Agilent Mammalian ChIP-on-chip

Protocol

Version 9.0, August 2006

Agilent Technologies

Notices

Edition

Version 9.0, August 2006

Printed in USA

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Santa Clara, CA 95051

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1 Sample Preparation

The steps in this protocol and the required amount of time are listed in Table 1 on page 6.

Table 1 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 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	4 hr
Microarray hybridization	1 hr, then 40 hr
Microarray washing	1 hr

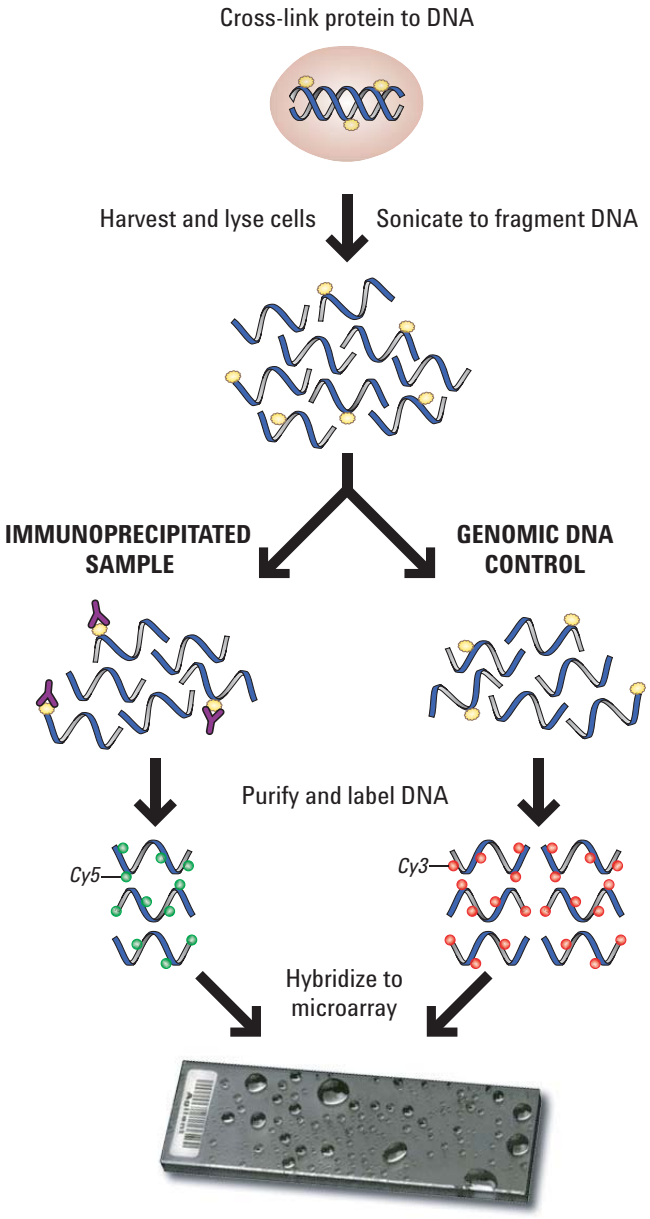

Figure 1 ChIP-on-chip overview

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

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

Adherent cells:

1. Add 1/10 of the cell culture volume of fresh 11% Formaldehyde Solution (see Table 2 on page 9) 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 twice with 5 mL 1x PBS. Harvest cells using a silicon scraper.
5. Pour the cells into the required number of 50 mL conical tubes and spin at 1,350 x 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 1x PBS per 10^8 cells. Transfer 5 x 10^7 to 1 x 10^8 cells to 15 mL conical tubes and spin at 1,350 x 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. 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 2 on page 9) 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 the cells at 1,350 x 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 1X PBS, spin at 1,350 x 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 per 10^8 cells. Transfer 5 x 10^7 to 1 x 10^8 cells to 15 mL conical tubes and spin at 1,350 x 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 Flash freeze the cells in liquid nitrogen and store the pellets at –80°C.

Table 2 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
37% Formaldehyde	14.9 mL	11%
ddH_2O	31.5 mL	

Step 2. Prepare the magnetic beads

Bind the antibody to the beads in a cold room or on ice.

1 Add 100 µL Dynal magnetic beads to a 1.5 mL microfuge tube.

   Set up 1 tube for each immunoprecipitate.

   The exact type of Dynal bead (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.

2 Add 1 mL Block Solution (see Table 3 on page 11.)

3 Gently mix the beads in Block Solution.

4 Put the tubes into a magnetic device (such as Dynal MPC-E magnet or equivalent).

5 Remove the supernatant.

6 Wash the beads 2x with 1.5 mL Block Solution:

   a Add 1.5 mL 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.

