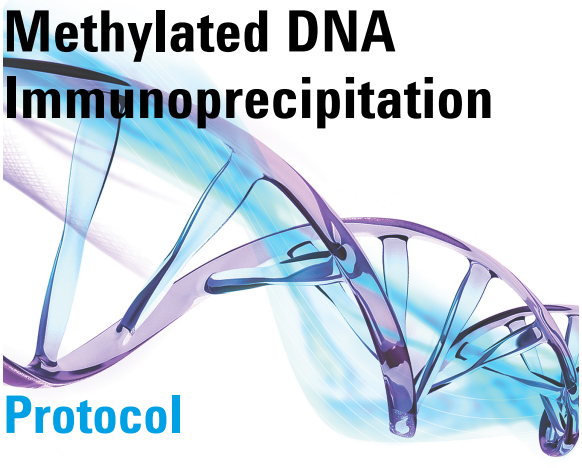
Agilent Microarray Analysis of Methylated DNA Immunoprecipitation



Protocol

Version 2.3.1, August 2015

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Before you begin, view hands-on videos of SurePrint procedures at
http://www.agilent.com/genomics/protocolvideos.

Agilent Technologies

Notices

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In This Guide...

This protocol describes the use of methylation microarrays for the analysis of Methylated DNA Immunoprecipitation (MeDIP).

1 Before You Begin

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

2 Sample Preparation

This chapter describes the standard method to process DNA prior to labeling.

3 Sample Labeling

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

4 Microarray Processing and Feature Extraction

This chapter describes the steps to hybridize, wash and scan Agilent DNA Methylation microarrays and to extract data using the Agilent Feature Extraction Software for use in Agilent Genomics Workbench.

5 Reference

This chapter contains reference information related to the amplification, labeling, hybridization and wash kits, and the protocol.

What's new in 2.3

- Corrected fluorescent labeling step to remove preparation of amplified reference and IP gDNA.
- Updated temperature for denaturing step.
- Updated product labeling statement.

What's new in 2.2

- Updated location to find design files.
- Corrected starting volume for amplified WCE and IP DNA for fluorescent labeling.
- Updated loading instructions for hybridization oven.
- Added reference to compatibility matrix for non-Agilent scanners.

What's new in 2.1

- Updated description of G4811A microarray.
- Updated Required Reagents list.
- Restored instructions to prepare amplified WCE and IP gDNA.
- Added note to calibrate hybridization oven on a regular basis for accuracy of the collected data.

What's new in 2.0

- SureTag DNA Labeling Kit replaces Genomic DNA Enzymatic Labeling Kit.
- Support for SureScan microarray scanner.

Content

1 Before You Begin

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

Procedural Notes

- To prevent contamination of reagents by nucleases, always wear powder-free laboratory gloves, and use dedicated solutions and pipettors with nuclease-free aerosol-resistant tips.
- Maintain a clean work area.
- Do not mix stock solutions and reactions containing gDNA or enzymes on a vortex mixer. Instead, mix the solutions and reactions by gently tapping the tube with your finger.
- Avoid repeated freeze-thaw cycles of solutions containing gDNA or enzymes.
- When preparing frozen reagent stock solutions for use:
  1. Thaw the aliquot as quickly as possible without heating above room temperature.
  2. Mix briefly on a vortex mixer, and then spin in a microcentrifuge for 5 to 10 seconds to drive the contents off the walls and lid.
  3. Store on ice or in a cold block until use.
- In general, follow Biosafety Level 1 (BL1) safety rules.

Safety Notes

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

WARNING

- **Cyanine reagents are considered hazardous by the OSHA Hazard Communication Standard (29 CFR 1910.1200). Contains material that causes damage to the following organs: kidneys, liver, cardiovascular system, respiratory tract, skin, eye lens or cornea, stomach. May be harmful if swallowed. Avoid contact with eyes, skin and clothing.**
- **2× HI-RPM Hybridization Buffer is considered hazardous by the OSHA Hazard Communication Standard (29 CFR 1910.1200). Contains material that causes damage to the following organs: skin, central nervous system. May be harmful if swallowed. Avoid contact with eyes, skin and clothing.**
- **Triton is harmful if swallowed. Risk of serious damage to eyes. Wear suitable PPE. Triton is a component of the Agilent 2× HI-RPM Hybridization Buffer.**
- **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 Methylation Microarray Kit Contents

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

Catalog SurePrint HD Methylation Microarray Kit

- One or two microarrays printed on each 1-inch × 3-inch glass slide
- Available as a 5-slide kit, as indicated in Table 1.

Design files can be downloaded from http://www.agilent.com/genomics/suredesign.

See Table 1 for available designs. For more information on Methylation designs, go to http://www.genomics.agilent.com. Under **Products**, click **Epigenetic & Specialty Microarrays**.

Table 1 Catalog SurePrint HD Methylation Microarray Kits

Part Number	Description
G4492A	Human CpG Island Microarray 5-slide Kit, 1×244K (HD)
G4811A	Mouse CpG Island Microarray, 2×105K (HD)

Unrestricted SurePrint HD Methylation Microarrays

- One microarray printed on each 1-inch × 3-inch glass slide
- Number of microarray slides vary per kit and per order

Design files can be downloaded from http://www.agilent.com/genomics/suredesign.

See Table 2 for available designs.

Table 2 Unrestricted SurePrint Methylation Microarrays

Part Number	Description
G4495A, AMAIDID 023795	Human DNA Methylation Microarray, 1×244K (HD)

Custom SurePrint HD and G3 Microarrays

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

See Table 3 for available formats.

Table 3 Custom SurePrint HD and G3 Methylation Microarrays

Part Number	Description
G4819A	Custom SurePrint G3 ChIP-on-chip/DNA Methylation, 1×1M
G4820A	Custom SurePrint G3 ChIP-on-chip/DNA Methylation, 2×400K
G4821A	Custom SurePrint G3 ChIP-on-chip/DNA Methylation, 4×180K
G4496A	Custom ChIP-on-chip/DNA Methylation, 1×244K
G4498A	Custom ChIP-on-chip/DNA Methylation, 2×105K
G4497A	Custom ChIP-on-chip/DNA Methylation, 4×44K

Required Equipment

Table 4 Required equipment

Description	Vendor and part number
200 µL Thin-Wall Tube	Agilent p/n 410091 or equivalent
Agilent Microarray Scanner Bundle for 1×244K, 2×105K, or 4×44K, *or* for 1×1M, 2×400K, or 4×180K	 Agilent p/n G4900DA, G2565CA or G2565BA Agilent p/n G4900DA or G2565CA
Hybridization Chamber, stainless	Agilent p/n G2534A
Hybridization gasket slides, 5-pack (20 and 100 packaging sizes are available) for 1-pack microarrays *or* for 2-pack microarrays *or* for 4-pack microarrays	 Agilent p/n G2534-60003 Agilent p/n G2534-60002 Agilent p/n G2534-60011
Hybridization oven; temperature set at 67°C	Agilent p/n G2545A
Hybridization oven rotator for Agilent Microarray Hybridization Chambers	Agilent p/n G2530-60029
Ozone-barrier slide covers (box of 20)[1]	Agilent p/n G2505-60550
1.5 mL RNase-free Microfuge Tube (sustainable at 98°C)	Ambion p/n AM12400 or equivalent
Magnetic stir plate (×1 or ×3)[2]	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
Tube rotator	Labquake p/n 56264-306 or equivalent
DynaMag-2 Magnet	Life Technologies p/n 123-21D
Sterile storage bottle	Nalgene 455-1000 or equivalent
UV-VIS spectrophotometer	NanoDrop 8000 or 2000, or equivalent
P10, P20, P200 and P1000 pipettes	Pipetman P10, P20, P200, P1000 or equivalent
1.5 L glass dish	Pyrex p/n 213-R or equivalent
MaXtract High Density 2-mL tube	Qiagen p/n 129056

