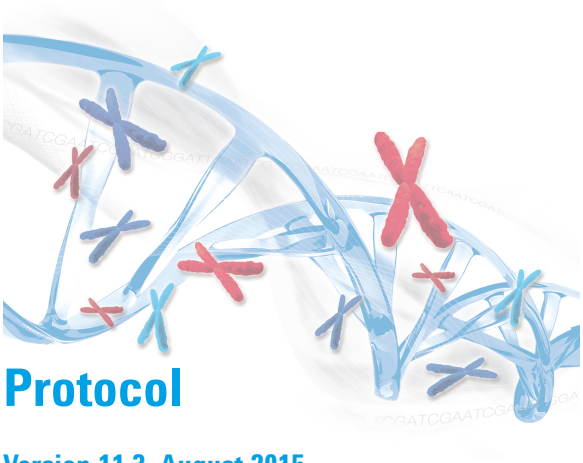
Agilent
ChIP-on-chip Analysis



Protocol

Version 11.3, August 2015

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

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

Agilent Technologies

Notices

Manual Part Number

G4481-90010

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CAUTION

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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.

In This Guide...

This guide describes the Agilent recommended operational procedures to analyze gene expression of genome (other than yeast) using Chromatin Immunoprecipitation (ChIP-on-chip) microarrays. For yeast genome, see the G4493-90010 *Agilent Yeast ChIP-on-chip Analysis Protocol*.

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 ChIP-on-chip 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 11.3

- Updated product labeling statement.

What's new in 11.2

- Removed obsolete microarray kits from list of supported kits.
- 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 11.1

- Updated Wash Buffer 2 temperature to 31°C.
- Clarified starting volume of amplified WCE and IP DNA for fluorescent labeling.
- 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 11.0

- 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 ChIP-on-chip 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 and G3 ChIP-on-chip Microarray Kit

- One or two microarrays printed on each 1-inch × 3-inch glass slide
- Available as a single slide or set, or in a 5-slide or 5-set 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 ChIP-on-chip designs, go to http://www.genomics.agilent.com. Under **Products**, click **Epigenetic & Specialty Microarrays**.

Table 1 Catalog SurePrint HD and G3 ChIP-on-chip Microarray Kits

Part Number	Description
G4873A	SurePrint G3 Human Promoter Microarray Slide or 5-slide Kit, 1×1M
G4874A	SurePrint G3 Human Promoter Microarray Slide or 5-slide Kit, 2×400K

Unrestricted SurePrint HD and G3 ChIP-on-chip 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 the tables that follow for available designs.

Table 2 Unrestricted SurePrint ChIP-on-chip Microarrays

Part Number	Description
G4495A, AMADID 014792	Human ENCODE ChIP-on-chip Microarray 1×244K
G4815A, AMAIDID 028383	SurePrint G3 Mouse Promoter Microarray, 1×1M
G4495A, AMAIDID 014798 & 014799	Arabidopsis Genome Microarray Kit, 2-Design Set, 1×244K
G4495A, AMAIDID 014793 & 014794	C. elegans Genome Microarray Kit, 2-Design Set, 1×244K
G4495A, AMAIDID 014816 & 014817	Drosophila Genome Microarray Kit, 2-Design Set, 1×244K
G4495A, AMAIDID 021203 & 021204	Zebrafish Promoter Microarray Kit, 2-Design Set, 1×244K

Custom SurePrint HD and G3 Microarrays

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

See Table 3 for available formats.

Table 3 Custom SurePrint HD and G3 ChIP-on-chip 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
G4822A	Custom SurePrint G3 ChIP-on-chip/DNA Methylation, 8×60K
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
G4499A	Custom ChIP-on-chip/DNA Methylation, 8×15K

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, 4×44K or 8×15K, *or* for 1×1M, 2×400K, 4×180K or 8×60K	 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 *or* for 8-pack microarrays	 Agilent p/n G2534-60003 Agilent p/n G2534-60002 Agilent p/n G2534-60011 Agilent p/n G2534-60014
Hybridization oven; temperature set at 65°C	Agilent p/n G2545A
Hybridization oven rotator for Agilent Microarray Hybridization Chambers	Agilent p/n G2530-60029
Ozone-barrier slide covers (box of 20)*	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)†	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
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
Vacuum Concentrator	Thermo Scientific Savant SpeedVac p/n DNA120-115 or equivalent

Table 4 Required equipment **(continued)**

Description	Vendor and part number
Magnetic stir bar, 7.9 × 38.1 mm (×2 or ×4)†	VWR p/n 58948-150 or equivalent
250 mL capacity slide-staining dish, with slide rack (×3 or ×5)†	Wheaton p/n 900200 *or* Thermo Shandon p/n 121
Circulating water baths or heat blocks set to 16°C, 37°C, 55°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	

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

† 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 ChIP-on-chip sample preparation

Item	Vendor and part number
Phase Lock Gel	Fisher p/n FP2302820
dNTP	GE Healthcare p/n 28-4065-51
oligo JW102 5′-GCGGTGACCCGGGAGATCTGAATTC-3′	Integrated DNA Technologies
oligo JW103 5′-GAATTCAGATC-3′	Integrated DNA Technologies
Formaldehyde 37% Solution	J.T. Baker p/n 2106-01
Dynabeads Pan Mouse IgG	Life Technologies p/n 110-41
Nuclease-free distilled water	Life Technologies p/n 10977-015
10× PBS, pH 7.2	Life Technologies p/n 70013-032
RNase A	Life Technologies p/n 12091-021
Proteinase K Solution	Life Technologies p/n 25530-049
T4 DNA Ligase Buffer	Life Technologies p/n 46300-018
AmpliTaq DNA Polymerase (5U/µL), includes: • Buffer II • $MgCl_2$ Solution	Life Technologies p/n N808-0156
UltraPure 0.5M EDTA, pH 8.0	Life Technologies p/n 15575-020
Glycerol	Life Technologies p/n 15514-011
UltraPure 1M Tris-HCl, pH 8.0	Life Technologies p/n 15568-025
UltraPure 10% SDS	Life Technologies p/n 15553-027
T4 DNA Polymerase, which includes: • T4 DNA Polymerase buffer • BSA	NEB p/n M0203S or M0203L
T4 DNA Ligase	NEB p/n M0202S or M0202L
• 10× ThermoPol Reaction Buffer	NEB p/n B9004S
NEBuffer 2	NEB p/n B7002S
BSA	NEB p/n B9001S

Table 6 Required reagents for ChIP-on-chip sample preparation (continued)

Item	Vendor and part number
Complete Protease Inhibitor Cocktail Tablet	Roche p/n 11 697 498
Glycogen	Roche p/n 901 393
Ethanol (95% to 100% molecular biology grade)	Sigma-Aldrich p/n E7023-6×500ML
Glycine	Sigma-Aldrich p/n G7126
BSA, powder	Sigma-Aldrich p/n A7906
NaCl	Sigma-Aldrich p/n S7653
0.5M EGTA, pH 8.0	Sigma-Aldrich p/n E3889
IGEPAL CA-630	Sigma-Aldrich p/n I8896
Triton X-100	Sigma-Aldrich p/n T8787
Sodium Deoxycholate	Sigma-Aldrich p/n D6750
N-Lauroylsarcosine sodium salt	Sigma-Aldrich p/n 61743
5M LiCl	Sigma-Aldrich p/n L4408
Phenol-chloroform-isoamyl alcohol	Sigma-Aldrich p/n 77617
Sodium acetate buffer solution	Sigma-Aldrich p/n S7899
7.5M Ammonium acetate solution	Sigma-Aldrich p/n A2706
$CaCl_2$ (Calcium Chloride)	Sigma-Aldrich p/n C5670 or equivalent
0.5 mL Thin Wall (PP) Tube	USA Scientific p/n 1405-4400
Antibody	Various
1M Hepes-KOH, pH 7.5	
ddH_20	
1M Tris-HCl, pH 7.9	

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*	Agilent p/n 5190-3400
Purification Column†(50 units)	Agilent p/n 5190-3391
1×TE (pH 8.0), Molecular grade	Promega p/n V6231

* Kit content is listed in "Reagent Kit Components" on page 76.

