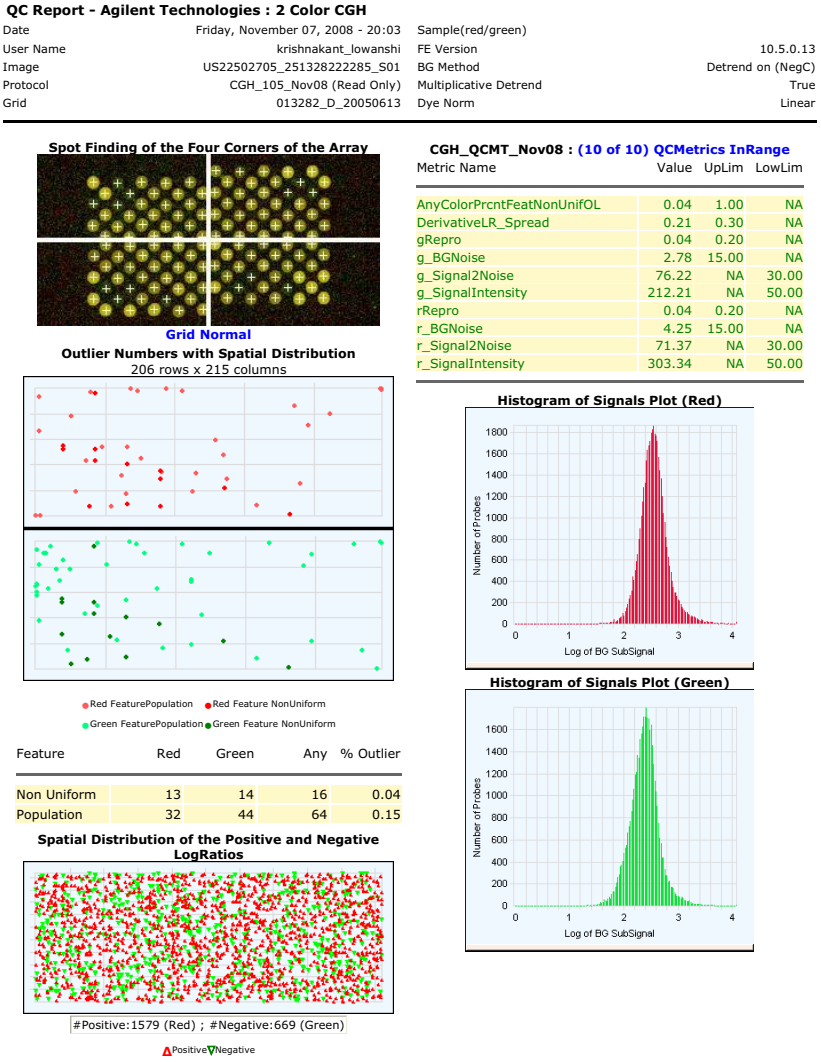

Figure 6 CGH QC report generated from Feature Extraction software v10.5, page 1

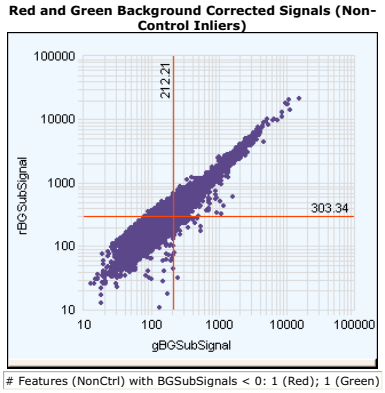

Figure 7 CGH QC report generated from Feature Extraction software v10.5, page 2

6 Troubleshooting

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

If you have an OD260/230 or OD260/280 value below 1.8

A low OD260/230 value can indicate contaminants, such as residual phenol or salt. A low OD260/280 value indicates residual protein. Either condition can result in low specific activity (pmol dye/µg DNA) or Degree of Labeling. See "To determine yield , degree of labeling or specific activity" on page 53.

- ✔ Repurify the DNA using the Qiagen DNeasy protocol. See "DNA Isolation" on page 21. This procedure includes a proteinase K treatment.
- ✔ If you must do a phenol/chloroform DNA extraction, do not get too close to the interface.

