Agilent Microarray Analysis of Methylated DNA Immunoprecipitation

Protocol

Version 1.0, May 2008

Research Use Only. Not for Diagnostic Procedures

Agilent Technologies

Notices

Manual Part Number

G4170-90012

Edition

Version 1.0, May 2008

Agilent Technologies, Inc.
5301 Stevens Creek Blvd.
Santa Clara, CA 95051

Research Use Only. Not for diagnostic procedures.

To provide feedback to this protocol, please send e-mail to feedback_genomics@agilent.com.

Warranty

The material contained in this document is provided "as is," and is subject to being changed, without notice, in future editions. Further, to the maximum extent permitted by applicable law, Agilent disclaims all warranties, either express or implied, with regard to this manual and any information contained herein, including but not limited to the implied warranties of merchantability and fitness for a particular purpose. Agilent shall not be liable for errors or for incidental or consequential damages in connection with the furnishing, use, or performance of this document or of any information contained herein. Should Agilent and the user have a separate written agreement with warranty terms covering the material in this document that conflict with these terms, the warranty terms in the separate agreement shall control.

Technology Licenses

The hardware and/or software described in this document are furnished under a license and may be used or copied only in accordance with the terms of such license.

Restricted Rights Legend

U.S. Government Restricted Rights. Software and technical data rights granted to the federal government include only those rights customarily provided to end user customers. Agilent provides this customary commercial license in Software and technical data pursuant to FAR 12.211 (Technical Data) and 12.212 (Computer Software) and, for the Department of Defense, DFARS 252.227-7015 (Technical Data - Commercial Items) and DFARS 227.7202-3 (Rights in Commercial Computer Software or Computer Software Documentation).

Safety Notices

CAUTION

A **CAUTION** notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in damage to the product or loss of important data. Do not proceed beyond a **CAUTION** notice until the indicated conditions are fully understood and met.

WARNING

A WARNING notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in personal injury or death. Do not proceed beyond a WARNING notice until the indicated conditions are fully understood and met.

What's in this Protocol?

This new protocol describes:

- Use of the G4492A Human CpG Island Arrays and the Mouse G4811A CpG Island Arrays for the analysis of Methylated DNA Immunoprecipitation (MeDIP).
- Process for isolating methylated DNA from Purified Genomic DNA samples.
- Labeling, hybridization and wash of microarrays.
- Array scanning with Agilent G2565BA Microarray Scanner
- Feature extraction with Agilent G4460AA Feature Extraction software v9.3.5 or higher

Contents

1 Sample Preparation

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

The reagents, enzymes and buffers that are used in this protocol are listed in "Reagents, Enzymes, Buffers and Equipment" on page 42.

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 hr, then 40 hr
Microarray washing	1 hr