7 Resuspend the beads in 250 µL Block Solution and add 10 µg of antibody.

8 Cool the bead mixture overnight on a rotating platform at 4 °C.

9 The next day, wash the beads 3x with 1 mL Block Solution (as described in step 6 above).

10 Resuspend the beads in 100 µL Block Solution.

Table 3 Block Solution

Stock	For 100 mL	Final Concentration
10x PBS	10.0 mL	1x
BSA	500.0 mg	0.5% BSA (w/v)
ddH_2O	90.0 mL	
Total	100.0 mL	

Step 3. Lyse the cells

Add protease inhibitors (final concentration 1x) to all lysis buffers before use. (Dissolve one Complete Protease Inhibitor Cocktail Tablet (Roche) in 2 mL H_2O to make a 25x 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 4 on page 13). Rock at 4°C for 10 min. Spin at 1,350 x 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 5 on page 13). Rock gently at room temperature for 10 min. Pellet nuclei in tabletop centrifuge by spinning at 1,350 x g for 5 minutes at 4°C.
3. Discard the supernatant.
4. Resuspend each pellet in 3 mL Lysis Buffer 3 (LB3) (Table 6 on page 13).
5. Transfer cells to a 15mL 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.

   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. Split into two 1.5 mL microfuge tubes. Spin at 20,000 x g for 10 minutes at 4°C in a microfuge to pellet debris.
8. Combine supernatants from the two 1.5 mL microfuge tubes 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 4 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	0.2 mL	1 mM
50% glycerol	20.0 mL	10%
10% NP-40	5.0 mL	0.5%
10% Triton X-100	2.5 mL	0.25%
ddH_2O	64.5 mL	

Table 5 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_2O	94.7 mL	

Table 6 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% Na-Deoxycholate	1.0 mL	0.1%
20% N-lauroylsarcosine	2.5 mL	0.5%
ddH_2O	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 10 to the cell lysate from "Step 3. Lyse the cells" on page 12.
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.

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 7 on page 15). 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 TE that contains 50 mM NaCl.
6. Spin at 960 x g for 3 minutes at 4°C in a centrifuge and remove any residual TE buffer.

Do these steps at room temperature

1. Add 210 µL of elution buffer.
2. Elute 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 x g for 1 minute at room temperature.
4. Remove 200 µL of supernatant and transfer it to a new 1.5 mL microfuge tube.

5 Reverse the cross-links by incubating at 65°C overnight.

6 Thaw 50 µL of WCE reserved after sonication, add 3 volumes of 150 µL of elution buffer (Table 8), and mix. Reverse the cross-links by incubating at 65°C overnight.

Table 7 Wash Buffer (RIPA)

Stock	For 250 mL	Final Concentration
1M Hepes-KOH, pH 7.6	12.5 mL	50 mM
5M LiCl	25.0 mL	500 mM
0.5M EDTA, pH 8.0	0.5 mL	1 mM
10% NP-40	25.0 mL	1%
10% Na-Deoxycholate	17.5 mL	0.7%
ddH_2O	169.5 mL	

Table 8 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_2O	83.0 mL	

Step 6. Digest the cellular protein and RNA

1. Add 200 µL of TE to each tube of IP and WCE DNA to dilute SDS in elution buffer.
2. Add 8 µL of 10 mg/mL RNaseA (0.2 mg/mL final concentration).
3. Mix and incubate at 37°C for 2 hours.
4. Add 7 µL of $CaCl_2$ stock solution (300 mM $CaCl_2$ in 10mM Tris pH 8.0) to each sample, followed by 4 µL of 20 mg/mL proteinase K (0.2 mg/mL final concentration).
5. Mix and incubate at 55°C for 30 minutes.
6. Add 400 µL phenol:chloroform:isoamyl alcohol to each tube.
7. Mix the sample on a vortex mixer.
8. Prepare one Phase Lock Gel tube for each IP and WCE sample by spinning tube at 12,000 to 16,000 x g at room temperature for 30 seconds.
9. Add the sample to the Phase Lock tube.
10. Spin the sample in a centrifuge at 12,000 to 16,000 x 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 5M NaCl (200 mM final concentration)
    - 1.5 µL of 20 µg/µL glycogen (30 µg total)
    - 880 µL EtOH
13. Cool the mixture for 30 minutes at -80°C.
14. Spin the mixture at 20,000 x g for 10 minutes at 4°C to create DNA pellets.
15. Wash the pellets with 500 µL of 70% EtOH.
16. Dry the pellets for 10 minutes with a vacuum dessicator, 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) and dilute the WCE DNA to 100 ng/µL.