If you have poor sample quality due to residual RNA

The input amount of DNA for the experimental labeling reaction must be the same as for the reference sample labeling reaction. RNA absorbs at the same wavelength as DNA, which makes an accurate measurement of the DNA concentration in an RNA-contaminated sample impossible.

- ✔ Repurify the DNA using the Qiagen DNeasy protocol. See "DNA Isolation" on page 21. This procedure includes a RNase A treatment.

If you get poor sample quality due to degradation

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

- ✔ Check DNA on a 1 to 1.5% agarose gel, if DNA that was isolated from cells, blood or frozen tissue, is degraded then repurify the DNA using the Qiagen DNeasy protocol. See "DNA Isolation" on page 21.
- ✔ For processing FFPE samples, refer to the *Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis (ULS Labeling for Blood, Cells, Tissues or FFPE) Protocol* v3.0 (p/n G4410-90020).

If the estimated concentration is too high or low

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

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

- ✔ Make sure that the gDNA is completely in solution by pipetting up and down. If needed, incubate at 37°C for 30 minutes. If the gDNA concentration is > 350 ng/µL, dilute 1:2 in water and re-quantitate to make sure quantitation is accurate.
- ✔ If needed, repurify the DNA using the Qiagen DNeasy protocol. See "DNA Isolation" on page 21.

If you have low specific activity or degree of labeling not due to poor sample quality

Low specific activity or degree of labeling can result from sub-optimal labeling conditions such as Cyanine dUTP with too many freeze thaws, enzyme degradation due to being left warm for too long, wrong temperatures or times, volume mistakes, or too much exposure to light or air.

- ✔ Store Cyanine dUTP at 4°C. Keep enzymes on ice and return to -20°C as quickly as possible.
- ✔ Double check incubation times and temperatures (use a calibrated thermometer), and use a thermocycler with heated lid.
- ✔ Evaporation can be a problem when you process samples in 96-well plates. Use a plate heat sealer (Eppendorf p/n 951023078) to avoid evaporation.
- ✔ Make sure that the pipettors are not out of calibration.
- ✔ Make sure that the gDNA, reagents, and master mixes are well mixed. Tap the tube with your finger or use a pipette to move the entire volume up and down. Then spin in a microcentrifuge for 5 to 10 seconds to drive the contents off the walls and lid. Do not mix the stock solutions and reactions that contain gDNA or enzymes on a vortex mixer.

If you have low yield not due to poor sample quality

Possible sample loss during clean-up after labeling.

- ✔ See "Step 2. Clean-up of Labeled Genomic DNA" on page 46 to remove unreacted dye. Many other columns result in the loss of shorter fragments.

If you have post-labeling signal loss

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

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

- ✔ Check oven and Wash 2 temperature.
- ✔ Check that Wash 2 was not accidentally used instead of Wash 1.
- ✔ Wash and scan slides in an ozone controlled environment (<5 ppb), such as an ozone tent.
- ✔ Use small batches that can be washed and scanned in about 40 minutes to minimize exposure to air.
- ✔ Use the Agilent Ozone-Barrier Slide Cover (p/n G2505-60550).
- ✔ Use the Stabilization and Drying Solution as described in "Wash Procedure B (with Stabilization and Drying Solution)" on page 69.

If you have high BGNoise values

High BGNoise can cause lower signal-to-noise values (see Table 38 on page 78 for thresholds) and higher DLRSD values. BGNoise is defined as the standard deviation of the signals on the negative controls. If the BGNoise is high, examine the array image for visible non-uniformities. High BGNoise is often introduced during the washes.

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

If you have poor reproducibility

Poor reproducibility (see Table 38 on page 78 for thresholds), defined as high CVs of signals of replicated probes may indicate that the hybridization volume was too low or that the oven stopped rotating during the hybridization. Only very high scores on this metric will affect the DLRSD.

- ✔ When setting up the gasket-slide hybridization sandwich dispense the hybridization sample mixture slowly in a “drag and dispense” manner to prevent spills.
- ✔ Check that the oven is rotating.

7 Reference

This chapter contains reference information that pertains to this protocol.