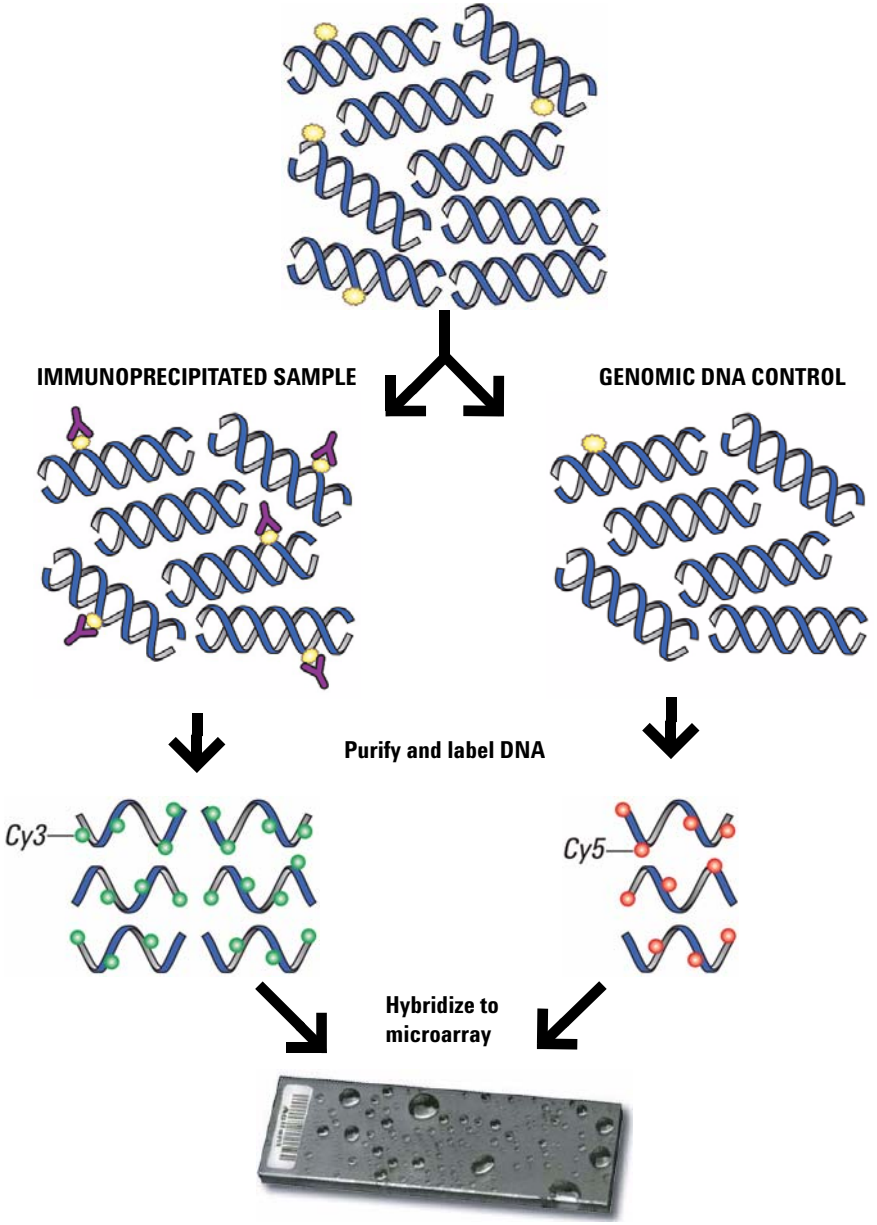


Figure 1 Methylated Genomic DNA overview

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 1 tube for each immunoprecipitation.

2 Vigorously mix bottle of pan-mouse IgG Dynal (Invitrogen) magnetic beads in a vortex mixer to resuspend. Dynal beads will have settled during storage.

3 Immediately add 50 µL Dynal magnetic beads to a 1.5 mL microfuge tube.

4 Place on a magnetic separation stand, such as a Dynal Magnetic Particle Concentrator (product number 120.20).

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 Block Solution 1 and resuspend gently. See Table 2 on page 10.

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 Block Solution 2 (see Table 3 on page 10) and add 5 µg of 5-methylcytosine antibody (Eurogentec, catalog number BI-MECY-1000) per immunoprecipitation.

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 (such as a Labquake Mixer, p/n 56264-306) 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 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.

Table 2 Block Solution 1

Stock	Amount	Final Concentration
PBS	100 mL	1X
BSA	500 mg	0.5% BSA (weight/volume)

Table 3 Block Solution 2*

Stock	Amount	Final Concentration
PBS	100 mL	1X
BSA	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.

Step 2. Prepare DNA for Immunoprecipitation

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

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 600 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.

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 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 9 to the 1.5 mL tube containing the 200 µL of sheared genomic DNA from "Step 2. Prepare DNA for Immunoprecipitation" on page 11.

2 Add 250 µL of 2X IP Buffer from Table 4.

Table 4 2X IP Buffer

	Final concentration in 2X buffer	Volume
1% Triton in PBS	0.05%	200 µL
Yeast tRNA (Invitrogen cat#15401-011)	50 µg/ml	8 µL
Phosphate Buffered Saline		3.8 mL
		4 mL (Total)

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 IP Wash Buffer (see Table 5) to each tube. Remove tubes from magnetic device and shake or agitate tube gently to resuspend beads. Rotate beads for 3 minutes at 4°C. Replace tubes in magnetic device to collect beads. 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

3 Remove any residual IP Wash Buffer with a pipette.

Elution

1 Add 150 µL of Elution Buffer (see Table 6) and resuspend beads.

Table 6 Elution Buffer 1% SDS in TE

	Volume
TE Buffer	45 mL
10% SDS	5 mL

2 Elute by placing 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 microfuge tube.

The supernatant contains your IP DNA. Do not discard.