† Included in the SureTag DNA Labeling Kit. Order additional columns when processing more than 25 8-pack microarrays.

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*	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 *or* • Rat Hybloc	Agilent p/n 5190-3393 Life Technologies p/n 18440-016 Applied Genetics p/n RHB
DNase/RNase-free distilled water	Life Technologies p/n 10977-015
Milli-Q ultrapure water	Millipore
Acetonitrile*	Sigma-Aldrich p/n 271004-1L

* 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.

2
Sample Preparation

The steps in this protocol and the estimated amounts of time required are listed in Table 9 on page 18.

Table 9 Overview and time requirements.

Step	Time Requirement
Formaldehyde cross-linking of cells	1.5 hr
Binding of antibody to magnetic beads	0.5 hr, then overnight
Cell sonication	1 hr
Chromatin immunoprecipitation	0.5 hr, then overnight
Wash, elution, and cross-link reversal	2 hr, then overnight
Digestion of cellular protein and RNA	4 hr
T4 DNA polymerase fill-in and blunt-end ligation	2 hr, then overnight
DNA amplification using ligation-mediated PCR (LM-PCR)	4 hr
Cy3/Cy5 labeling of IP and WCE material	3 hr
Microarray hybridization	1 hr, then 40 hr
Microarray washing	1 hr

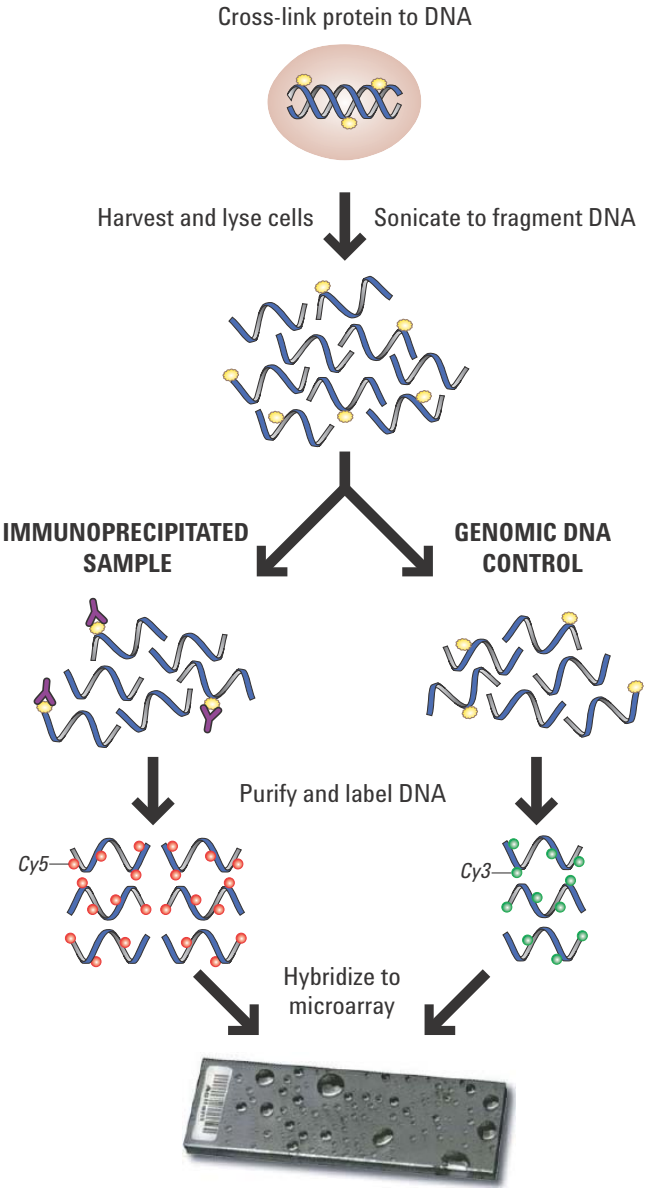

Figure 1 ChIP-on-chip overview

Step 1. Prepare the cells and cross-link proteins to DNA

Use 5×10^7 to 1×10^8 cells for each immunoprecipitation.

Adherent cells:

1. Add 1/10 of the cell culture volume of fresh 11% Formaldehyde Solution (see Table 10 on page 21) to the plates or flasks.

   Formaldehyde Solution can be added directly to culture media or to PBS.
2. Swirl the plates or flasks briefly and let them sit at room temperature for 10 minutes.
3. Add 1/20 volume of 2.5 M Glycine to plates or flasks to quench the formaldehyde.
4. Rinse the cells with 5 mL of a 1× solution of 10× PBS, pH 7.2. Add another 5 mL of 1× solution of 10× PBS, pH 7.2. Harvest cells using a silicone scraper.
5. Pour the cells into the required number of 50 mL conical tubes and spin at 1,350×g for 5 minutes at 4°C in a table-top centrifuge with swinging bucket rotor, such as a Sorvall Legend RT. Discard the supernatant.
6. Resuspend pellet in 10 mL of 1× solution of 10× PBS, pH 7.2 per 10^8 cells. Transfer 5×10^7 to 1×10^8 cells to 15 mL conical tubes and spin at 1,350×g for 5 minutes at 4°C in a table-top centrifuge with swinging bucket rotor, such as a Sorvall Legend RT. Discard the supernatant.
7. If you are not going to use the cells immediately, flash freeze the cells in liquid nitrogen and store the pellets at –80°C.

Suspension cells:

1. Add 1/10 of the cell culture volume of fresh 11% Formaldehyde Solution (see Table 10 on page 21) directly to the culture media in the flasks.
2. Swirl flasks briefly and let them sit at room temperature for 20 minutes.
3. Add 1/20 of the cell culture volume of 2.5 M Glycine to flasks to quench the formaldehyde.
4. Spin down the cells at 1,350×g for 5 minutes at 4°C in a table-top centrifuge with swinging bucket rotor, such as a Sorvall Legend RT.
5. Resuspend the pellets in 50 mL of 1× solution of 10× PBS, pH 7.2, spin at 1,350×g for 5 minutes at 4°C in a table-top centrifuge with swinging bucket rotor, such as a Sorvall Legend RT. Discard supernatant. Repeat once.

6 Resuspend in 10 mL of 1× solution of 10× PBS, pH 7.2 per 10^8 cells. Transfer 1×10^8 cells to 15 mL conical tubes and spin at 1,350×g for 5 minutes at 4°C in a table-top centrifuge with swinging bucket rotor, such as a Sorvall Legend RT. Discard the supernatant.

7 If you are not going to use the cells immediately, flash freeze the cells in liquid nitrogen and store the pellets at –80°C.

Table 10 Formaldehyde Solution

Stock	For 50 mL	Final Concentration
1M Hepes-KOH, pH 7.5	2.5 mL	50 mM
5M NaCl	1.0 mL	100 mM
0.5M EDTA, pH 8.0	100.0 µL	1 mM
0.5M EGTA, pH 8.0	50.0 µL	0.5 mM
Formaldehyde 37% Solution	14.9 mL	11%
ddH_20	31.5 mL	

Step 2. 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. Vigorously resuspend 100 µL of Dynabeads Pan Mouse IgG. Dynal beads will have settled during storage.
2. Add the 100 µL of Dynabeads Pan Mouse IgG to a 1.5 mL RNase-free Microfuge Tube.

   Set up 1 tube for each immunoprecipitate.