Table 4 Required equipment **(continued)**

Description	Vendor and part number
Vacuum Concentrator	Thermo Scientific Savant SpeedVac p/n DNA120-115 or equivalent
Magnetic stir bar, 7.9 × 38.1 mm (×2 or ×4)[2]	VWR p/n 58948-150 or equivalent
250 mL capacity slide-staining dish, with slide rack (×3 or ×5)[2]	Wheaton p/n 900200 *or* Thermo Shandon p/n 121
Circulating water baths or heat blocks set to 37°C, 65°C, and 95°	
Ice bucket	
Clean forceps	
Powder-free gloves	
Sterile, nuclease-free aerosol barrier pipette tips	
Timer	
Vacuum desiccator or N_2 purge box for slide storage	
Vortex mixer	
Sonicator machine	

[1] Optional. Recommended when processing arrays with a G2565CA scanner in environments in which ozone levels are 5 ppb or higher.

[2] The number varies depending on if wash procedure A or B is selected.

Table 5 Optional. Recommended when using high-throughput method on 2-pack microarrays.

Description	Vendor and part number
Tall Chimney PCR plate	ABgene p/n AB-1184

Required Reagents

Table 6 Required reagents for DNA Methylation sample preparation

Item	Vendor and part number
PBS (Phosphate Buffered Saline)	Amresco p/n K812-500 mL
5-Methylcytidine, Monoclonal Antibody, purified	Eurogentec p/n BI-MECY-1000
Dynabeads Pan Mouse IgG	Life Technologies p/n 110-41
Nuclease-free distilled water	Life Technologies p/n 10977-015
Yeast tRNA	Life Technologies p/n 15401-011
UltraPure 10% SDS	Life Technologies p/n 15553-027
Glycogen	Roche p/n 901 393
Ethanol (95% to 100% molecular biology grade)	Sigma-Aldrich p/n E7023-6×500ML
BSA, powder	Sigma-Aldrich p/n A7906
NaCl	Sigma-Aldrich p/n S7653
Triton X-100	Sigma-Aldrich p/n T8787
Phenol-chloroform-isoamyl alcohol	Sigma-Aldrich p/n 77617

Table 7 Required reagents for enzymatic sample prep and labeling with the SureTag DNA Labeling Kit

Description	Vendor and part number
SureTag DNA Labeling Kit[1]	Agilent p/n 5190-3400
1×TE (pH 8.0), Molecular grade	Promega p/n V6231

[1] Kit content is listed in "Reagent Kit Components" on page 62.

Table 8 Required reagents for hybridization and wash

Description	Vendor and part number
Oligo aCGH/ChIP-on-chip Wash Buffer Kit *or*	Agilent p/n 5188-5226
Oligo aCGH/ChIP-on-chip Wash Buffer 1 *and* Oligo aCGH/ChIP-on-chip Wash Buffer 2	Agilent p/n 5188-5221 Agilent p/n 5188-5222
Stabilization and Drying Solution[1]	Agilent p/n 5185-5979
Oligo aCGH/ChIP-on-chip Hybridization Kit	Agilent p/n 5188-5220 (25) or p/n 5188-5380 (100)
Cot-1 DNA (1.0 mg/mL)	
• Human Cot-1 DNA *or* • Mouse Cot-1 DNA	Agilent p/n 5190-3393 Life Technologies p/n 18440-016
DNase/RNase-free distilled water	Life Technologies p/n 10977-015
Milli-Q ultrapure water	Millipore
Acetonitrile[1]	Sigma-Aldrich p/n 271004-1L
Deionized Formamide	Sigma-Aldrich p/n F9037-100ML

[1] Optional components recommended if wash procedure B is selected.

Required Hardware and Software

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

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2 Sample Preparation

The steps in this protocol and the estimated amounts of time required are listed in Table 1.

Table 1 Overview and time requirements.

Step	Time Requirement
Binding of antibody to magnetic beads	0.5 hour, then overnight
DNA sonication	1 hour
Methylated DNA immunoprecipitation	0.5 hour, then overnight
Wash, elution	2 hours
DNA Purification with phenol:chloroform:Isoamyl alcohol	2 hours
Cyanine 3/cyanine 5 labeling of immunoprecipitate and reference material	3 hours
Microarray hybridization	1 hour, then 40 hours
Microarray washing	1 hour

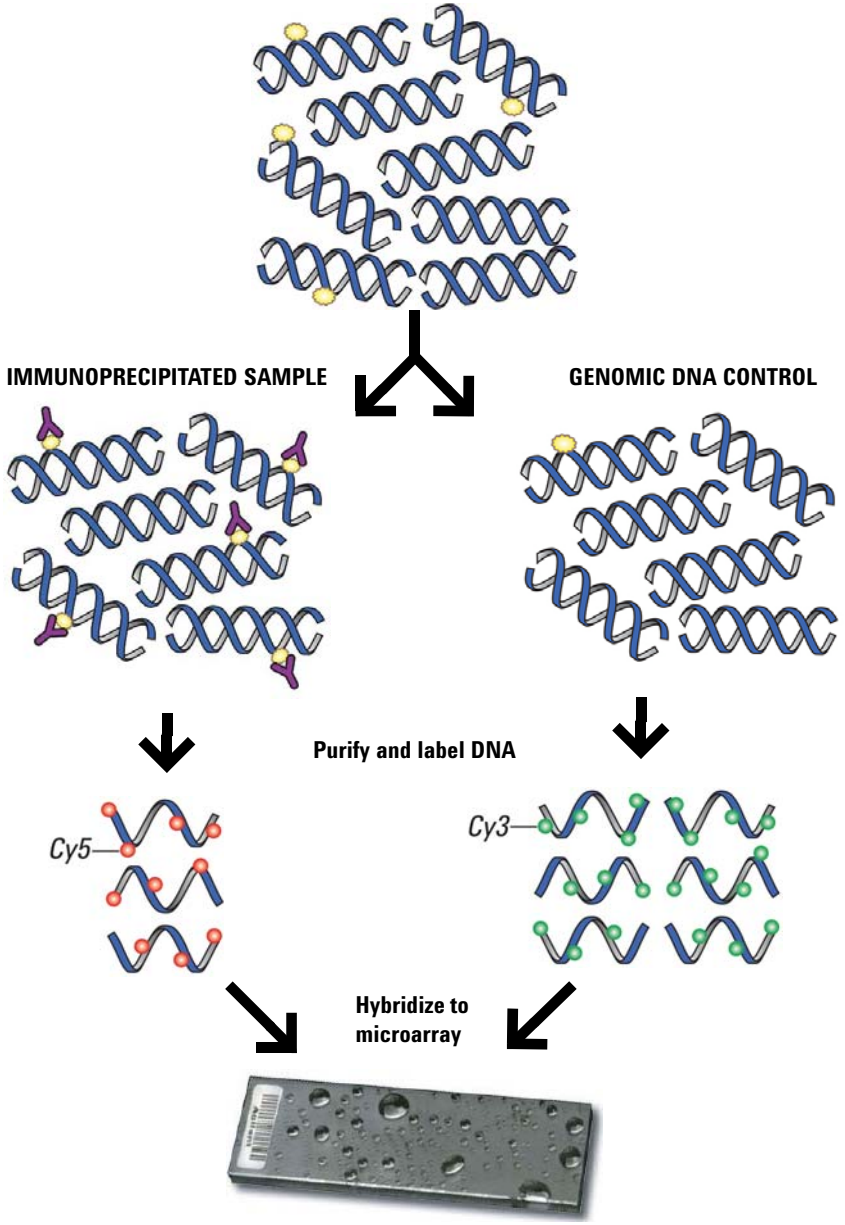

Figure 1 Methylated Genomic DNA overview

Step 1. Prepare the magnetic beads

The following steps to bind the antibody to the beads are to be done in a cold room or on ice.