7 Repeat elution 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 11, for a total volume of 300 µL.
2. Add 300 µL phenol:chloroform:isoamyl alcohol to each immunoprecipitate and reference tube.
3. Mix the samples on a vortex mixer for 20 seconds.
4. Spin on a centrifuge one 1.5 mL Maxtract high density extraction column (Fischer Scientific catalog number FP2302820) 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 column.
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 microfuge tube and dispose the extraction column.
8. To each tube that contains the aqueous layer, add:
   - 16 µL of 5M NaCl (200 mM final concentration)
   - 1.5 µL of 20 µg/µL glycogen (Invitrogen)
   - 880 µL EtOH
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 EtOH.
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, resuspend your DNA in 31 µL of nuclease-free water, then keep 5 µL for additional sample analysis. Otherwise, resuspend in 26 µL of nuclease-free water.

2 Sample Labeling

The Agilent Genomic DNA Labeling Kit PLUS (Agilent p/n 5188-5309) uses random primers and the exo- Klenow fragment to differentially label genomic DNA samples with fluorescent-labeled nucleotides. For Agilent's Methylation application, the IP 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 in the green channel (Cyanine 3) and the IP sample is labeled in the red channel (Cyanine 5).

Step 1. Label DNA with fluorescent dye

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.

For 1x244K, 2x105K and 4x44K Microarrays

1 Equilibrate water baths to 95°C, 37°C and 65°C. You can also use a thermocycler for these heating steps.

2 Add 5 µL of Random Primers (supplied with Agilent Genomic DNA Labeling Kit PLUS) to each reaction tube containing 26 µL of either reference or immunoprecipitated DNA from "Step 5. Extract immunoprecipitated and reference DNA with phenol-chloroform" on page 15 to make a total volume of 31 µL. Use a pipette to draw in and release the mixture several times to gently mix well.

3 Transfer sample tubes to a circulating water bath or heat block at 95°C. Incubate at 95°C for 3 minutes, then move to ice and incubate for 5 minutes.

You may want to use protective tube locks so that the tubes do not open while in the 95°C incubation.

4 Prepare the Labeling Master Mix by mixing the components in Table 7 on ice in the order indicated.

Table 7 Preparation of Labeling Master Mix (for 1x244k, 2x105K and 4x44K microarrays)

Component	Per reaction (µL)	× 6 rxns (µL) (including excess)	× 12 rxns (µL) (including excess)
5X Buffer	10.0	65.0	125.0
10X dNTP	5.0	32.5	62.5
Cyanine 3-dUTP (1.0 mM) *or* Cyanine 5-dUTP (1.0 mM)	3.0	19.5	37.5
Exo-Klenow fragment	1.0	6.5	12.5
Final volume of Labeling Master Mix	**19.0**	**123.5**	**237.5**

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

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

7 Transfer sample tubes to a circulating water bath at 65°C. Incubate at 65°C for 10 minutes to inactivate the enzyme, then move to ice.

8 Reactions can be stored overnight at -20°C in the dark.

Step 2. Clean up labeled genomic DNA

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

1. Transfer samples to a 1.5 mL nuclease-free microfuge tube.
2. Add 430 µL of 1X TE (pH 8.0) to each reaction tube.
3. For each labeled sample, place a Microcon YM-30 size exclusion filter into the supplied 1.5-mL microfuge tube and load each labeled reference sample or IP sample 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 12 minutes at 8,000 × g in a microcentrifuge at room temperature. Discard the flow-through.

NOTE This spin time can be adjusted to meet your sample volume needs in step 6. Increase spin time for less volume, decrease for more volume.

5. Invert the filter into a new 1.5-mL microfuge tube (supplied). Spin in a centrifuge for 1 minute at 8,000 × g at room temperature to collect purified sample.

   Keep the filter until you are certain that you have collected all of the sample.
6. Measure and record volume (µL) of each eluate. If sample volume exceeds the target volume listed in Table 8, return sample to its filter and spin in a centrifuge for 1 minute at 8,000 × g at room temperature. Discard the flow-through.