   The exact type of Dynabeads Pan Mouse IgG (Protein A, Protein G, sheep anti-mouse IgG, sheep anti-rabbit IgG, etc.) depends on the antibody being used. Other brands or bead types have not been tested by Agilent Technologies, so you may need to make adjustments to the protocol to optimize your results.
3. Add 1 mL of Block Solution (see Table 11 on page 23.)
4. Gently mix the Dynabeads Pan Mouse IgG in Block Solution.
5. Put tubes on a magnetic separation stand, such as a DynaMag-2 Magnet.
6. Remove the supernatant.
7. Wash the beads 2× with 1.5 mL of Block Solution:
   a. Add 1.5 mL of Block Solution to the beads.
   b. Remove the tubes from the magnetic stand and gently resuspend beads in the block solution.
   c. Use a magnetic device to collect the beads against the side of tube and remove the supernatant.
   d. Repeat one more time.
8. Resuspend the beads in 250 µL of Block Solution and add 10 µg of antibody.
9. Cool the bead mixture overnight on a rotating platform at 4°C.
10. The next day, wash the beads 3x with 1 mL of Block Solution (as described in step 7 above).

11 Spin for 1 minute at 4°C at 17,000×g to collect beads and remove the supernatant.

12 Resuspend the beads in 100 µL of Block Solution.

Table 11 Block Solution

Stock	For 100 mL	Final Concentration
10× PBS, pH 7.2	10 mL	1×
BSA, powder	500 mg	0.5% BSA (weight/volume)
ddH_20	90 mL	
Total	**100 mL**	

Step 3. Lyse the cells

Add protease inhibitors (final concentration 1×) to all lysis buffers before use. (Dissolve one Complete Protease Inhibitor Cocktail Tablet in 2 mL of Nuclease-free distilled water to make a 25× solution. Store in aliquots at -20°C.)

1. Resuspend each pellet of approximately 10^8 cells in 5 mL of Lysis Buffer 1 (LB1) (Table 12 on page 25). Rock at 4°C for 10 minutes. Spin at 1,350 × g for 5 minutes at 4°C in a tabletop centrifuge. Discard the supernatant.
2. Resuspend each pellet in 5 mL of Lysis Buffer 2 (LB2) (Table 13 on page 25). Rock gently at room temperature for 10 minutes. Pellet nuclei in tabletop centrifuge by spinning at 1,350 × g for 5 minutes at 4°C.
3. Discard the supernatant.
4. Resuspend each pellet in 3 mL of Lysis Buffer 3 (LB3) (Table 14 on page 25).
5. Transfer cells to a 15-mL polypropylene tube that has been cut at the 7 mL mark (to make sonification easier).
6. Sonicate the suspension with a microtip attached to sonicator. Samples should be kept in an ice water bath during sonication.

   If you use a Misonix 3000, initially set output power to 4 and increase manually to final power (7) during the first burst. Keep the power output at 7 for the remainder of the sonication. Sonicate 7 cycles of 30 seconds ON and 60 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 100 to 600 bp in size. Shearing varies greatly depending on cell type, growth conditions, quantity, volume, cross-linking, and equipment. Depending on the specific experiment, and using power settings as high as 9, you can use anywhere from 3 to 12 cycles and variable ratios of time ON and time OFF.

7. Add 300 µL of 10% Triton X-100 to the sonicated lysate and mix by pipetting up and down several times. Split into two 1.5 mL RNase-free Microfuge Tube. Spin at 20,000×g for 10 minutes at 4°C in a microcentrifuge to pellet debris.
8. Combine supernatants from the two 1.5 mL RNase-free Microfuge Tube into a new 15 mL conical tube for immunoprecipitation.

9 Save 50 µL of cell lysate from each sample as Whole Cell Extract (WCE) DNA. Store at -20°C.

Table 12 Lysis Buffer 1 (LB1)

Stock	For 100 mL	Final Concentration
1M Hepes-KOH, pH 7.5	5.0 mL	50 mM
5M NaCl	2.8 mL	140 mM
0.5M EDTA, pH 8.0	0.2 mL	1 mM
50% Glycerol	20.0 mL	10%
10% IGEPAL CA-630	5.0 mL	0.5%
10% Triton X-100	2.5 mL	0.25%
ddH_20	64.5 mL	

Table 13 Lysis Buffer 2 (LB2)

Stock	For 100 mL	Final Concentration
1M Tris-HCl, pH 8.0	1.0 mL	10 mM
5M NaCl	4.0 mL	200 mM
0.5M EDTA, pH 8.0	0.2 mL	1 mM
0.5M EGTA, pH 8.0	0.1 mL	0.5 mM
ddH_20	94.7 mL	

Table 14 Lysis Buffer 3 (LB3)

Stock	For 100 mL	Final Concentration
1M Tris-HCl, pH 8.0	1.0 mL	10 mM
5M NaCl	2.0 mL	100 mM
0.5M EDTA, pH 8.0	0.2 mL	1 mM
0.5M EGTA, pH 8.0	0.1 mL	0.5 mM
10% Sodium Deoxycholate	1.0 mL	0.1%

Table 14 Lysis Buffer 3 (LB3)

Stock	For 100 mL	Final Concentration
20% N-Lauroylsarcosine sodium salt	2.5 mL	0.5%
ddH_20	93.2 mL	

Step 4. Immunoprecipitate the chromatin

1. Add 100 µL antibody/magnetic bead mixture from "Step 2. Prepare the magnetic beads" on page 22 to the 15 mL conical tube containing the cell lysate from "Step 3. Lyse the cells" on page 24.
2. Gently mix overnight on rotator or rocker at 4°C.

Step 5. Wash, elute, and reverse the cross-links

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

1. Pre-chill one 1.5 mL microfuge tube for each immunoprecipitate.
2. Transfer half the volume of an immunoprecipitate to a pre-chilled tube.
3. Let tubes sit in magnetic device to collect the beads. Remove supernatant and add remaining immunoprecipitation reaction (IP). Let tubes sit again in magnetic device to collect the beads.
4. Add 1 mL Wash Buffer (RIPA) to each tube (Table 15 on page 27). Remove tubes from magnetic device and shake or agitate tube gently to resuspend beads. Replace tubes in magnetic device to collect beads. Remove supernatant. Repeat this wash 3 to 7 more times.

   You may need to optimize the number of washes for each antibody, depending on the quality of the immunoprecipitating antibody. You may want to start with 7 washes.
5. Wash once with 1 mL of 1×TE (pH 8.0) that contains 50 mM NaCl.
6. Spin at 960×g for 3 minutes at 4°C in a centrifuge and remove any residual 1×TE (pH 8.0) with a pipette.

Elution and Reversing Cross-Links

1 Add 210 µL of Elution Buffer and resuspend beads.

2 Incubate in water bath at 65°C for 15 minutes. During elution, resuspend beads every 2 minutes by mixing briefly on a vortex mixer.

3 Spin down the beads at 16,000 × g for 1 minute at room temperature.

4 Remove 200 µL of supernatant and transfer it to a new 1.5 mL RNase-free Microfuge Tube.

5 Reverse the cross-links by incubating in a water bath at 65°C overnight.

6 Thaw 50 µL of WCE reserved after sonication, add 3 volumes (150 µL) of Elution Buffer (Table 16), and mix. Reverse the cross-links by incubating in a water bath at 65°C overnight.