1 Set up and label one 1.5 mL RNase-free Microfuge Tube for each immunoprecipitation.

2 Vigorously mix bottle of Dynabeads Pan Mouse IgG in a vortex mixer to resuspend. Dynal beads will have settled during storage.

3 Immediately add 50 µL of Dynabeads Pan Mouse IgG to a 1.5 mL RNase-free Microfuge Tube.

4 Put tubes on a magnetic separation stand, such as a DynaMag-2 Magnet.

5 Remove the supernatant with a pipette.

NOTE Beads can also be processed as a batch in a 15 mL conical tube. Scale the volumes accordingly.

6 Add 750 µL of Block Solution 1 and resuspend gently.

Table 2 Block Solution 1

Stock	For 100 mL	Final Concentration
PBS	100 mL	1×
BSA, powder	500 mg	0.5% BSA (weight/volume)

7 Gently mix the beads in Block Solution 1 for 5 minutes at 4°C on a Tube rotator.

8 Remove the supernatant with a pipette and magnetic separation stand.

9 Repeat step 6 to step 8 for a second wash.

10 Spin in a centrifuge at 4°C at 1500 × g for 3 minutes.

11 Resuspend the beads in 230 µL of Block Solution 2 (Table 3 on page 20) and add 5 µg of 5-Methylcytidine, Monoclonal Antibody, purified per immunoprecipitation.

Table 3 Block Solution 2*

Stock	For 100 mL	Final Concentration
PBS	100 mL	1×
BSA, powder	50 mg	0.05% BSA (weight/volume)

* You can also make a 1:10 dilution of Block Solution 1 in PBS to create Block Solution 2. Prepare Blocking Solution fresh each time.

The first time the antibody is used, divide the unused 5-Methylcytidine, Monoclonal Antibody, purified into 5 µg aliquots and store at -80°C to limit freeze/thaw cycles.

NOTE Beads for up to 6 immunoprecipitations can be combined into one 1.5 mL tube or 8 in a 2 mL tube.

12 Place tubes on a Tube rotator at 4°C for a minimum of 6 hours to bind the Antibody to the beads. You can also leave the tubes on the Tube rotator overnight.

13 The next day quick, spin to collect fluid at the bottom of the tube. Use a magnetic separation stand to collect the beads against the side of tube and remove the supernatant:

- **a** Add 750 µL of Block Solution 2 to the beads.
- **b** Remove the tubes from the magnetic stand and gently resuspend beads.
- **c** Use a magnetic separation stand to collect the beads against the side of tube and remove the supernatant with a pipette.
- **d** Repeat wash two more times.

14 Remove the last wash with a 1 mL pipette.

15 Resuspend the beads in 50 µL of Block Solution 2.

The beads are now ready for the IP step.

NOTE If beads for multiple IPs were processed together, scale the wash volume accordingly and resuspend the washed beads in 50 µL of Block Solution 2 per IP.

Step 2. Prepare DNA for Immunoprecipitation

1 For each sample, resuspend 5 µg of purified genomic DNA in 250 µL of PBS.

Do *not* use lower volumes and compensate with a whole genome amplification (WGA) as this will bias the data.

2 Sonicate the suspension with a microtip attached to sonicator. Keep samples in an ice water bath during sonication.

If you use a Branson Digital Sonifier Model 450, set output power to 57%. Sonicate 5 cycles of 5 seconds ON and 10 seconds OFF to decrease foaming.

NOTE

You may need to optimize sonication conditions. Use the lowest settings that result in sheared DNA that ranges from 200 to 1000 bp in size. Shearing varies greatly depending on quantity, volume, and equipment. Depending on the specific experiment, and using power settings as high as 70%, you can use anywhere from 3 to 8 cycles and variable ratios of time ON and time OFF. You can determine the degree with the use of the Agilent 2100 Bioanalyzer. See "QC Metrics" on page 71.

3 Check volumes with a pipette and bring the volume of each of your samples to 250 µL with PBS.

4 Transfer 50 µL from each sheared sample into a new 1.5 mL RNase-free Microfuge Tube to use as the reference sample. (Store the sample at -20°C until use.) With the remaining 200 µL, go to the next step.

Step 3. Immunoprecipitate the methylated DNA

1 Add 50 µL antibody/magnetic bead mixture from "Step 1. Prepare the magnetic beads" on page 19 to the 1.5 mL RNase-free Microfuge Tube that contains the 200 µL of sheared genomic DNA from "Step 2. Prepare DNA for Immunoprecipitation" on page 21.

2 Add 250 µL of 2× IP Buffer.

Table 4 2× IP Buffer

	Final concentration in 2X buffer	Volume
1% Triton X-100 in PBS	0.05%	200 µL
Yeast tRNA	50 µg/ml	8 µL
PBS		3.8 mL
Total		**4 mL**

3 Gently mix tubes overnight on a Tube rotator at 4°C.

Step 4. Wash and elute methylated DNA from beads

Do these steps in a 4°C cold room or on ice.

1 Place tubes in a magnetic separation stand to collect the beads. Remove supernatant with a pipette. Keep as many magnetic beads as you can.

2 Add 1 mL of IP Wash Buffer to each tube.

3 Remove tubes from magnetic device and shake or agitate tube gently to resuspend beads.

4 Rotate beads for 3 minutes at 4°C.

5 Replace tubes in magnetic device to collect beads.

6 Remove supernatant. Repeat this wash 2 more times.

Table 5 IP Wash Buffer

Stock	Volume
PBS	48.75 mL
1% Triton X-100 in PBS	1.25 mL

7 Remove any residual IP Wash Buffer with a pipette.

Elution

1 Add 150 µL of Elution Buffer and resuspend beads.

Table 6 Elution Buffer

Component	Volume
1×TE (pH 8.0)	45 mL
10% SDS	5 mL

2 Incubate in water bath at 65°C for 2.5 minutes.

3 Remove tubes and quickly mix on a vortex mixer to resuspend beads.

4 Return to 65°C water bath and incubate for an additional 2.5 minutes.

5 Place tubes on magnetic separation stand.

6 Transfer 150 µL of supernatant to a new labeled 1.5 mL RNase-free Microfuge Tube.

The supernatant contains your IP DNA. Do not discard.