Table 8 Target Sample Volume

Microarray Format	Volume
1x244K	42 µL
2x105K	22 µL
4x44K	13 µL

7. Repeat step 5 and step 6 until each sample volume is less than or equal to the target sample volume in Table 8.

8 Bring your total sample volume up to the appropriate volume listed in Table 8 with 1X TE (pH 8.0) buffer.

9 Take 1.5 µL of each sample and use the NanoDrop ND-1000 UV-VIS Spectrophotometer to determine the yield and specific activity of each of your samples:

DNA yield	> 2.5 µg per reaction
Cyanine 5	7 to 20 pmol/µg
Cyanine 3	18 to 25 pmol/µg

10 Combine the appropriate cyanine 5-labeled sample and cyanine 3-labeled sample for a total mixture volume listed in Table 9 in a new 1.5-mL heat-resistant microfuge tube.

Table 9 Total Mixture Volume

Microarray Format	Volume
1x244K	80 µL
2x105K	79 µL
4x44K	39 µL

Labeled samples can be stored overnight at -20°C in the dark.

3 Hybridization and Wash

In this section, you hybridize and wash Methylation microarrays.

Hybridization and Wash

This section contains information to help you hybridize and wash your microarray using the SureHyb Hybridization Chamber. If you need more information, please refer to the *Oligonucleotide Array-Based CGH for Genomic DNA Analysis Protocol*, part number G4410-900010.

Step 1. Hybridize the microarray

1 Prepare the 10X Blocking Agent:

   a Add 1350 µL of nuclease-free water to the vial containing lyophilized 10X Blocking Agent (supplied with Agilent Oligo aCGH Hybridization Kit).

   b If you need to reconstitute the sample, incubate at room temperature for 60 minutes. Make sure that the Blocking agent is completely reconstituted before use.

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

NOTE Use aluminum foil and amber tubes to keep samples in the dark as much as possible.

The next steps are an abbreviated version of the Agilent aCGH array hybridization protocol. Please refer to the *Oligonucleotide Array-based CGH for Genomic DNA Analysis User Guide* (G4410-90010) if you require additional details.

2 Equilibrate water baths or heat blocks to 95°C and 37°C.

3 Combine Cy5- and Cy3-labeled samples with ddH_2O in a 1.5 mL microfuge tube for a total volume as indicated in Table 10.

Table 10

	4x44K Array Format	2x105K Array Format	1x244K Array Format
Cy5-labeled Samples	2.5 to 5.0 µg	2.5 to 5 µg	2.5 to 5 µg
Cy3-labeled Samples	2.5 to 5.0 µg	2.5 to 5 µg	2.5 to 5 µg
Total Volume with ddH_20	22.5 µL	40 µL	80 µL

4 Add the following in the order indicated to the microfuge tube:

Table 11 Hybridization Mix

Stock	4x44K Array Format (µL)	2x105K Array Format (µL)	1x244K Array Format (µL)
Cyanine 5- and cyanine 3-labeled gDNA mixture	22.5	40	80
Cot-1 DNA	5	25	50
Agilent 10X Blocking Solution	11	26	52
De-ionized Formamide	16.5	39	78
Agilent 2X Hyb Buffer	55	130	260

NOTE When you first open the deionized formamide, separate into smaller aliquots and store at -80°C.

5 Mix contents and quick spin to collect.

6 Heat samples for 3 minutes at 95°C.

7 Immediately transfer the sample tubes to a circulating water bath or heat block at 37°C and incubate for 30 minutes.

8 Spin at 17,900 × g for 1 minute at room temperature to collect the sample.

9 Load a clean gasket slide into the Agilent SureHyb chamber base with the 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 misaligned.

Refer to the *Agilent Microarray Hybridization Chamber User Guide* (G2534-90001) for in-depth instructions on how to load slides, assembly and disassembly of chambers, as well as other helpful tips. This user guide is available with the Agilent Microarray Hybridization Chamber Kit (G2534A) and can also be downloaded from the Agilent Web site at www.agilent.com/chem/dnamanuals-protocols.

10 Slowly dispense hybridization sample onto the gasket well in a "drag and dispense" manner in this amount:

4x44K format	100 µL/array (4 individual samples of 100 µL each)
2x105K format	250 µL (2 individual samples of 240 µL each)
1x244K format	490 µL

11 Place a microarray "active side" down onto the SureHyb gasket slide, so the numeric barcode side is facing up and the "Agilent" barcode is facing down. Verify that the sandwich-pair is properly aligned.