Table 15 Wash Buffer (RIPA)

Stock	For 250 mL	Final Concentration
1M Hepes-KOH, pH 7.5	12.5 mL	50 mM
5M LiCl	25.0 mL	500 mM
0.5M EDTA, pH 8.0	0.5 mL	1 mM
10% IGEPAL CA-630	25.0 mL	1%
10% Sodium Deoxycholate	17.5 mL	0.7%
ddH_20	169.5 mL	

Table 16 Elution Buffer

Stock	For 100 mL	Final Concentration
1M Tris-HCl, pH 8.0	5.0 mL	50 mM
0.5M EDTA, pH 8.0	2.0 mL	10 mM
10% SDS	10.0 mL	1%
ddH_20	83.0 mL	

Step 6. Digest the cellular protein and RNA

1. Add 200 µL of 1×TE (pH 8.0) to each tube of IP and WCE DNA to dilute 10% SDS in Elution Buffer.
2. Add 8 µL of 10 mg/mL RNase A (0.2 mg/mL final concentration).
3. Mix and incubate in a circulating water bath for 2 hours at 37°C.
4. Add 7 µL of $CaCl_2$ stock solution (300 mM $CaCl_2$ in 10mM Tris-HCl, pH 8.0) to each sample, followed by 4 µL of 20 mg/mL Proteinase K Solution (0.2 mg/mL final concentration).
5. Mix and incubate in a water bath at 55°C for 30 minutes.
6. Add 400 µL of Phenol-chloroform-isoamyl alcohol to each tube.
7. Mix the sample on a vortex mixer.
8. Prepare one Phase Lock Gel for each IP and WCE sample by spinning the tube at 14,000 × g at room temperature for 30 seconds.
9. Add the sample to the Phase Lock Gel tube.
10. Spin the sample in a centrifuge at 14,000 × g for 5 minutes at room temperature.

    If the WCE DNA remains cloudy, repeat the Phenol-chloroform-isoamyl alcohol extraction one more time.
11. Transfer the aqueous layer to a new 1.5 mL microfuge tube.
12. Add:
    - 16 µL of NaCl (200 mM final concentration)
    - 1.5 µL of 20 µg/µL Glycogen (30 µg total)
    - 880 µL of Ethanol
13. Cool the mixture for 30 minutes at -80°C.
14. Spin the mixture at 20,000 × g for 10 minutes at 4°C to create DNA pellets.
15. Wash the pellets with 500 µL of 70% ice-cold Ethanol.
16. Dry the pellets for 10 minutes with a vacuum desiccator, such as a Savant Speed Vac, and resuspend each pellet in 70 µL of 10mM Tris-HCl, pH 8.0.
17. Save 15 µL of the IP sample for future checkpoints or verification.
18. Measure the DNA concentration of WCE with NanoDrop (NanoDrop Technologies). Expected concentration is between 200 to 300 ng/µL. Dilute the WCE DNA to 100 ng/µL.

Step 7. Prepare linkers for LM-PCR

1 Resuspend each of oligo JW102 5'-GCGGTGACCCGGGAGATCTGAATTC-3' and oligo JW103 5'-GAATTCAGATC-3' in ddH_20 to 40µM.

2 Mix the components in Table 17.

Table 17 Oligo mix

Component	Volume
1M Tris-HCl, pH 7.9	250 µL
40µM oligo JW102 5'-GCGGTGACCCGGGAGATCTGAATTC-3'	375 µL
40µM oligo JW103 5'-GAATTCAGATC-3'	375 µL

NOTE Neither oligo requires 5' phosphorylation.

3 Put 100 µL of the mixture into PCR tubes.

4 Place the tubes in a thermal cycler and run this program:

Step	Temperature	Time
Step 1	95°C	5 minutes
Step 2	70°C	1 minutes
Step 3	Ramp down to 4°C (0.4°C/min)	
Step 4	4°C	HOLD

5 Store the linkers at -20°C.

NOTE Prepared linkers are temperature sensitive. Thaw them on ice.

Step 8. Blunt the DNA ends

Use T4 DNA polymerase to blunt the DNA ends.

Keep the sample on ice while you do the first 7 steps.

1 Put 2 µL (200 ng) WCE DNA into a PCR tube (0.2 to 0.5 mL) and add 53 µL of ddH_20.

   Set up one WCE for each IP sample you have.

2 Put 55 µL of each IP sample into separate PCR tubes (0.2 to 0.5 mL) on ice.

3 Make Blunting Mix on ice (55 µL of mix per reaction):

   If you are using a Master Mix for multiple samples, include 10% extra volume.

Table 18 Blunting Mix

Stock	1× Mix	Final Concentration*
10× NEBuffer 2	11.0 µL	1×
10 µg/µL BSA	0.5 µL	5 µg
10mM each dNTP	1.1 µL	100 µM
3U/µL T4 DNA Polymerase	0.5 µL	1.5 U
ddH_20f	41.9 µL	
Total	**55 µL**	

* The Final Concentration is the reagent concentration in the final reaction and *not* the master mix.

4 Add 55 µL of Blunting Mix to all samples.

5 Cool for 20 minutes at 12°C in a thermal cycler.

6 Place tubes on ice.

7 Add 11.5 µL of cold 3M Sodium acetate buffer solution and 0.5 µL of 20 µg/µL Glycogen (10 µg total) to the sample.

8 Add an equal volume (120 µL) of cold Phenol-chloroform-isoamyl alcohol to sample. Keep on ice.

9 Thoroughly mix the sample by pipetting the sample up and down.

10 Prepare one Phase Lock Gel tube for each IP and WCE sample by spinning the tube at 14,000×g at room temperature for 30 seconds.

11 Transfer the sample to the Phase Lock Gel tube.

12 Spin in a centrifuge at 14,000 × g for 5 minutes at room temperature.

13 Transfer the aqueous layer to a 1.5 mL microcentrifuge tube.

14 Add 250 µL of 100% Ethanol.

15 Chill the sample for 30 minutes at -80°C.

16 Spin at 20,000 × g for 10 minutes at 4°C to pellet the DNA.

17 Wash the pellets with 500 µL of ice-cold 70% Ethanol.

18 Dry the pellets for 10 minutes with a vacuum desiccator, such as a Savant Speed Vac, and resuspend each pellet in 25 µL of ddH_20. Chill on ice.

Step 9. Ligate the blunt-end

1 Make Ligase Mix on ice (25 µL of mix per reaction):

If you are using a Master Mix for multiple samples, include 10% extra volume.

Table 19 Ligase Mix

Component	1× Mix	Final Concentration*
5x T4 DNA Ligase Buffer	10.0 µL	1×
15 µM linkers (see "Step 7. Prepare linkers for LM-PCR" on page 29)	6.7 µL	2 µM
400U/µL T4 DNA Ligase	0.5 µL	200U
ddH_20	7.8 µL	
Total	**25.0 µL**	

* The Final Concentration is the reagent concentration in the final reaction and *not* the master mix.

2 Add 25 µL of Ligase Mix to 25 µL of sample. Use a pipette to mix the reaction.

3 Cool for 16 hours in 16°C water bath or a thermal cycler.

4 Add 6 µL of 3M Sodium acetate buffer solution.

5 Add 130 µL of 100% Ethanol.

6 Chill the sample for 30 minutes at -80°C.

7 Spin at 20,000 × g for 10 minutes at 4°C.

8 Wash the pellets with 500 µL of ice-cold 70% Ethanol.

9 Dry the pellets for 10 minutes in a vacuum desiccator, such as a Savant Speed Vac, and resuspend each pellet in 25 µL of ddH_20.

Step 10. Amplify the IP and WCE samples

NOTE PCR methods and reagents may be covered by one or more third-party patents. It is the user's responsibility to obtain any necessary licenses and/or licensed PCR reagents for such patents.

This protocol enables large-scale amplification of IP and WCE samples. After 15 cycles of PCR-based amplification, the reaction is diluted and used as template for a second round of 25 cycles. Remaining template can be stored long-term at -20°C.

1 Put 25 µL each of IP and WCE DNA into separate PCR tubes (0.2 to 0.5 mL).

2 Make two buffer mixes:

Table 20 Mix A

Stock	1× Mix	Final Concentration*
10× ThermoPol Reaction Buffer	4.00 µL	1×
dNTP (2.5 mM each)	5.00 µL	250 µM
oligo JW102 (40 µM)	1.25 µL	1 µM
ddH_20	4.75 µL	
Total	**15.00 µL**	

* The Final Concentration is the reagent concentration in the final reaction and *not* the master mix.