Step 7. Blunt the DNA ends and ligate the linkers

Use T4 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 ddH_2O.

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 9 Blunting Mix

Stock	1x Mix	Final Concentration*
10X NE Buffer 2	11.0 µL	1x
10 µg/µL BSA (NEB)	0.5 µL	5 µg
10mM each dNTP	1.1 µL	100 µM
3U/µL T4 DNA polymerase (NEB)	0.5 µL	1.5 U
ddH_2O	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 3 M sodium acetate and 0.5 µL of 20 µg/µL glycogen (10 µg total) to the sample. Keep on ice.

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

9 Thoroughly mix the sample by using a pipette to move the sample up and down.

10 Prepare one Phase Lock Gel tube for each IP and WCE sample by spinning the tube at 12,000 to 16,000 x g at room temperature for 30 seconds.

11 Transfer the sample to the Phase Lock Gel tube.

12 Spin in a centrifuge at 12,000 to 16,000 x g for 5 minutes at room temperature.

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

14 Add 250 µL EtOH.

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

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

17 Wash the pellets with 500 µL of 70% EtOH.

18 Dry the pellets for 10 minutes with a vacuum dessicator, such as a Savant Speed Vac, and resuspend each pellet in 25 µL H_2O. Chill on ice.

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 10 Ligase Mix

Stock	1x Mix	Final Concentration*
5x ligase buffer (Invitrogen)	10.0 µL	1x
15 µM linkers (see "To prepare linkers for LM-PCR" on page 27)	6.7 µL	2 µM
400U/µL T4 DNA ligase (NEB)	0.5 µL	200U
ddH_2O	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.

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

4 Add 6 µL of 3 M sodium acetate and 130 µL EtOH.

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

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

7 Wash the pellets with 500 µL of 70% EtOH.

8 Dry the pellets for 10 minutes in a vacuum dessicator, such as a Savant Speed Vac, and resuspend each pellet in 25 µL H_2O.

Step 8. 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 11 Mix A

Stock	1x Mix	Final Concentration*
10X Thermopol buffer (NEB)	4.00 µL	1x
dNTP mix (2.5 mM each)	5.00 µL	250 µM
oligo JW102 (40 µM)	1.25 µL	1 µM
ddH_2O	4.75 µL	
Total	15.00 µL	

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

Table 12 Mix B

Stock	1x Mix	Final Concentration*
10X Thermopol buffer (NEB)	1.0 µL	1x
Taq polymerase (5U/µL)	0.5 µL	0.25 U
ddH_2O	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 thermocycler:

a Start the program below.

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.

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:	GO TO Step 4 x 14 times	
Step 8:	72°C	5 minutes
Step 9:	4°C	HOLD

5 Transfer the product to a 1.5 mL microfuge tube and add 475 µL 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 13 PCR Mixture

Stock	1x Mix	Final Concentration*
10x Thermopol buffer (NEB)	5.00 µL	1x
dNTP mix (2.5 mM each)	5.00 µL	250 µM
oligo oJW102 (40 µM)	1.25 µL	1 µM
Taq polymerase (5U/µL)	0.25 µL	1.25 U
ddH_2O	33.50 µL	
Total	45.00 µL	

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

8 Put 45 µL of PCR mix to individual PCR tubes.

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

Step 1:	95°C	2 minutes
Step 2:	95°C	30 seconds
Step 5:	60°C	30 seconds
Step 6:	72°C	1 minute
Step 7:	GO TO Step 2 x 24 times	
Step 8:	72°C	5 minutes
Step 9:	4°C	HOLD

10 Make the precipitation mix:

Table 14 Precipitation Mix

Stock	1x Mix	Final Concentration*
7.5 M Ammonium acetate	25.0 µL	625 mM
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 microfuge tube.

12 Add 250 µL precipitation mix to each tube.

13 Cool for 30 minutes at -80°C.

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

15 Wash the pellets with 500 µL of 70% EtOH.

16 Dry the pellets for 10 minutes with a vacuum dessicator, such as a Savant Speed Vac, and resuspend each pellet in 50 µL H_2O.

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

Step 9. Label the IP and WCE

In this step, you use the random-primed, Klenow-based extension protocol that is used with Invitrogen's CGH Labeling kit. This protocol varies from the instructions provided by Invitrogen in both reaction volume and reagent concentrations (yielding 20 reactions per "30 reaction" Invitrogen kit, p/n 18095-011). A pair of reactions, one for each dye, yields enough material for 1 to 2 hybridizations. To scale up for more arrays, increase the number, not the volume, of individual reactions.