NOTE

Multi-pack users: When you form the sandwich pair, keep the array parallel to the gasket as you lower it so that the gasket surfaces contact the active side of the slide evenly.

12 Place the SureHyb chamber cover onto the sandwiched slides and slide on the clamp assembly. Hand-tighten the clamp onto the chamber.

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

14 Place assembled slide chamber in rotisserie hybridization oven set to 67°C. Hybridize as follows:

4x44K format	Hybridize at 20 RPM for 24 hours
1x244K or 2x105K formats	Hybridize at 20RPM for 40 hours

Step 2. Prepare Wash Buffer 2

Warm the **Oligo aCGH/ChIP-on-chip Wash Buffer 2** to 37°C as follows:

1. Dispense 1000 mL of Oligo aCGH/ChIP-on-chip Wash Buffer 2 directly into a sterile 1000-mL bottle. Repeat until you have enough prewarmed Wash Buffer 2 solution for your experiment.
2. Tightly cap the 1000-mL bottle and place in a 37°C water bath the night before washing arrays. Alternatively, remove the plastic cubitainer from the box and place it in a 37°C water bath the night before washing the arrays.
3. Put a slide-staining dish into a 1.5 L glass dish three-fourths filled with water. Warm to 37°C by storing overnight in an incubator set to 31°C.

Step 3. Prepare the equipment

Always use clean equipment when doing the hybridization and wash steps. Designate and dedicate dishes to two-color experiments. The acetonitrile wash is only necessary if the staining dishes, racks and stir bars were used in previous experiments with the Agilent Stabilization and Drying Solution. Otherwise proceed to "Milli-Q water wash" on page 28.

Acetonitrile wash

Wash staining dishes, racks and stir bars that were used in previous experiments with the Agilent Stabilization and Drying Solution with acetonitrile to remove any remaining residue.

WARNING **Conduct acetonitrile washes in a vented fume hood.**

1. Add the slide rack and stir bar to the staining dish.
2. Transfer the staining dish with the slide rack and stir bar to a magnetic stir plate.
3. Fill the staining dish with 100% acetonitrile.
4. Turn on the magnetic stir plate and adjust the speed to a setting of 4 (medium speed).
5. Wash for 5 minutes.

6 Discard the acetonitrile as is appropriate for your site.

7 Repeat steps step 1 through step 6.

8 Air dry the staining dish in the vented fume hood.

9 Proceed to "Milli-Q water wash" below.

Milli-Q water wash

Wash all dishes, racks, and stir bars with Milli-Q water.

1 Run copious amounts of Milli-Q water through the staining dish.

2 Empty out the water collected in the dish.

3 Repeat steps 1 and 2 at least 5 times, as it is necessary to remove any traces of contaminating material.

4 Discard the Milli-Q water.

CAUTION Some detergents may leave fluorescent residue on the dishes. Do not use any detergent in the washing of the staining dishes. If detergent is used, all traces must be removed by copiously rinsing with Milli-Q water.

Step 4. Wash the microarray slides

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 is designed to protect against ozone-induced degradation of cyanine dyes. Use this solution when working with Agilent oligo-based microarrays in high ozone environments.

NOTE When setting up the apparatus for the washes, be sure to do so near the water bath containing the pre-warmed Wash 2 solutions.

Before you begin, determine which wash procedure to use:

- If you are washing slides in an ozone-controlled environment, and the ozone level is 5 ppb or less, then use the standard wash procedure, which *does not* include the optional steps listed in Table 12.
- If you are washing slides in an environment in which the ozone level exceeds 5 ppb, do the optional 3rd and 4th washes listed in Table 12. These steps are described in "Stabilization and Drying Solution (optional)" on page 32.