7 Repeat elution step 1 through step 6 with additional 150 µL of Elution Buffer. Combine the two elutions into one tube.

Step 5. Extract immunoprecipitated and reference DNA with phenol-chloroform

1. Add 250 µL of elution buffer to each of your reference samples, which you set aside from "Step 2. Prepare DNA for Immunoprecipitation" on page 21, for a total volume of 300 µL.
2. Add 300 µL of Phenol-chloroform-isoamyl alcohol to each immunoprecipitate and reference tube.
3. Mix the samples on a vortex mixer for 20 seconds.
4. On a centrifuge, spin one MaXtract High Density 2-mL tube for each immunoprecipitated and reference DNA sample at 14,000 × g for 30 seconds at room temperature.
5. Transfer all 600 µL of the sample to the MaXtract High Density 2-mL tube.
6. Spin the sample in a centrifuge at 14,000 × g for 5 minutes at room temperature.

   If any samples remains cloudy, repeat the Phenol-chloroform-isoamyl alcohol extraction one more time.
7. Transfer the aqueous layer (top) to a new 1.5 mL RNase-free Microfuge Tube and dispose the MaXtract High Density 2-mL tube.
8. To each tube that contains the aqueous layer, add:
   - 16 µL of NaCl (200 mM final concentration)
   - 1.5 µL of 20 µg/µL Glycogen
   - 880 µL Ethanol
9. Mix on a vortex mixer.
10. Cool the mixture for 30 minutes at -80°C.
11. Spin the mixture in a centrifuge at 20,000 × g for 10 minutes at 4°C to create DNA pellets.
12. Carefully remove the supernatant and retain the pellets.
13. Wash the pellets with 500 µL of 70% ice-cold Ethanol.
14. Spin the mixture in a centrifuge at 12,000 × g for 3 minutes.
15. Discard the supernatant while you retain the pellets.
16. Dry the pellets for 10 minutes with a vacuum desiccator, such as a Savant Speed Vac.

17 If you want to keep a part of this sample for more analysis, such as quantification, resuspend the DNA in 31 µL of nuclease-free water. Otherwise, resuspend in 26 µL of nuclease-free water.

18 Use the Nanodrop spectrophotometer to measure the yield of the IP and reference samples using the reserved 5 µL of the sample.

Yield from a typical tissue ranges from 500 to 700 ng. Hypomethylated or hypermethylated abnormal tissues can produce slightly different yields.

3 Sample Labeling

The SureTag DNA Labeling Kit contains sufficient two-color labeling reaction reagents for 25 microarrays (25 reactions of each color).

It also contain clean-up columns for 25 reactions of each color.

The kit uses random primers and the exo-Klenow fragment to differentially label IP and reference DNA samples with fluorescent-labeled nucleotides. For the Agilent DNA Methylation 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. Typically, the reference sample is labeled with Cy3 and the IP is labeled with Cy5.

Step 1. Fluorescent Labeling of 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.

CAUTION The test/reference sample pairs must be treated identically when they are processed, or else the quality of your data can be adversely affected. The best way to ensure that the sample pairs are exposed to the same temperature during the denaturation step is to use a water bath.

1. Equilibrate heat blocks or water baths to 98°C, 37°C and 65°C, or use a thermal cycler.
2. Spin the samples in a centrifuge for 1 minute at 6,000 × g to drive the contents off the walls and lid.
3. Add Random Primer:
   - For 1-pack, 2-pack, and 4-pack microarrays, add 5 µL of Random Primer to each reaction tube containing 26 µL of reference or IP DNA to make a total volume of 31 µL. Mix well by pipetting up and down gently.
4. Transfer sample tubes to a circulating water bath or heat block at 98°C. Incubate at 98°C for 3 minutes, then move to ice and incubate on ice for 5 minutes.

   or

   Transfer sample tubes to a thermal cycler. Program the thermal cycler according to Table 7 and run the program.

Table 7 DNA denaturation using a thermal cycler

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

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

6 Prepare Labeling Master Mix:

a Mix the components in Table 8 on ice in the order indicated to prepare one cyanine-3 and one cyanine-5 Labeling Master Mix.

Table 8 Labeling Master Mix (for 1-pack, 2-pack and 4-pack microarrays)

Component	Per reaction (µL)	× 8 rxns (µL) (including excess)	× 24 rxns (µL) (including excess)	× 48 rxns (µL) (including excess)
5× Reaction Buffer	10.0	85	250	500
10× dNTPs	5.0	42.5	125	250
Cyanine 3-dUTP *or* Cyanine 5-dUTP	3.0	25.5	75	150
Exo (-) Klenow	1.0	8.5	25	50
Final volume of Labeling Master Mix	**19.0**	**161.5**	**475**	**950**

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

7 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 thermal cycler. Program the thermal cycler according to Table 9 and run the program.

Table 9 DNA labeling using a thermal cycler

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

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

Step 2. Purification of labeled DNA

Labeled DNA is purified using the reaction Purification Column provided with the SureTag DNA Labeling Kit. The Purification Column includes:

- column
- 2-mL collection tube

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

1. Spin the labeled reference or IP DNA samples in a centrifuge for 1 minute at 6,000 × g to drive the contents off the walls and lid.
2. Add 430 µL of 1×TE (pH 8.0) to each reaction tube.
3. For each reference or IP DNA sample to be purified, place a column into a 2-mL collection tube and label the column appropriately. Load each labeled reference or IP DNA onto a column.
4. Cover the column with a cap and spin for 10 minutes at 14,000 × g in a microcentrifuge at room temperature. Discard the flow-through and place the column back in the 2-mL collection tube.
5. Add 480 µL of 1×TE (pH 8.0) to each column. Spin for 10 minutes at 14,000 × g in a microcentrifuge at room temperature. Discard the flow-through.
6. Invert the column into a fresh 2-mL collection tube that has been appropriately labeled. Spin for 1 minute at 1,000 × g in a microcentrifuge at room temperature to collect purified sample.
7. Add 1×TE (pH 8.0), or use a concentrator to bring the sample volume to that listed in Table 10. Do not excessively dry the DNA because the pellets will become difficult to resuspend.
8. Mix thoroughly. If the sample has dried or precipitated after concentration, incubate the tube that contains DNA sample on ice for 5 minutes, and then pipette the solution up and down 10 times.
9. Take 1.5 µL of each sample to determine yield and specific activity. See "To determine yield and specific activity" on page 33. Refer to Table 11 on page 33 for expected yield of labeled DNA and specific activity after labeling and clean-up.

10 In a fresh 1.5 mL RNase-free Microfuge Tube or 200 µL Thin-Wall Tube, 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 10. Use the appropriate container listed in Table 10.

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

Table 10 Sample volume and total mixture volumes

Microarray	Cy3 or Cy5 sample volume after purification	Total mixture volume after Nanodrop and combining	Container
1-pack	42 µL	80 µL	1.5 mL RNase-free Microfuge Tube
2-pack	22 µL	40 µL	1.5 mL RNase-free Microfuge Tube or Tall Chimney PCR plate
4-pack	13.25 µL	22.5 µL	1.5 mL RNase-free Microfuge Tube or Tall Chimney PCR plate

To determine yield and specific activity

Use the NanoDrop 8000 or 2000 UV-VIS Spectrophotometer to measure yield and specific activity.