1 Open the required number of Invitrogen CGH kits and consolidate the stocks of 2.5x Random Primer Solution, 10x dUTP Nucleotide Mix, Klenow, and Stop Buffer.

2 Put the LM-PCR product into a PCR tube (0.2 to 0.5 mL) and add random primer solution and water as follows:

Table 15

Stock	1x Mix	Final Concentration*
LM-PCR product (100 ng/µL)	20.0 µL	2 µg
2.5x random primer solution	35.0 µL	1x
ddH_2O	20.0 µL	
Total	75.0 µL	

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

3 Mix on a vortex mixer for 30 seconds.

4 Place the tubes in a thermal cycler preheated to 95°C and incubate for 5 minutes.

5 Immediately transfer the tubes to an ice-water bath and cool for 5 minutes.

6 While the reactions are cooling, create the Labeling Mix (see Table 16).

Typically Cy5 mix is used for IP DNA and Cy3 for WCE DNA.

Table 16 Labeling Mix

Stock	1x Mix	Final Concentration
10X dUTP Nucleotide Mix	8.2 µL	112/56 nM
Cy5- or Cy3-dUTP (1 mM)	1.5 µL	17 µM
Klenow (40 U/µL)	1.5 µL	60 U
ddH_2O	1.8 µL	
Total	13.0 µL	

Keep the reactions in dark as much as possible to minimize degradation of Cy dyes.

7 Mix the mixture on a vortex mixer for 30 seconds.

8 Put 13 µL of the label mix in each tube. Mix by pipetting up and down multiple times.

9 Incubate for 3 hours at 37°C. Keep the samples in the dark.

10 Add 9 µL stop buffer to each tube and mix.

11 Transfer each sample to a 1.5 mL microfuge tube.

12 Clean up the samples using Invitrogen's CGH column as follows:

- **a** Add 0.4 mL of Purification Buffer A to each tube and mix with a vortex mixer for 30 seconds.
- **b** Place the Purification Column into a 2 mL collection tube.
- **c** Use a pipette to transfer the sample to the Purification Column.
- **d** Spin the column in a centrifuge at 8,000 × g for 1 minute at room temperature.
- **e** Add 0.6 mL of Purification Buffer B to the column (make sure Ethanol has been added to Buffer B).
- **f** Spin the sample in a centrifuge at 8,000 × g for 1 minute at room temperature. Discard the flow-through from the collection tube, and place the column back in the tube.
- **g** Add 0.2 mL of Purification Buffer B to the column.

h Spin the sample in a centrifuge at 8,000 × g for 1 minute at room temperature. Discard the flow-through.

i Place the Purification Column in a new, sterile 1.5-mL collection tube.

j Add 50 µL of sterile water and incubate at room temperature for 1 minute.

k Spin the sample in a centrifuge at 8,000 × g for 1 minute at room temperature. The flow-through contains the purified labeled DNA sample.

13 Measure Cy label and total DNA yield using the NanoDrop.

Expect >3.5 pmol/µL Cy3 label incorporation, >2.5 pmol/µL Cy5 label incorporation, and >5 µg total DNA per reaction (approximately 100 ng/µL).

To prepare linkers for LM-PCR

1 Mix the following

250 µL	Tris-HCl (1M) pH 7.9
375 µL	oligo JW102 (40 µM stock)
375 µL	oligo JW103 (40 µM stock)

oligo JW102 5'-GCGGTGACCCGGGAGATCTGAATTC-3'

oligo JW103 5'-GAATTCAGATC-3'

Order these oligos desiccated, then resuspend in ddH_20 to 40 µM.

2 Put 100 µL of the mixture into PCR tubes.

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

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

4 Store the linkers at -20°C.

2
Hybridization and Wash

In this section, you hybridize and wash the microarrays.

Step 1. Hybridize the microarray

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

This step is an abbreviated version of the Agilent aCGH array hybridization protocol. Please refer to aCGH protocol for details, notes, and warnings.

1 Combine Cy5- and Cy3-labeled samples with ddH_2O in a 1.5 mL microfuge tube for a total volume as indicated in Figure 17.