Table 12 Wash conditions

	Dish	Wash Buffer	Temperature	Time
Disassembly	1	Oligo aCGH/ChIP-on-chip	Room temperature	
1st wash	2	Oligo aCGH/ChIP-on-chip	Room temperature	5 minutes
2nd wash	3	Oligo aCGH/ChIP-on-chip	37°C	1 minutes
3rd wash (optional)	4	Wash Buffer: ACN	Room temperature	10 seconds
4th wash (optional)	5	Wash Buffer: Stabilization and Drying Solution	Room temperature	30 seconds

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

2 Place a slide rack into slide-staining dish #2. Add a magnetic stir bar. Fill slide-staining dish #2 with enough Oligo aCGH/ChIP-on-chip Wash Buffer 1

at room temperature to cover the slide rack. Place this dish on a magnetic stir plate.

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

4 Prepare the hybridization chamber disassembly.

   a Place the hybridization chamber assembly on a flat surface and loosen the thumbscrew, turning counterclockwise.

   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/ChIP-on-chip Wash Buffer 1.

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

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

   b Gently turn the forceps upwards or downwards to separate the slides.

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

   d Remove the microarray slide and place into slide rack in the slide-staining dish #2 containing Oligo aCGH/ChIP-on-chip Wash Buffer 1 at room temperature. Minimize exposure of the slide to air. *Touch only the barcode portion of the microarray slide or its edges!*

   More effort is needed to separate the multi-pack than the single-pack sandwiched slides.

6 Repeat steps step 3 through step 5 for up to seven additional slides in the group. For uniform washing, do up to a maximum of eight disassembly procedures yielding eight microarray slides.

7 When all slides in the group are placed into the slide rack in slide-staining dish #2, stir using setting 4 for 5 minute.

8 During this wash step, remove Oligo aCGH/ChIP-on-chip Wash Buffer 2 from the 37°C water bath and pour into the slide-staining dish #3.

9 Approximately 1 min before the end of Wash1, 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/ChIP-on-chip Wash Buffer 2 (warmed to 37°C). Add a magnetic stir bar. Turn on the heating element and maintain temperature of Oligo aCGH/ChIP-on-chip Wash Buffer 2 at 37°C. Monitor using a thermometer.

10 After the 5 minute wash, transfer the slide rack into slide staining dish #3, which contains Oligo aCGH/ChIP-on-chip Wash Buffer 2 at 37°C.

11 Wash for 1 minute.

12 Slowly remove the slide rack to minimize droplets on the slides. Take 5 to 10 seconds to remove the slide rack.

13 If you are washing slides in an environment in which the ozone level exceeds 5 ppb, continue at "Stabilization and Drying Solution (optional)" on page 32.

14 Discard used Oligo aCGH/ChIP-on-chip Wash Buffer 1 and Oligo aCGH/ChIP-on-chip Wash Buffer 2.

15 Repeat step 1 through step 14 for the next group of eight slides using fresh Oligo aCGH/ChIP-on-chip Wash Buffer 1 and Oligo aCGH/ChIP-on-chip Wash Buffer 2 pre-warmed to 37°C.

16 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.

NOTE

Use fresh Oligo aCGH/ChIP-on-chip Wash Buffer 1 and 2 for each wash group (up to 8 slides).

Stabilization and Drying Solution (optional)

Cy5 has been shown to be sensitive to ozone degradation. Ozone levels as low as 5 ppb (approximately 10 µg/m^3) can affect Cy5 signal and compromise microarray results. The Agilent Stabilization and Drying Solution is designed to protect against ozone-induced degradation of Cy dyes. Use this solution when working with Agilent oligo-based microarrays in high ozone environments.

Acetonitrile wash removes any remaining residue of Agilent Stabilization and Drying Solution from slide-staining dishes, slide racks and stir bars.

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

Step 1. Prewarm Stabilization and Drying Solution

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 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 Warm the solution slowly in a water bath set to 37°C to 40°C in a closed container with sufficient head space to allow for expansion. Warm the solution only in a controlled and contained area that meets local fire code requirements.

   The original container can be used to warm the solution. Container volume is 700 mL and contains 500 mL of liquid. If a different closed container is used, maintain or exceed this headspace/liquid ratio. The time needed to completely redissolve the precipitate is dependent on the amount of precipitate present, and may require overnight warming if precipitation is heavy.