Supporting User Guides

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

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

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

Agilent Microarray Layout and Orientation

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

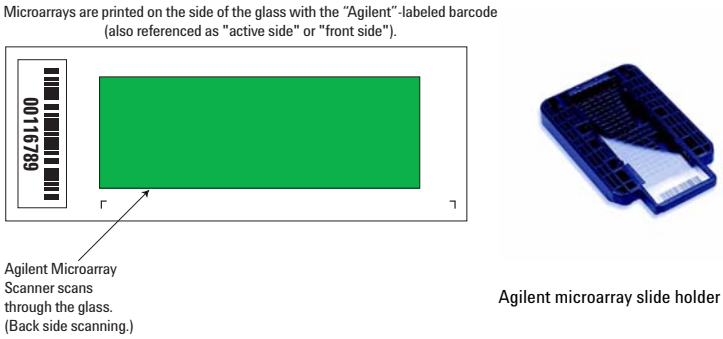

Figure 8 Agilent microarray slide and slide holder

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

Figure 8 depicts how the Agilent microarray scanner reads the microarrays and how this relates to the "microarray design files" that Agilent generates during the manufacturing process of its *in situ*-synthesized oligonucleotide microarrays. Thus, if you have a scanner that reads microarrays from the "front side" of the glass slide, the collection of microarray data points will be different in relation to the "microarray design files" supplied with the Agilent oligo microarray kit you purchased. Therefore, please take a moment to become familiar with the microarray layouts for each of the Agilent oligo microarrays and the layout information as it pertains to scanning using a "front side" scanner.

Non-Agilent Front Side Microarray Scanners

When scanning Agilent oligo microarray slides, the user must determine:

- If the scanner images the microarrays by reading them on the “front side” of the glass slide (“Agilent”-labeled barcode side of the slide) and
- If the microarray image produced by the non-Agilent scanner is oriented in a “portrait” or “landscape” mode, and “Agilent”-labeled barcode is on the left-side, right-side, up or down, as viewed as an image in the imaging software (see Figure 9).

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

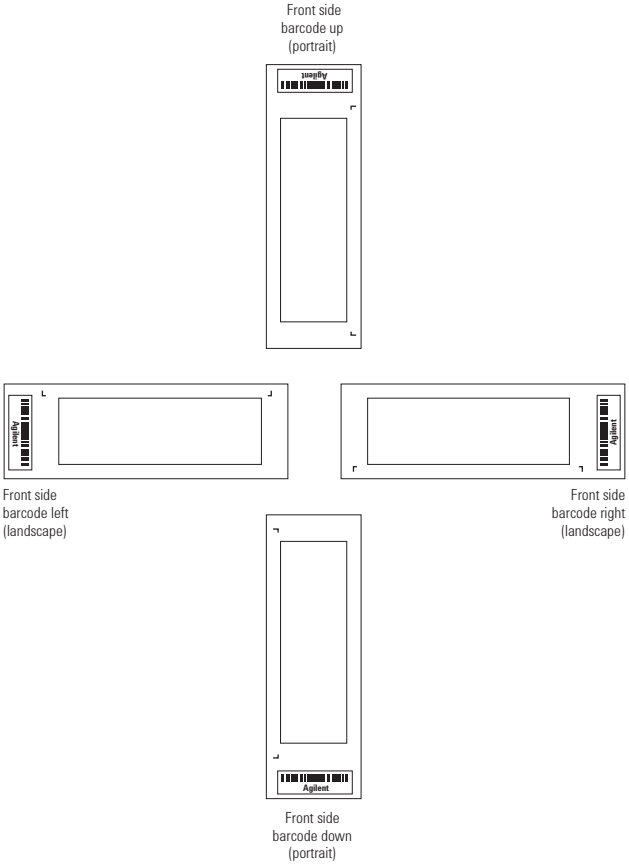

Figure 9 Microarray slide orientation

Array/Sample tracking on a 8x array slide

Use the form below to make notes to track your samples on a 8-pack array slide.

Arrays

BARCODE	Array 1_1	Array 1_2	Array 1_3	Array 1_4
	Sample: _____ _____ _____	**Sample:** _____ _____ _____	**Sample:** _____ _____ _____	**Sample:** _____ _____ _____
	Sample: _____ _____ _____	**Sample:** _____ _____ _____	**Sample:** _____ _____ _____	**Sample:** _____ _____ _____
	Array 2_1	**Array 2_2**	**Array 2_3**	**Array 2_4**

Barcode Number _____

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

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In This Book

This guide contains information to run the Oligonucleotide Array-Based CGH for Genomic DNA Analysis (enzymatic labeling) protocol.

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