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

2 Use 1.5 µL of 1×TE (pH 8.0) to blank the instrument.

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

4 Calculate the Specific Activity of the labeled DNA:

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

*pmol dyes per µg DNA

The Specific Activity is Degree of Labeling divided by 0.034.

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

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

Refer to Table 11 for expected yield of labeled DNA and specific activity after labeling and purification.

Table 11 Expected Yield and Specific Activity after Labeling and Purification

Yield (µg)	Specific activity of cyanine 3 labeled sample (pmol/µg)	Specific activity of cyanine 5 labeled sample (pmol/µg)
> 2.5	18 to 25	7 to 20

The cyanine-3 and cyanine-5 yield after labeling should be the same.

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4 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.

Hybridization

If you are new to microarray processing, refer to the "Running a microarray experiment" training presentation, which you can find when you go to http://www.genomics.agilent.com and search on the title of the presentation ("Running a microarray experiment"). This presentation shows you how to hybridize, wash and scan microarray slides.

To practice hybridization, prepare a 1:1 2× HI-RPM Hybridization Buffer and water mix and use a microscope slide or used microarray slide, and a gasket slide. You can use the same slide to practice wash and placement of slide in the slide holder.

Before you begin, make sure you read and understand "Microarray Handling Tips" on page 64.

Step 1. Prepare the 10× Blocking Agent

1 Add 1,350 µL of DNase/RNase-free distilled water to the vial containing lyophilized 10× aCGH Blocking Agent (included in the Oligo aCGH/ChIP-on-chip Hybridization Kit).

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

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

Step 2. Prepare labeled DNA for hybridization

1 Equilibrate water baths or heat blocks to 95°C and 37°C or use a thermal cycler.

2 Mix the components according to the microarray format to prepare the Hybridization Master Mix. Refer to Table 12 through Table 14.

Table 12 Hybridization Master Mix for **1-pack** microarray

Component	Volume (µL) per hybridization	× 8 rxns (µL) (including excess)	× 24 rxns (µL) (including excess)	× 48 rxns (µL) (including excess)
Cot-1 DNA (1.0 mg/mL)*	50	425	1,250	2,500
10× aCGH Blocking Agent†	52	442	1,300	2,600
2× HI-RPM Hybridization Buffer†	260	2,210	6,500	13,000
Deionized Formamide	78	663	1950	3900
Final Volume of Hybridization Master Mix	440	3,740	11,000	22,000

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

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

Table 13 Hybridization Master Mix for **2-pack** microarray

Component	Volume (µL) per hybridization	× 8 rxns (µL) (including excess)	× 24 rxns (µL) (including excess)	× 48 rxns (µL) (including excess)
Cot-1 DNA (1.0 mg/mL)*	25	212.5	625	1,250
10× aCGH Blocking Agent†	26	221	650	1,300
2× HI-RPM Hybridization Buffer†	130	1,105	3,250	6,500
Deionized Formamide	39	331.5	975	1950
Final Volume of Hybridization Master Mix	220	1,870	5,500	11,000

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

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

Table 14 Hybridization Master Mix for **4-pack** microarray

Component	Volume (µL) per hybridization	× 8 rxns (µL) (including excess)	× 24 rxns (µL) (including excess)	× 48 rxns (µL) (including excess)
Cot-1 DNA (1.0 mg/mL)*	5	42.5	125	250
10× aCGH Blocking Agent†	11	93.5	275	550
2× HI-RPM Hybridization Buffer†	55	467.5	1,375	2,750
Deionized Formamide	16.5	140.25	412.25	824.5
Final Volume of Hybridization Master Mix	87.5	743.75	2187.25	4374.5

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

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

3 Add the appropriate volume of the Hybridization Master Mix to the 1.5 mL RNase-free Microfuge TubeTall Chimney PCR plate well that contains the labeled reference or IP DNA to make the total volume listed in Table 15.

Table 15 Volume of Hybridization Master Mix per hybridization

Microarray format	Volume of Hybridization Master Mix	Total volume
1-pack	440 µL	520 µL
2-pack	220 µL	260 µL
4-pack	87.5 µL	110 µL

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

5 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 thermal cycler. Program the thermal cycler according to the following table and run the program:

Table 16 Thermal cycler program

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

6 Remove sample tubes from the water bath, heat block, or thermal cycler. 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.

CAUTION The samples must be hybridized immediately. If not, keep the temperature of hybridization sample mixtures as close to 37°C as possible on a heat block, thermal cycler or in an oven.

Step 3. Prepare the hybridization assembly

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.

Before you begin, make sure you read and understand "Microarray Handling Tips" on page 64.

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 hybridization sample mixture onto the gasket well in a "drag and dispense" manner:
   - 490 µL (for 1-pack microarray)
   - 245 µL (for 2-pack microarray)
   - 100 µL (for 4-pack microarray)

   For multi-pack microarray formats (2-pack or 4-pack), load all gasket wells before you load the microarray slide. For multi-pack formats, refer to "Agilent Microarray Layout and Orientation" on page 65.

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

3. Put a microarray slide "active side" down onto the gasket slide, so the numeric barcode side is facing up and the "Agilent"-labeled barcode is facing down. Assess that the sandwich-pair is properly aligned.
4. Put the SureHyb chamber cover onto the sandwiched slides and slide the clamp assembly onto both pieces.
5. Hand-tighten the clamp firmly onto the chamber.
6. Vertically rotate the assembled chamber to wet the slides and assess the mobility of the bubbles. Tap the assembly on a hard surface if necessary to move stationary bubbles.

Step 4. Hybridize

1 Load each assembled chamber into the oven rotator rack. Start from the center of the rack (position 3 or 4 when counting from the left). Set your hybridization rotator to rotate at 20 rpm.

2 Hybridize at 67°C for:

- 24 hours (4-pack microarrays)
- 40 hours (1-pack and 2-pack microarrays)

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

CAUTION You must calibrate the hybridization oven regularly for accuracy of the collected data. Refer to *Agilent G2545A Hybridization Calibration Procedure* (p/n G2545-90002) for more information.

NOTE The Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 that is used in the microarray wash procedure needs to be warmed overnight. While you are waiting for the microarray slides to hybridize, do the steps in "Step 1. Prewarm Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 (overnight)" on page 43.

Microarray Wash

NOTE

The microarray wash procedure must be done in environments where ozone levels are 5 ppb or less. For Scanner C and Scanner B, if ozone levels are between 5 to 10 in your laboratory, use the Agilent Ozone Barrier Slide Cover. SureScan microarray scanner uses a slide holder with a built-in ozone barrier. If ozone levels exceed 10 ppb, use the Stabilization and Drying Solution together with the ozone barrier.

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

Before you begin, determine which wash procedure to use:

Table 17 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 46	No
> 5 ppb < 10 ppb	"Wash Procedure A (without Stabilization and Drying Solution)" on page 46	Yes
> 10 ppb	"Wash Procedure B (with Stabilization and Drying Solution)" on page 48	Yes

CAUTION

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

- Always use clean equipment when conducting the wash procedures.
- Use only dishes that are designated and dedicated for use in Agilent Methylation experiments.

Step 1. Prewarm Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 (overnight)

The temperature of Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 must be at 37°C for optimal performance.

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

2 Put a slide-staining dish with a lid, a 1.5 L glass dish, and one to two liters of Milli-Q ultrapure water in an incubator or water bath set at 37°C to warm overnight.