Table 21 Mix B

Stock	1× Mix	Final Concentration*
10× ThermoPol Reaction Buffer	1.0 µL	1×
AmpliTaq DNA Polymerase (5U/µL)	0.5 µL	0.25 U
ddH_20	8.5 µL	
Total	**10.0 µL**	

* The Final Concentration is the reagent concentration in the final reaction and *not* the master mix.

3 Add 15 µL of Mix A to each sample.

4 Run an LM-PCR program in a thermal cycler:

a Start the program in Table 22.

b Midway through Step 1, pause the program.

c Add 10 µL Mix B to each tube to hot start the reactions.

Maintain the tubes at 55°C while adding Mix B.

d Continue the program.

Table 22 LM-PCR Program

Step	Temperature	Time
Step 1	55°C	4 minutes
Step 2	72°C	3 minutes
Step 3	95°C	2 minutes
Step 4	95°C	30 seconds
Step 5	60°C	30 seconds
Step 6	72°C	1 minute
Step 7		Repeat Step 4 through Step 6 for a total of 15 times.
Step 8	72°C	5 minutes
Step 9	4°C	HOLD

5 Transfer the product to a 1.5 mL RNase-free Microfuge Tube and add 475 µL of ddH_20 (total volume approximately 525 µL).

6 Put 5 µL of the resulting PCR product into a PCR tube (0.2 to 0.5 mL) for a second expansion.

7 Make the PCR Mixture:

Table 23 PCR Mixture

Stock	1× Mix	Final Concentration*
10× ThermoPol Reaction Buffer	5.00 µL	1×
dNTP (2.5 mM each)	5.00 µL	250 µM
oligo JW102 (40 µM)	1.25 µL	1 µM
AmpliTaq DNA Polymerase (5U/µL)	0.25 µL	1.25 U
ddH_20	33.50 µL	
Total	**45.00 µL**	

* The Final Concentration is the reagent concentration in the final reaction and *not* the master mix.

8 Add 45 µL of PCR Mixture to each reaction tube.

9 Run the LM-PCR program below in a thermocycler:

Table 24 LM-PCR

Step	Temperature	Time
Step 1	95°C	2 minutes
Step 2	95°C	30 seconds
Step 3	60°C	30 seconds
Step 4	72°C	1 minute
Step 5		Repeat Step 2 through Step 4 for a total of 24 times.
Step 6	72°C	5 minutes
Step 7	4°C	HOLD

10 Make the Precipitation Mix:

Table 25 Precipitation Mix

Stock	1× Mix	Final Concentration*
7.5M Ammonium acetate solution	25.0 µL	625 mM
100% Ethanol	225.0 µL	75%
Total	**250.0 µL**	

* The Final Concentration is the reagent concentration in the final reaction and *not* the master mix.

11 Transfer the product to a 1.5 mL RNase-free Microfuge Tube.

12 Add 250 µL Precipitation Mix to each tube.

13 Cool for 30 minutes at -80°C.

14 Spin at 20,000 × g for 10 minutes at 4°C to pellet DNA.

15 Wash the pellets with 500 µL of ice-cold 70% Ethanol.

16 Dry the pellets for 10 minutes with a vacuum desiccator, such as a Savant Speed Vac, and resuspend each pellet in 50 µL of ddH_20.

17 Measure DNA concentration with NanoDrop (NanoDrop Technologies) (use 10-fold dilutions, if necessary) and normalize all samples to 100 ng/µL.

3 Sample Labeling

The SureTag DNA Labeling Kit contains sufficient two-color labeling reaction reagents for:

- 25 1-pack, 2-pack, or 4-pack microarrays *or*
- 50 8-pack microarrays

It also contain clean-up columns for 25 reactions of each color. Order additional columns when processing more than 25 8-pack microarrays.

The kit uses random primers and the exo-Klenow fragment to differentially label IP and WCE DNA samples with fluorescent-labeled nucleotides. For the Agilent ChIP-on-chip 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 WCE sample is labeled with Cy3 and the IP is labeled with Cy5.

Step 1. Fluorescent Labeling of Amplified 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 95°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. Prepare amplified WCE and IP gDNA:
   - For 1-pack, 2-pack, and 4-pack microarrays, add 2 µg of amplified WCE or IP gDNA to a 1.5 mL RNase-free Microfuge Tube per reaction. Bring the volume up to 26 µL with Nuclease-free distilled water.
   - For 8-pack microarrays, add 1 µg of amplified WCE or IP gDNA to a 1.5 mL RNase-free Microfuge Tube per reaction. Bring the volume up to 13 µL with Nuclease-free distilled water.
4. 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 amplified WCE or IP DNA to make a total volume of 31 µL. Mix well by pipetting up and down gently.
   - For 8-pack microarrays, add 2.5 µL of Random Primer to each reaction tube that contains 13 µL of WCE or IP DNA to make a total volume of 15.5 µL. Mix well by pipetting up and down gently.
5. 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 on ice for 5 minutes.

   or

   Transfer sample tubes to a thermal cycler. Program the thermal cycler according to Table 26 and run the program.

Table 26 DNA denaturation using a thermal cycler

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

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

7 For 1-pack, 2-pack and 4-pack microarrays:

a Mix the components in Table 27 on ice in the order indicated to prepare one cyanine-3 and one cyanine-5 Labeling Master Mix.

Table 27 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 WCE or IP DNA to make a total volume of 50 µL. Mix well by gently pipetting up and down.

8 For 8-pack microarrays:

a Mix the components in Table 28 on ice in the order indicated to prepare one cyanine-3 and one cyanine-5 Labeling Master Mix.

Table 28 Labeling Master Mix (for 8-pack microarrays)

Component	Per reaction (µL)	× 8 rxns (µL) (including excess)	× 24 rxns (µL) (including excess)	× 48 rxns (µL) (including excess)
5× Reaction Buffer	5.0	42.5	125	250
10× dNTPs	2.5	21.25	62.5	125
Cyanine 3-dUTP *or* Cyanine 5-dUTP	1.5	12.75	37.5	75
Exo (-) Klenow	0.5	4.25	12.5	25
Final volume of Labeling Master Mix	**9.5**	**80.75**	**237.5**	**475**

b Add 9.5 µL of Labeling Master Mix to each reaction tube that contains the amplified WCE or IP DNA to make a total volume of 25 µL. Mix well by gently pipetting up and down.

9 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 29 and run the program.

Table 29 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 Amplified DNA

Labeled amplified 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 amplified DNA samples separated throughout this clean-up step.

1. Spin the labeled amplified WCE 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 WCE or IP DNA sample to be purified, place a column into a 2-mL collection tube and label the column appropriately. Load each labeled WCE 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 30. 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 44. Refer to Table 31 on page 44 for expected yield of labeled amplified 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 30. Use the appropriate container listed in Table 30.

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

Table 30 Sample volume and total mixture volumes

Microarray	Cy3 or Cy5 sample volume after purification	Total mixture volume after Nanodrop and combining	Container
1-pack	80.5 µL	158 µL	1.5 mL RNase-free Microfuge Tube
2-pack	41 µL	79 µL	1.5 mL RNase-free Microfuge Tube or Tall Chimney PCR plate
4-pack	21 µL	39 µL	1.5 mL RNase-free Microfuge Tube or Tall Chimney PCR plate
8-pack	10 µL	16 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

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

Refer to Table 31 for expected yield of labeled amplified DNA and specific activity after labeling and purification.

Table 31 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)
>5	15	18

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

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 78.

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 amplified 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 32 through Table 35.