2 Gently shake the container to obtain a homogenous solution.

3 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 2. Wash microarray with Stabilization and Drying Solution

1. In the fume hood, fill slide-staining dish #1 approximately three-fourths full with acetonitrile. Add a magnetic stir bar and place this dish on a magnetic stir plate.
2. In the fume hood, fill slide-staining dish #2 approximately three-fourths full with Stabilization and Drying Solution. Add a magnetic stir bar and place this dish on a magnetic stir plate.
3. If you are hybridizing with the Tecan HS Pro Hybridization Station, remove the slides from the MTP slide adapter and put them in a slide rack.
4. Immediately transfer the slide rack to slide-staining dish #1 containing acetonitrile, and stir using setting 4 for 10 seconds.
5. Transfer slide rack to slide-staining dish #2 filled with Stabilization and Drying Solution, and stir using setting 4 for 30 seconds.
6. Slowly remove the slide rack while you minimize droplets on the slides. Take 5 to 10 seconds to remove the slide rack.

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). 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.

7. 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.
8. Dispose of acetonitrile and Stabilization and Drying Solution as flammable solvents.
9. If you are hybridizing with the SureHyb Hybridization Chamber, return to step 15 on page 31.

4 Scanning and Feature Extraction

In this section, you scan and extract data from Methylation microarrays.

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

Step 1. Scan the slides

Agilent Scanner Settings

1 Assemble the slides into an appropriate slide holder, either version B or A. Place the slides into the slide holder such that the numeric barcode side is visible (*not* the "Agilent"-labeled barcode side). Refer to "General Microarray Layout and Orientation" on page 49.

2 Place assembled slide holders into scanner carousel.

3 Verify scan settings for two-color scans.

Table 13 Scan Settings

	For 1x244K, 2x105K and 4x44K Formats
Scan region	Scan Area (61 x 21.6 mm)
Scan resolution (µm)	5
Dye channel	Red&Green
Green PMT	100%
Red PMT	100%

To change any settings, click **Settings > Modify Default Settings**. A window pops up from which you can change the settings.

4 Select settings for the automatic file naming

- **Prefix 1** is set to **Instrument Serial Number**
- **Prefix 2** is set to **Array Barcode**

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.

Gene Pix scanner settings

Only GenePix 4000A and 4000B scanners are supported for scanning Agilent microarrays.

Refer to the manufacturer's user guide for appropriate scanner settings.

Refer to "General Microarray Layout and Orientation" on page 49 for appropriate slide layout and orientation in GenePix scanner.

NOTE

Agilent 1x244K, 2x105K, and 4x44K microarrays require 5 µm scan resolution, which is not supported by the GenePix 4000A.

Step 2. Extract data using Agilent Feature Extraction Software

Feature Extraction is the process by which information from probe features is extracted from microarray scan data, allowing researchers to measure gene expression in their experiments. To get the most recent Feature Extraction software for gene expression, go to the Agilent Web site at www.agilent.com/chem/fe.

Feature Extraction (FE) 9.5.3 supports extraction of two-color .tif images of Agilent microarrays scanned on Agilent Scanner or GenePix (Axon/Molecular Devices) scanner.

After generating the microarray scan images, extract .tif images using the Feature Extraction software.

1 Open the Agilent Feature Extraction (FE) software version 9.5.3.

   To get the most recent Feature Extraction protocols for gene expression, go to the Agilent Web site at www.agilent.com/chem/FEprotocols.

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 or GenePix (4000A or 4000B) and have an Agilent barcode.
   - For auto assignment of the ChIP-on-Chip FE protocol, the **default Methylation protocol** must be selected 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** text box.

   c In the **Input** section, verify that at least the following default settings as shown in Figure 2 below are selected.

For outputs that can be imported into Rosetta Resolver, select MAGE and JPEG.

⊟ General	
Operator	Unknown
⊟ Input	
Number of Extraction Sets Included	0
⊟ Output and Data Transfer	
⊟ Outputs	
⊞ MAGE	None
JPEG	None
⊞ TEXT	Local file only
Visual Results	Local file only
Grid	Local file only
QC Report	Local 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	True
⊟ Other	
QC Metric Set	
External DyeNorm List File	
Overwrite Previous Results	False

Figure 2 Default settings

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**. Browse 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.

NOTE The grid template file for Human and Mouse CpG Island arrays have been modified to exclude probes with high Tms from analysis by the Feature Extraction Software.