Step 2. Wash with Milli-Q ultrapure water

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

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

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

3 Repeat step 1 and step 2 until all traces of contaminating material are removed.

Step 3. Clean with Acetonitrile (Wash Procedure B Only)

Acetonitrile wash removes any remaining residue of Stabilization and Drying Solution from slide-staining dishes, slide racks and stir bars that were used in previous experiments with "Wash Procedure B (with Stabilization and Drying Solution)" on page 48.

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

1. Add the slide rack and stir bar to the slide-staining dish, and transfer to a magnetic stir plate.
2. Fill the slide-staining dish with 100% Acetonitrile.
3. Turn on the magnetic stir plate and adjust the speed to a setting of 4 (medium speed).
4. Wash for 5 minutes at room temperature.
5. Discard the Acetonitrile as is appropriate for your site.
6. Repeat step 1 through step 5.
7. Air dry everything in the vented fume hood.
8. Continue with the Milli-Q ultrapure water wash as previously instructed.

Step 4. Prewarm Stabilization and Drying Solution (Wash Procedure B Only)

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

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

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

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

1 Put a clean magnetic stir bar into the Stabilization and Drying Solution bottle and recap.
2 Partially fill a plastic bucket with hot water at approximately 40°C to 45°C (for example from a hot water tap).
3 Put the Stabilization and Drying Solution bottle into the hot water in the plastic bucket.
4 Put the plastic bucket on a magnetic stirrer (*not a hot-plate*) and stir.
5 The hot water cools to room temperature. If the precipitate has not all dissolved replenish the cold water with hot water.
6 Repeat step 5 until the solution is clear.
7 After the precipitate is completely dissolved, allow the solution to equilibrate to room temperature prior to use.

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

Step 5. Wash microarrays

Wash Procedure A (*without* Stabilization and Drying Solution)

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

Table 18 lists the wash conditions for the Wash Procedure A without Stabilization and Drying Solution.

Table 18 Wash conditions

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

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

3 Prepare dish #3:

   a Put the prewarmed 1.5 L glass dish on a magnetic stir plate with heating element.

   b Put the slide-staining dish #3 into the 1.5 L glass dish.

   c Fill the 1.5 L glass dish with pre-warmed Milli-Q ultrapure water.

   d Fill the slide-staining dish #3 approximately three-fourths full with Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 (warmed to 37°C).

   e Add a magnetic stir bar.

   f Turn on the heating element and maintain temperature of Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 at 37°C. Monitor with a thermometer.

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

5 Prepare the hybridization chamber disassembly.

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

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

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

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

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

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

   b Gently twist the forceps to separate the slides.

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

   d Remove the microarray slide, grasp it from the upper corners with thumb and forefinger, and quickly put into slide rack in the slide-staining dish #2 containing Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1 at room temperature. Minimize exposure of the slide to air. *Touch only the barcode portion of the microarray slide or its edges!*

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

8 When all slides in the group are put into the slide rack in slide-staining dish #2, stir at 350 rpm for 5 minutes. Adjust the setting to get good but not vigorous mixing.

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

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

   a Activate the magnetic stirrer.

   b Wash microarray slides for at least 1 minute and no more than 2 minutes.

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

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

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

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

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

Wash Procedure B (*with* Stabilization and Drying Solution)

Cyanine reagents are susceptible to degradation by ozone. Use this wash procedure if the ozone level exceeds 10 ppb in your laboratory.

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

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

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

Table 19 lists the wash conditions for the Wash Procedure B with Stabilization and Drying Solution.

Table 19 Wash conditions

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

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

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

3 Do step 1 through step 9 in "Wash Procedure A (without Stabilization and Drying Solution)" on page 46.

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

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

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

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

NOTE

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

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

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

Step 6. Put slides in a slide holder

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

For SureScan microarray scanner

1. Carefully place the end of the slide without the barcode label onto the slide ledge.
2. Gently lower the microarray slide into the slide holder. Make sure that the active microarray surface faces up, toward the slide cover.
3. Close the plastic slide cover, pushing on the tab end until you hear it click.

For more detailed instruction, refer to the *Agilent G4900DA SureScan Microarray Scanner System User Guide*.

Figure 2 Slide in slide holder for SureScan microarray scanner

For Agilent Scanner C

- In environments in which the ozone level exceeds 5 ppb, immediately put the slides with Agilent barcode facing up in a slide holder. Make sure that the slide is not caught up on any corner. Put an ozone-barrier slide cover on top of the array as shown in Figure 3. Refer to the *Agilent Ozone-Barrier Slide Cover User Guide* (p/n G2505-90550), included with the slide cover, for more information.

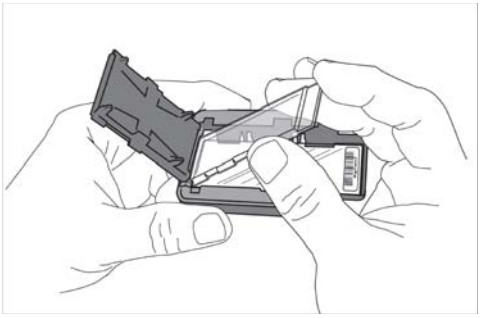

Figure 3 Inserting the ozone-barrier slide cover

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

Microarray Scanning and Feature Extraction

Step 1. Scan the microarray slides

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

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

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

Agilent SureScan Microarray Scanner

1. Put assembled slide holders into the scanner cassette.
2. Select **Protocol AgilentG3_CGH** for G3 microarrays. Select **Protocol AgilentHD_CGH** for HD microarrays.
3. Verify that the Scanner status in the main window says Scanner Ready.
4. Click **Start Scan**.

Agilent C Scanner Settings

1. Put assembled slide holders with or without the ozone-barrier slide cover into scanner carousel.
2. Select Start Slot *m* End Slot *n* where the letter *m* represents the Start slot where the first slide is located and the letter *n* represents the End slot where the last slide is located.
3. Select **Profile AgilentG3_CGH** for G3 microarrays. Select **Profile AgilentHD_CGH** for HD microarrays.
4. Verify scan settings. See Table 20.

Table 20 C Scanner Scan Settings

	For HD Microarray Formats	For G3 Microarray Formats
Dye channel	R+G (*red and green*)	R+G (*red and green*)
Scan region	Agilent HD (61 x 21.6 mm)	Agilent HD (61 x 21.6 mm)
Scan resolution	5 µm	3 µm
Tiff file dynamic range	16 bit	16 bit
Red PMT gain	100%	100%
Green PMT gain	100%	100%
XDR	<No XDR>	<No XDR>

5 Check that **Output Path Browse** is set for desired location.

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

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

Agilent B Scanner Settings

Agilent Scanner Control software v7.0.03 is recommended for 5 µm scans of HD format microarrays.

1 Put assembled slide holders, with or without the ozone-barrier slide cover, into scanner carousel.

2 Verify Default Scan Settings (click **Settings > Modify Default Settings**).

Table 21 B Scanner Scan Settings

	For HD Microarray Formats
Scan region	Scan Area (61 x 21.6 mm)
Scan resolution (µm)	5
eXtended Dynamic range	(cleared)
Dye channel	Red&Green
Red PMT	100%
Green PMT	100%

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

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

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

Step 2. Extract data using the Feature Extraction program

The Feature Extraction software v10.5 or higher supports extraction of microarray TIFF images (.tif) of Agilent Methylation microarrays scanned on the Agilent SureScan or C Scanner.