Table 32 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
Final Volume of Hybridization Master Mix	362	3,077	9,050	18,100

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

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

Table 33 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
Final Volume of Hybridization Master Mix	181	1,538.5	4,525	9,050

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

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

Table 34 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
Final Volume of Hybridization Master Mix	71	603.5	1,775	3,550

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

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

Table 35 Hybridization Master Mix for **8-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)*	2	17	50	100
10× aCGH Blocking Agent†	4.5	38.25	112.5	225
2× HI-RPM Hybridization Buffer†	22.5	191.25	562.5	1,125
Final Volume of Hybridization Master Mix	29	246.5	725	1,450

* Use Cot-1 DNA (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 Tube or Tall Chimney PCR plate well that contains the labeled amplified WCE or IP DNA to make the total volume listed in Table 36.

Table 36 Volume of Hybridization Master Mix per hybridization

Microarray format	Volume of Hybridization Master Mix	Total volume
1-pack	362 µL	520 µL
2-pack	181 µL	260 µL
4-pack	71 µL	110 µL
8-pack	29 µL	45 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 37 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 78.

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)
   - 40 µL (for 8-pack microarray)

   For multi-pack microarray formats (2-pack, 4-pack or 8-pack microarray), load all gasket wells before you load the microarray slide. For multi-pack formats, refer to "Agilent Microarray Layout and Orientation" on page 79.

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 65°C for:

- 24 hours (4-pack and 8-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 53.

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 38 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 56	No
> 5 ppb < 10 ppb	"Wash Procedure A (without Stabilization and Drying Solution)" on page 56	Yes
> 10 ppb	"Wash Procedure B (with Stabilization and Drying Solution)" on page 58	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 ChIP-on-chip 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 31°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 31°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 31°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 58.

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

Table 39 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	31°C	5 minutes

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 31°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 31°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 31°C:

   a Activate the magnetic stirrer.

   b Wash microarray slides for at least 5 minutes and no more than 6 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 31°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 40 lists the wash conditions for the Wash Procedure B with Stabilization and Drying Solution.

Table 40 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	31°C	5 minutes
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 56.

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 31°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 (with "Agilent"-labeled barcode) 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 active microarray surface ("Agilent"-labeled 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.

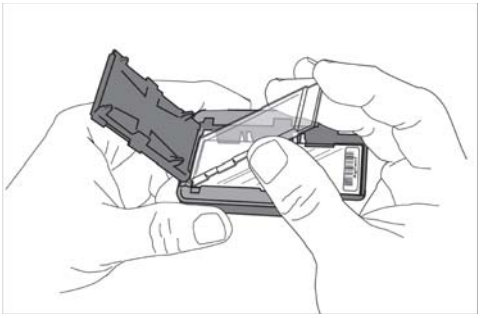

Figure 3 Inserting the ozone-barrier slide cover

- 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 41.

Table 41 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 42 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 ChIP-on-chip microarrays scanned on the Agilent SureScan or C Scanner.

The Feature Extraction software v9.5 supports extraction of microarray TIFF images (.tif) of Agilent ChIP-on-chip 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 69 for configuration.

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

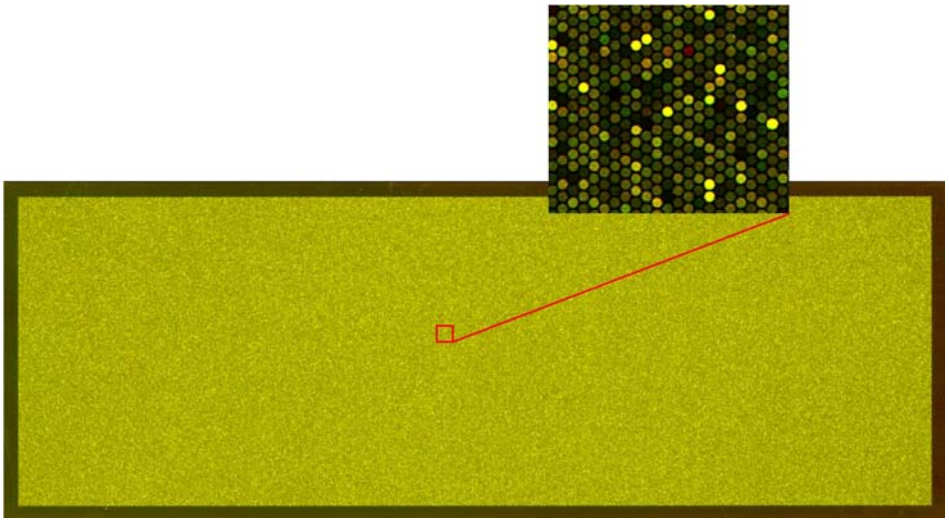

Figure 4 Agilent SurePrint G3 1×1M microarray shown in red and green channels, full and zoomed view

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 69 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 69 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.

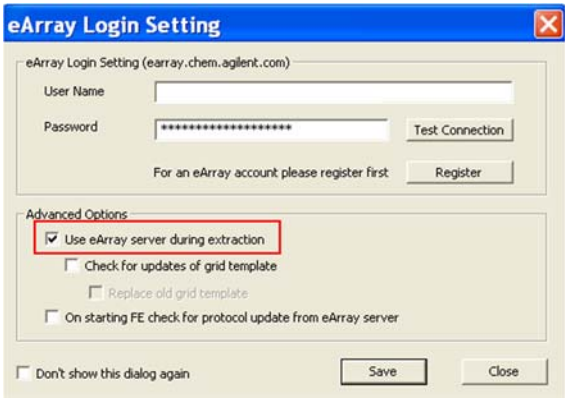

Figure 6 eArray Login Setting. You can mark the other two check boxes under Advanced Options if you want to get update of grid templates already in the database or to get protocol updates. See the Feature Extraction user guide for more information.

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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 43 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 44 Oligo aCGH/ChIP-on-chip Hybridization Kit

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

Table 45 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

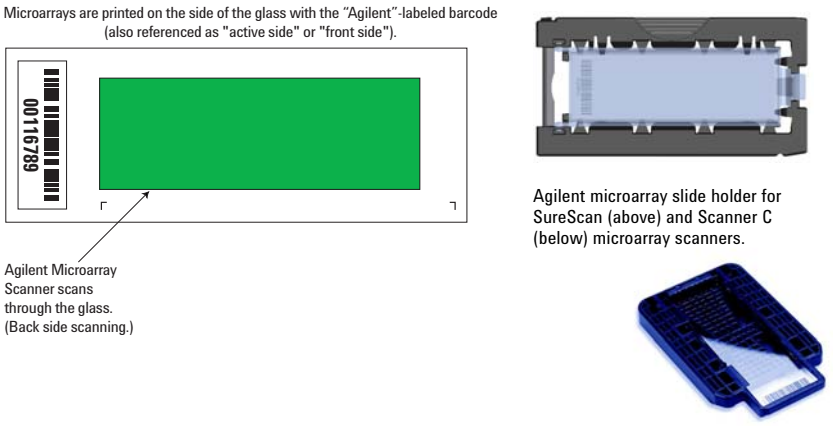

Figure 7 Agilent microarray slide and slide holder

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”.