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

c Verify that the protocol **ChIP-v1_95_May07** is assigned to each extraction set in the **Protocol Name** column. To assign a different protocol to an extraction set, select one from the pull-down menu.

The protocols automatically distinguish the formats for processing the data.

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 **FE Protocol Browser**, select **Import**. Browse for the FE protocol (.xml) and click **Open** to load the protocol into the FE database. Visit the Agilent Web site at www.agilent.com/chem/FEprotocols to download the latest protocols.

NOTE These FE Protocols were optimized using data from Agilent catalog arrays, which have many replicated probes and validated Negative Control probes. If custom arrays without enough replicated probes are used, or arrays with custom probes designated as Negative Control probes are used, the default FE Protocols may not be optimal.

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 at the Four Corners of the Array.

5 Reference

Reagents, Enzymes, Buffers and Equipment

This topic lists the reagents, enzymes, buffers and equipment that are used in this protocol.

Table 14 Immunoprecipitation

Item	Size	Vendor	Catalog #	Stock Concentration
Dynal Magnetic Beads	various	Invitrogen	Various	
5-Methylcytidine, Monoclonal Antibody, purified	various	Eurogentec	BI-MECY-1000	1 mg/mL
BSA, powder	50 g	Sigma	A7906-50G	
Phosphate Buffered Saline (PBS)	500 mL	Amresco	K812-500 mL	1X
Dynal Magnetic stand (MPC-S)	n/a	Dynal (Invitrogen)	120.20	n/a

Table 15 Cell Sonication and DNA Clean-up

Item	Size	Vendor	Catalog #	Stock Concentration
Sonicator machine	n/a	Various (see protocol)		n/a
Tube rotator	n/a	Various (see protocol)		n/a
YM-30 Columns	n/a	Millipore	42410	n/a

Table 16 Cy Labeling, Hybridization and Washing

Item	Size	Vendor	Catalog #	Stock Concentration
Agilent Genomic DNA Labeling Kit PLUS	50 reactions	Agilent	5188-5309	
Oligo aCGH/ChIP-on-chip Hybridization Kit	25 reactions	Agilent	5188-5220	
Deionized Formamide	500 mL	Sigma	F7503-500ML	
Human Cot-1 DNA	500 µg	Invitrogen	15279-011	
Oligo aCGH/ChIP-on-chip Wash Buffer 1	4 L	Agilent	5188-5221	
Oligo aCGH/ChIP-on-chip Wash Buffer 2	4 L	Agilent	5188-5222	
Acetonitrile	1 L or 4 L	J.T. Baker	9017-02 or 9017-03	n/a
Stabilization & Drying Solution	500 mL	Agilent	5185-5979	n/a

Table 17 General Use Buffers and Reagents

Item	Size	Vendor	Catalog #	Stock Concentration
NaCl	1 kg	Sigma	S7653	5 M
Triton X-100	250 mL	Sigma	T8787	10%
SDS	4 x 100 mL	Invitrogen	15553-027	10%
Glycogen	100 µL	Invitrogen	10814-010	20 mg/mL
Phenol/Chloroform/ Isoamyl Alcohol	100 mL	Fluka	77617	n/a
MaXtract Phase Lock Gel Tubes	200 tubes	Fisher Scientific	FP2302820	2 mL size

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.

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.

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 Agilent's Genomic Labeling Kit and cyanine dyes.
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 Microcon YM-30 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 65°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

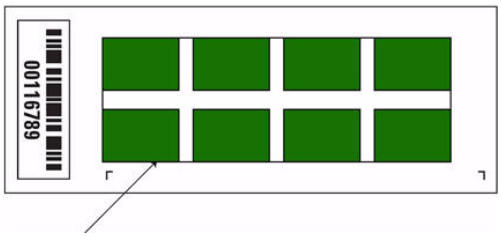
SOP

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

General Microarray Layout and Orientation

Agilent oligo microarray (8 microarray/slide format) as imaged on the Agilent microarray scanner (G2565BA)

Microarrays are printed on the side of the glass labeled with the "Agilent" bar code (also called the "active side" or "front side").



Agilent Microarray Scanner scans through the glass. (Back side scanning.)

Agilent microarray slide holder

Figure 3 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 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 3 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.

www.agilent.com

Version 1.0, May 2008

G4170-90012

Agilent Technologies