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

Feature Extraction version 10.7 or higher can automatically download Grid Templates, protocols and QC metrics (QCMT) from SureDesign if configured appropriately. See "Automatic Download from SureDesign" on page 59 for configuration.

Figure 4 shows an example of an Agilent SurePrint G3 1×1M microarray image opened in the Feature Extraction software.

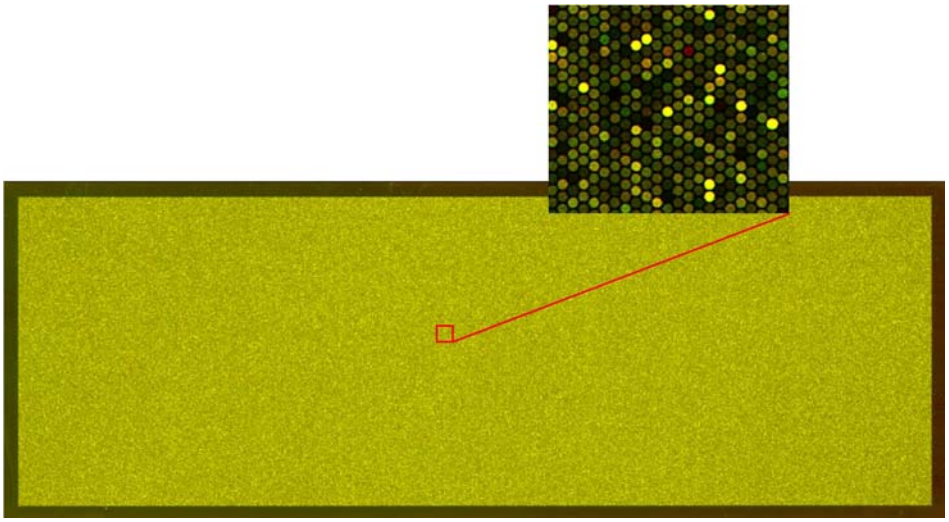

Figure 4 Agilent SurePrint G3 1×1M microarray shown in red and green channels, full and zoomed view

1 Open the Agilent Feature Extraction program.

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

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

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

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

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

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

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

3 Set Feature Extraction Project Properties.

a Select the **Project Properties** tab.

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

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

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

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

QC metrics updates are available automatically from SureDesign if configured appropriately. See "Automatic Download from SureDesign" on page 59 for configuration.

Project Properties | Extraction Set Configuration

General	
Operator	Unknown
Input	
Number of Extraction Sets Included	0
Output and Data Transfer	
Outputs	
MAGE	None
JPEG	None
TEXT	Local file only
Output Package	Compact
Visual Results	Local file only
Grid	None
QC Report	Local PDF file only
FTP Send Tiff File	False
Local File Folder	
Same As Image	True
Results Folder	
FTP Setting	
Automatic Protocol Assignment	
Highest Priority Default Protocol	Grid Template Default
Project Default Protocol	
Automatic Grid Template Assignment	
Use Grid file if available	False
External DyeNorm List File	
Overwrite Previous Results	False

Figure 5 Default settings in Feature Extraction 10.5

4 Check the Extraction Set Configuration.

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

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

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

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

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

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

      Protocols are also available automatically from SureDesign if configured appropriately. See "Automatic Download from SureDesign" on page 59 for configuration.

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

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

7 Select **Project > Start Extracting**.

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

Automatic Download from SureDesign

Feature Extraction version 10.7 or higher can automatically download Grid Templates, protocols and QC metrics (QCM or QCMT). To set this up, in the eArray Login Setting dialog box, under **Advanced Options**, click **Use eArray server during extraction**. See Figure 6.

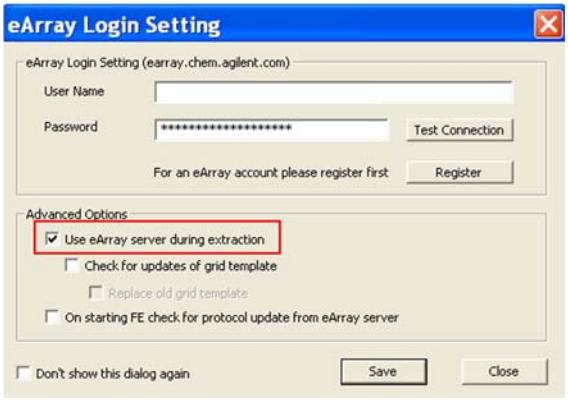

Figure 6 eArray Login Setting. You can mark the other two check boxes under Advanced Options if you want to get update of grid templates already in the database or to get protocol updates. See the Feature Extraction user guide for more information.

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5 Reference

This chapter contains reference information that pertains to this protocol.

Reagent Kit Components

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

Table 22 SureTag DNA Labeling Kit

Component
10× Restriction Enzyme Buffer*
BSA*
Alu I*
Rsa I*
Purification Column
Nuclease-Free Water
Exo (-) Klenow
5× Reaction Buffer
Cyanine 5-dUTP
Cyanine 3-dUTP
10× dNTPs
Random Primer

* Not used in this protocol.

Table 23 Oligo aCGH/ChIP-on-chip Hybridization Kit

Component
2× HI-RPM Hybridization Buffer
10× aCGH Blocking Agent

Table 24 Oligo aCGH/ChIP-on-chip Wash Buffer Kit

Component
Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1
Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2

Microarray Handling Tips

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

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

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

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

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

Agilent Microarray Layout and Orientation

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

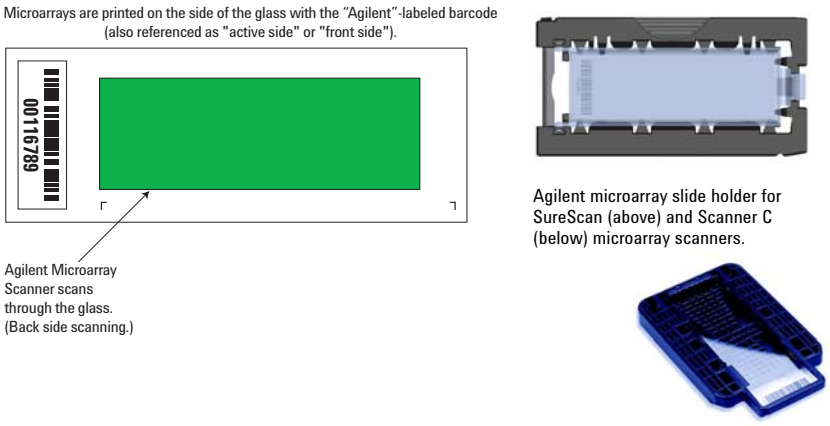

Figure 7 Agilent microarray slide and slide holder

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

Figure 7 depicts how the Agilent microarray scanner reads the microarrays and how this relates to the "microarray design files" that Agilent generates during the manufacturing process of its *in situ*-synthesized oligonucleotide microarrays. Thus, if you have a scanner that reads microarrays from the "front side" of the glass slide, the collection of microarray data points will be different in relation to the "microarray design files". Therefore, please take a

moment to become familiar with the microarray layouts for each of the Agilent oligo microarrays and the layout information as it pertains to scanning using a “front side” scanner.