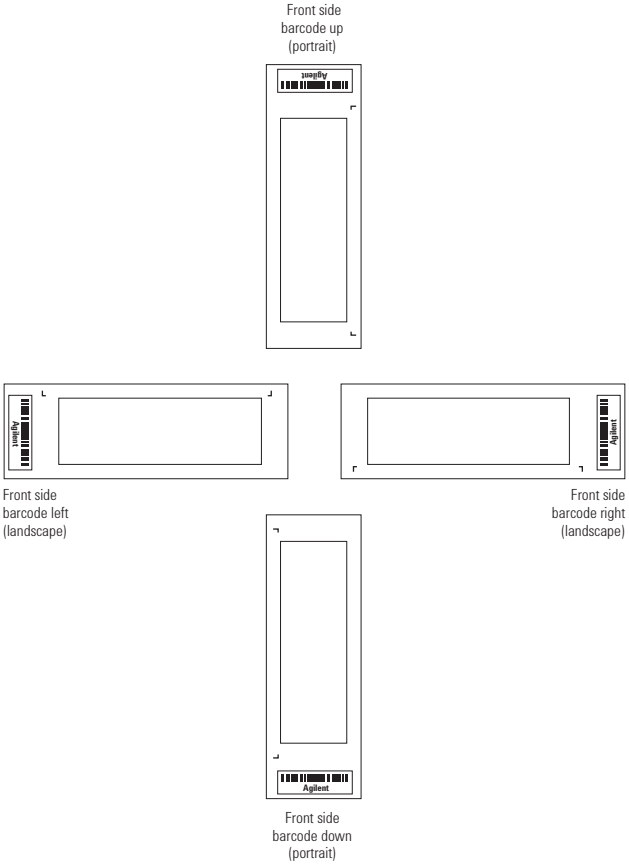

Figure 8 Microarray slide orientation

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

Arrays

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

Barcode Number _____

Figure 11 8-pack microarray slide

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Notes and Considerations

ChIP-on-chip enables investigators to capture DNA-binding proteins in action and identify the DNA sequences bound by these proteins across an entire genome *in vivo*. The protocol requires an antibody to the DNA-binding protein of interest that specifically immunoprecipitates the protein and associated DNA from a complex whole-cell mixture. The identity of the DNA immunoprecipitate is revealed using microarrays, allowing identification of precise binding coordinates.

The ChIP-on-chip protocol consists of eight general steps:

- Cell cross-linking and harvesting
- Cell lysis and chromatin shearing
- Chromatin immunoprecipitation
- Cross-link reversal and DNA isolation
- DNA amplification
- DNA labeling
- Microarray hybridization and washing

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- 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. Cell cross-linking and harvest

Goal Covalently link proteins to the DNA to create the protein-DNA complexes for an immunoprecipitation.

SOP

1. Treat approximately 10^8 cells with 1% formaldehyde for 10 minutes (for adherent cells) or 20 minutes (for suspension cells). The cells can be in either media or 1× PBS at the time of this treatment.
2. After incubation, neutralize the formaldehyde with 1/20 volume 2.5 M glycine.
3. Wash the cells with cold 1× PBS, make pellets from the cells, flash freeze the pellets, and store long term at –80°C.

Key variables

- Fixative type and concentration
- Time
- Temperature

QC Metrics None

Notes Too few or too many cross-links could theoretically reduce ChIP-on-chip performance. The optimal level of cross-linking must be determined. Changes in reaction time, temperature, and reagent concentrations have not been tested.

2. Cell lysis and chromatin shearing

Goal Lyse cells and shear chromatin to approximately 500 bp average.

SOP

1. Lyse approximately 10^8 cells using a series of three lysis buffers and resuspend in 3 mL of the final buffer.
2. Sonicate the cell solution with a microtip for a variety of durations (total 'on' time is usually 5 to 8 minutes with cooling breaks at least every 30 seconds).

Key variables

- Lysis buffer reagents concentration and volume
- Cell density
- Shearing instrument and parameters (power, duration, volume, temperature)

QC Metrics Lysate that was saved for the reference channel can be analyzed using the 2100 Bioanalyzer (Agilent) following cross-link reversal and DNA isolation (step 6). Fragments from the sonicated material should range from approximately 100bp to approximately 1kb.

Notes Overall, the goal is to obtain consistent results using sonication. Slight differences in volume, placement of probe tip in vessel, foaming, and other subtle changes may lead to different shearing results. Furthermore, some cell types are more difficult to sonicate to the desired DNA fragment size (e.g. U937) than others. We recommend the Misonix sonicator in this protocol with the appropriate settings. However, the optimal sonication conditions for different cell types may need to be determined empirically.

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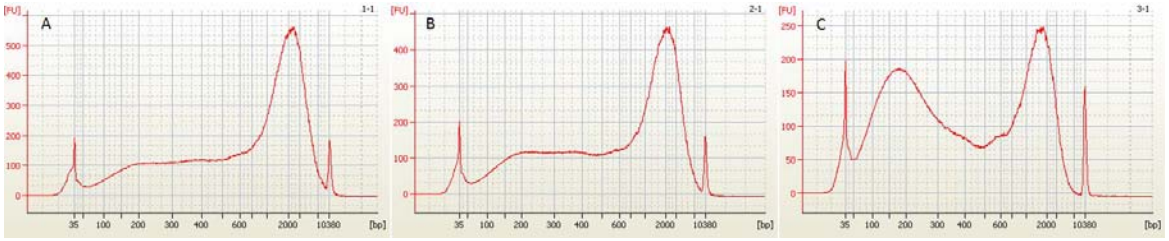

Figure 12 The effect of sonication on DNA fragment size. Samples of whole cell extract (WCE) were sonicated with increasing cycles on the Misonix 3000. Each sonication cycle consisted of: 30 seconds at level 7 followed by 60 seconds off. Panel A: 7 cycles, B: 10 cycles and C: 13 cycles. The resulting DNA fragment distributions were analyzed on the 2100 Bioanalyzer using the Agilent DNA high sensitivity kit. Panels A and B show consistent fragment size between 100 and 1000bp, which indicates good shearing. Panel C shows slight over-shearing with an increase in DNA fragments <300bp. The peak centered at 2000bp appears large because it represents all fragments between 1000 to 10,000bp.

3. Chromatin immunoprecipitation (ChIP)

Goal Use selective antibody bound to magnetic beads to specifically capture the DNA-binding factors with covalently tethered DNA.

SOP

1. Mix antibody bound to magnetic beads (Dynal) with cell lysate (approximately 3 mL).
2. Place at 4°C overnight on a rotating platform.
3. Isolate the beads containing the antibody bound to the DNA-protein complexes.
4. Wash 4 to 8 times with buffer to remove non-specific contaminants.
5. After the wash, heat the complexes for a few minutes to elute the DNA-protein complexes from the antibody and beads.

Key variables

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

QC Metrics After the cross-links are reversed and the DNA is isolated (step 6), gene-specific PCR can be done to determine the relative enrichment of *known* bound targets. We currently cannot recommend a particular method to validate the success of ChIP for factors that lack known targets.

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.

Some antibodies require different buffer conditions during incubation and may have differing binding efficiencies. You may need to optimize the buffer composition for your antibody.

The optimal number of washes may vary with different antibodies. The goal is to remove non-specific interactions from the beads and enrich for the targeted immunoprecipitate.

4. Cross-link reversal and DNA isolation

Goal Untether and purify DNA from associated proteins and RNA and protein contaminants.

SOP

1. Reverse the cross-links between DNA and protein overnight in mildly acidic Tris-HCl solution at 65°C.
2. Enzymatically digest proteins and RNA.
3. Purify the DNA via organic extraction and ethanol precipitation.

Key variables

- Temperature
- Time
- SDS concentration
- Type and amount of enzymes

QC Metrics None

5. DNA amplification

Goal Amplify the immunoprecipitated DNA to detectable quantities for microarray hybridization and detection.

SOP This protocol uses ligation-mediated PCR as the method for amplification of the immunoprecipitated DNA. Ligation-mediated PCR is a method in which short, blunt, duplex DNA fragments of a known sequences (linkers) are ligated to each of the blunted ends of the input DNA mixture. This places known and universal priming sites at these ends to which a universal primer can anneal for PCR amplification.

Key variables

- Blunting reaction time and temperature; linker composition, concentration
- Ligation time and temperature
- Number of PCR cycles

QC Metrics Nanodrop measurement of total DNA yield (at least 2 to 3 µg per 50 µL reaction) and visualization on an agarose gel provide assurance of success, but this does not necessarily indicate that the ChIP worked.

Notes LM-PCR is often difficult to perform consistently. It is important to take care in following the protocol carefully for LM-PCR.