Non-Agilent Front Side Microarray Scanners

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

- If the scanner images the microarrays by reading them on the “front side” of the glass slide (“Agilent”-labeled barcode side of the slide) and
- If the microarray image produced by the non-Agilent scanner is oriented in a “portrait” or “landscape” mode, and “Agilent”-labeled barcode is on the left-side, right-side, up or down, as viewed as an image in the imaging software (see Figure 8).

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

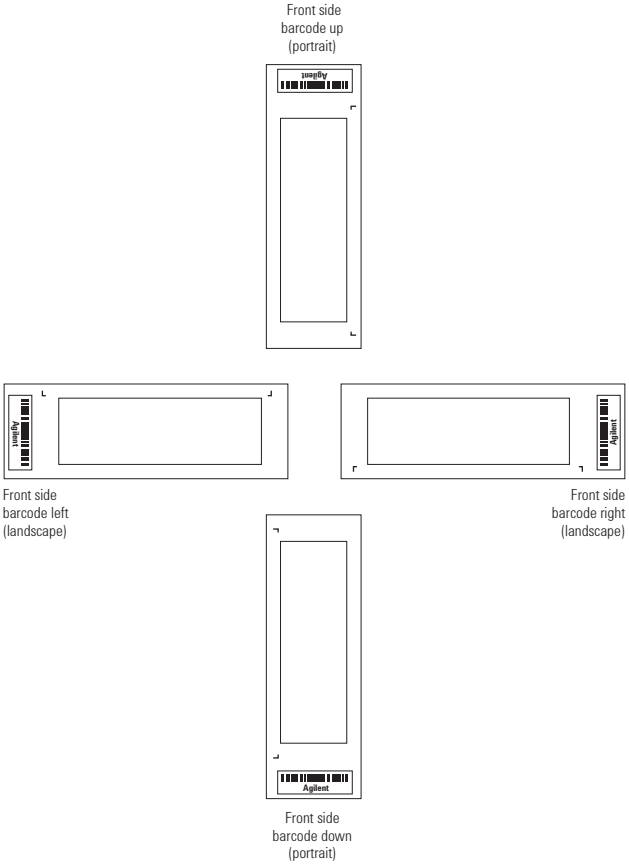

Figure 8 Microarray slide orientation

Array/Sample tracking on microarray slides

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

Arrays

	Array 1_1	**Array 1_2**
BARCODE	**Sample:**	**Sample:**

Barcode Number _____

Figure 9 2-pack microarray slides

Arrays

	Array 1_1	Array 1_2	Array 1_3	Array 1_4
BARCODE	Sample:	Sample:	Sample:	Sample:

Barcode Number _____

Figure 10 4-pack microarray slides

Notes and Considerations

Methylated DNA IP lets investigators capture DNA sequences and study their relative methylation levels across an entire genome. The protocol requires an antibody to 5-methyl cytosine that will immunoprecipitate methylated DNA from a DNA sample.

The Methylated DNA Immunoprecipitation protocol consists of five general steps:

- DNA shearing
- Methylated DNA immunoprecipitation
- DNA labeling
- Microarray hybridization and washing
- Microarray scanning and storage

This reference summarizes the goals and steps for this protocol. Other considerations outside of this protocol include initial probe and microarray design and the design and implementation of robust quantitative metrics that validate success at multiple steps of the protocol.

1. Chromatin immunoprecipitation (ChIP)

Goal Use selective antibody bound to magnetic beads to specifically capture the Methylated DNA.

SOP

1. Mix antibody bound to magnetic beads (Dynal) with DNA sample.
2. Place at 4°C overnight on a rotating platform.
3. Isolate the beads containing the antibody bound to the methylated DNA.
4. Wash 2 times with buffer to remove non-specific contaminants.
5. After the wash, heat the complexes for a few minutes with detergent to elute the methylated DNA from the antibody and beads.

Key variables

- Beads, type, and quantity
- Time
- Temperature
- Immunoprecipitation buffer, volume, and composition
- Wash buffer composition
- Number of washes

QC Metrics After the DNA is isolated (step 2), you can run the Bioanalyzer or gel equivalent to assess fragment size after shearing. Use 1 µL for the Bioanalyzer, or 5 µL on a 1.5% agarose gel.

Notes Magnetic beads coated with protein G are routinely used due to their ease-of-use and ability to bind a variety of antibodies. Other coatings (e.g. protein A) and bead types (e.g. agarose) are available but have not been validated by Agilent.

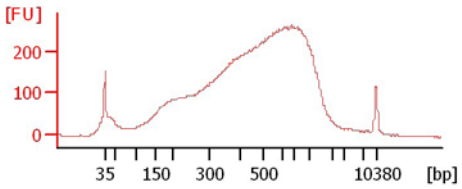

Figure 11 Human liver DNA (Biochain p/n D1234149) was sonicated following the procedure outlined in "Step 2. Prepare DNA for Immunoprecipitation" on page 21. 1 µL (~20 ng) of sheared DNA was analyzed using the Agilent 2100 Bioanalyzer in conjunction with the DNA High Sensitivity Kit (p/n 5067-4626). The expected size distribution is between 200 and 1000 bp.

2. DNA isolation

Goal Purify DNA from associated proteins and RNA and protein contaminants.

SOP

1 Purify the DNA via organic extraction and ethanol precipitation.

Key variables

- Temperature
- Time
- SDS concentration

QC Metrics None

3. DNA labeling

Goal Incorporate fluorescent-tagged nucleotides into the IP and reference DNA for hybridization.

SOP

1 Use the Agilent SureTag DNA Labeling Kit.

2 Do 1 Labeling reaction for each sample. Approximately 1 µg input per reaction for the reference channel cyanine 3, and all IP DNA for the cyanine 5.

3 Anneal random primers to the DNA.

4 Extend primers using high concentration exo- Klenow enzyme and fluorescent-labeled nucleotides.

5 Purify labeled DNA using the Amicon 30 kDa columns.

Key variables

- Reaction size
- Reagent quantity (input DNA material, Cy dye, enzyme) per reaction

QC Metrics Nanodrop measurement of total DNA yield (expect >2.5 µg per reaction); Nanodrop measurement of pmol/µL dye (expect >2 pmol/µL with Cy5-dUTP and >3 pmol/µL Cy3-dUTP).

4. Microarray hybridization and washing

Goal Hybridize material to and wash excess/nonspecific material from Agilent 60-mer oligo arrays to yield low background and high signal ("flat" background with high peaks)

SOP

1. Hybridize for 40 hours at 67°C in hybridization oven rotating at 20 rpm. Hybridization buffer contains a proprietary wetting agent (that keeps bubbles moving freely), approximately 5 µg labeled DNA per channel (10 µg total) and competitor nucleic acids.
2. Wash slides in a series of two buffers with an optional 3rd wash that contains ozone-scavenging reagents to help prevent premature dye degradation.

Key variables

- Hybridization duration
- Quantity of labeled material
- Temperature
- Type and quality of detergent
- Type and quantity of nucleic acid competitors

Notes These conditions are identical to those developed for Agilent aCGH hybridizations. Refer to the Bioreagent Wash/Dry Solution application note for more information. The wash conditions are specific for Agilent's ChIP-on-chip application.

5. Microarray scanning and storage

Goal Extract data from microarray; store microarray for possible future analysis

SOP

1. Use default settings on Agilent scanner.
2. Store used slides in N_2 box.

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