Other amplification procedures exist but have not been tested by Agilent Technologies.

6. DNA labeling

Goal Incorporate fluorescent-tagged nucleotides into the amplified DNA material for hybridization.

SOP

1. Use the Agilent Genomic DNA Enzymatic Labeling Kit and cyanine dyes.
2. For each array, perform 1 to 2 labeling reactions with 2 µg input DNA per reaction for both the ChIP (Cy5-dUTP) and WCE (Cy3-dUTP).
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 >5 µg per reaction); Nanodrop measurement of pmol/µL dye (expect >2 pmol/µL with Cy5-dUTP and >3 pmol/µL Cy3-dUTP).

7. 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 three buffers that include 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.

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8. 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 purged vacuum pack.

Gene-specific PCR for E2F4 ChIP in Human Cells

The success of chromatin immunoprecipitation (ChIP) requires both good technique and capable antibodies. To troubleshoot whether ChIP failure is due to improper technique or poor antibodies, you should include a positive control ChIP.

E2F4 is recommended as a positive control ChIP. This transcription factor has two advantages as a positive control. First, it is a regulator of the cell cycle that controls similar genes in all cell lines, types, and tissues tested so far. E2F4 is therefore an applicable control in most experimental contexts. Second, a chip-capable antibody is readily available (Santa Cruz, sc-1082).

Known gene targets of E2F4 include Rbl1/p107 and CDC25a. The enrichment of Rbl1/p107 and CDC25a in IP versus input DNA fractions in comparison to a reference (often the actin promoter, but can be any stretch of DNA not bound by E2F4) genomic locus reveals technical ChIP success.

Gene-specific guidelines using conventional PCR

- Use 1 to 2 µL of IP eluant per reaction.
- To extend linear range of gel analysis, use 5 WCE DNA dilutions (from approximately 2 ng to 25 pg using 3-fold dilutions to remain in linear range on gel)
- Primers are usually at 1 µM and reaction runs for 25 cycles – both can be adjusted as needed.
- Annealing temp for E2F4 primers is 60°C.
- Products can be visualized on PAGE or 2.5% agarose gels using an appropriate stain (Sybr Gold or ethidium), or ^{32}P.

Gene-specific guidelines using real time PCR

- Perform all reactions in duplicate or triplicate.
- Use 0.25 to 0.5 µL IP eluant per reaction.
- Annealing temp for E2F4 primers is 60°C.
- Compare IP to 1 ng, 100 pg, and 10 pg of input DNA.

General analysis guidelines:

- Normalize each test amplicon to reference amplicon (test/reference)

- Compare test/reference in IP vs. test/reference in WCE
- Enrichment is defined as the fold increase in IP (test/reference) over WCE (test/reference)

Primer sets for known E2F4 genomic targets

Rb11/p107(NM_002895)

Conventional PCR(product = 160 bp)
Left - GAGAAAAGCGGAGGCAGACTm = 63
Right - TTGTCCTCGAACATCCCTTCTm = 60

Real-time PCR(product = 65 bp)
Left - GCAGACGGTGGATGACAACATm = 59
Right - CAACCACCTGCGCCAAATm = 59

Promoter sequencechr17(-):35157700-35158000
.....CCAGCCAAGGGCCAAGGACAGGTCTTTCAGAATCTGAGGTACATC
TTCTTATCACATTTCCGGGGAGGGACTGCTAGGAGCTCCGGAGGAAAAAC
GGACTTTTTTTGAGGAGAAAAGCGGAGGCAGACGGTGGATGACAACACGT
CCCGCAGCTGCAGATTTTCGCGCGC**tttggcgc**AGGTGGTTGTGGGTAGC
GCGCCTGGGAGGGAGAAAGAAGTCGGGGGCCGTGGCGCGCAGCCCGCGGG
GCCTGAAGGGATGTTC[illegible]GCGGCGGTGG
TCGCCG.....

E2F4 target site is bold and in lower case, gene is green, amplification primers are colored red (conventional PCR) and blue (real-time PCR).

CDC25a (NM_001789)
Conventional PCR(product = 188 bp)
Left - CGCTTTCTTCTTCCCCTCTCTm = 62
Right - CACCTCTTACCCAGGCTGTCTm = 65

Real-time PCR(product = 72 bp)
Left - TCATTGGCCCAGCCTAGCTTm = 59
Right - CAAACGGAATCCACCAATCAGTm = 59

Promoter sequence chr3(-):48204760-48205135
.....TGACCTCTGCTCCCCCTCTCATTTTGATCCCCGCTCTTCTGCTC
TGGGCTCCGCCCCCTTCTGAGAGCCGATGACCTGGCAGAGTCCCGCGAGC
CGCTTTCTTCTTCCCCTCTCATTGGCCCAGCCTAGCTGCCATTCGGTTGA
GAGGAGGAGAAGTTGCTTACTGATTGGTGGATTCCG**tttagcgc**CAACTA
GGAAAGGGGGGCGGGGCAGCAGCTGGCCCCACTGAGCCGCTATTACCGCG
AAAGGCCGGCCTGGCTGCGACAGCCTGGGTAAGAGGTGTAGGTCGGCTTG
GTTTTCTGCTACCCGGAGCTGGGCAAGCGGGTGGGAGAACAGCGAAGACA
AGCGTGAGCCTGGGCCGTTGCCTCGAGGCTCTCGC.....

E2F4 target site is bold and in lower case, gene is green, amplification primers are colored red (conventional PCR) and blue (real-time PCR).

β-Actin (NM_001101)

Conventional and Real-time PCR(product = 77 bp)

Left - AGTGTGGTCCTGCGACTTCTAAGTm = 59

Right - CCTGGGCTTGAGAGGTAGAGTGTTm = 60

Promoter sequence chr7(-):5343400-5344200

.....ACCTCCAGCCACTGGACCGCTGGCCCCTGCCCTGTCCTGGGGAGT
GTGGTCCTGCGACTTCTAAGTGGCCGCAAGCCACCTGACTCCCCCAACAC
CACACTCTACCTCTCAAGCCCAGGTCTCTCCCTAGTGACCCACCCAGCAC
ATTTAGCTAGCTGAGCCCCACAGCCAGAGGTCCTCAGGCCCTGCTTTCAG
GGCAGTTGCTCTGAAGTCGGCAAGGGGGAGTGACTGCCTGGCCACTCCAT
GCCCTCCAAGAGCTCCTTCTGCAGGAGCGTACAGAACCCAGGGCCCTGGC
ACCCGTGCAGACCCTGGCCCACCCCACCTGGGCGCTCAGTGCCCAAGAGA
TGTCCACACCTAGGATGTCCCGCGGTGGGTGGGGGGCCCGAGAGACGGGC
AGGCCGGGGGCAGGCCTGGCCATGCGGGGCCGAACCGGGCACTGCCCAGC
GTGGGGCGCGGGGGCCACGGCGCGCGCCCCCAGCCCCCGGGCCCAGCACC
CCAAGGCGGCCAACGCCAAAACTCTCCCTCCTCCTCTTCCTCAATCTCGC
TCTCGCTCTTTTTTTTTTTCGCAAAAGGAGGGGAGAGGGGGTAAAAAAAT
GCTGCACTGTGCGGCGAAGCCGGTGAGTGAGCGGCGCGGGGCCAATCAGC
GTGCGCCGTTCCGAAAGTTGCCTTTTATGGCTCGAGCGGCCGCGGCGGCG
CCCTATAAAACCCAGCGGCGCGACGCGCCACCACCGCCGAGACCGCGTCC
GCCCCGCGAGCACAGAGCCTCGCCTTTGCCGATCCGCCGCCCGTCCACAC
CCGCCG.....

There are no E2F4 target sites in the promoter region. The gene sequence is green, and the amplification primers (both conventional and real-time) are colored red.

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