Table 17

	4x44K Array Format	1x244K Array Format
Cy5-labeled samples	2.5 µg to 5 µg	5 µg
Cy3-labeled samples	2.5 µg to 5 µg	5 µg
Total volume with ddH_2O	37.5 µL	150 µL

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

Table 18

Stock	4x44K Array Format	1x244 Array Format	Final Concentration
Human Cot-1 DNA (1.0 mg/mL)	12.5 µL	50 µL	0.1 mg/mL
Agilent Blocking Agent (10x)*	12.5 µL	50 µL	1x
Agilent Hybrdization Buffer (2x)*	62.5 µL	250 µL	1x

* Supplied in the Agilent Oligo aCGH/ChIP-on-chip Hybridization Kit

3 Mix contents and quick spin to collect.

4 Heat samples for 3 minutes at 95°C.

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

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

7 Load a clean gasket slide into the Agilent SureHyb chamber base with the label facing up and aligned with the rectangular section of the chamber base. Ensure that the gasket slide is flush with the chamber base and is not misaligned.

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

8 Slowly dispense hybridization sample onto the gasket well in a “drag and dispense” manner in this amount:

4x44K format	110 µL/array (4 individual samples of 110 µL each)
1x244K format	490 µL

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

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

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

12 Place assembled slide chamber in rotisserie hybridization oven set to 65°C. Hybridize at 10 rpm for 40 hours.

   You can use a higher rotation speed (up to 20 rpm) to enhance the overall assay signal intensity.

Step 2. Wash the microarray

NOTE

Cyanine 5 is susceptible to degradation by ozone. The following procedure is strongly recommended if the ozone levels exceed 5 ppb in your laboratory. For more information, visit www.agilent.com/chem/dnatechnicalnotes to download our technical note on "Improving Microarray Results by Preventing Ozone-Mediated Fluorescent Signal Degradation" (publication 5989-0875EN).

NOTE

Fresh Oligo aCGH/ChIP-on-chip Wash Buffers 1 and 2 should be used for each wash group (up to five slides). The acetonitrile and the Stabilization and Drying Solution may be reused for washing of up to four groups of slides (that is, a total of 20 slides).

WARNING

The Stabilization and Drying Solution must be set-up in a fume hood. For practical reasons, Oligo aCGH/ChIP-on-chip Wash Buffers 1 and 2 set-up areas should be placed close to, or preferably in, the same fume hood. Gloves and eye/face protection should be used in every step of the warming procedure.

Table 19 lists the wash conditions to wash the microarray.

Table 19 Wash Conditions

	Dish	Wash Buffer	Temperature	Time
Disassembly	1	Oligo aCGH/ ChIP-on-chip Wash Buffer 1	Room temperature	
1st wash	2	Oligo aCGH/ ChIP-on-chip Wash Buffer 1	Room temperature	5 minutes
2nd wash	3	Oligo aCGH/ ChIP-on-chip Wash Buffer 2	31°C	5 minutes
3rd wash	4	Acetonitrile	Room temperature	1 minute
4th wash	5	Stabilization and Drying Solution	Room temperature	30 seconds

1. Completely fill slide-staining dish #1 with Oligo aCGH/ChIP-on-chip Wash Buffer 1 at room temperature.
2. Place a slide rack into slide-staining dish #2. Add a magnetic stir bar. Fill slide-staining dish #2 with enough Oligo aCGH/ChIP-on-chip Wash Buffer 1 at room temperature to cover the slide rack. Place this dish on a magnetic stir plate.
3. Fill slide-staining dish #3 approximately three-fourths full with Oligo aCGH/ChIP-on-chip Wash Buffer 2 (prewarmed to 31°C). Add a magnetic stir bar and place this dish on a magnetic stir plate with heating element. Adjust heating element to maintain wash buffer temperature at 31°C; monitor using a thermometer.
4. Fill slide-staining dish #4 approximately three-fourths full with acetonitrile. Add a magnetic stir bar and place this dish on a magnetic stir plate.
5. Fill slide-staining dish #5 approximately three-fourths full with Stabilization and Drying Solution. Add a magnetic stir bar and place this dish on a magnetic stir plate.
6. Remove one hybridization chamber from incubator and record time. Record whether bubbles formed during hybridization, and if all bubbles are rotating freely.
7. Prepare the hybridization chamber disassembly.
   a. Place the hybridization chamber assembly on a flat surface and loosen the thumbscrew, turning counterclockwise.
   b. Slide off the clamp assembly and remove the chamber cover.
   c. With gloved fingers, remove the microarray-gasket sandwich from the chamber base by grabbing the slides from their ends. Keep the microarray slide numeric barcode facing up as you quickly transfer the sandwich to slide-staining dish #1.
   d. Without letting go of the slides, submerge the microarray-gasket sandwich into slide-staining dish #1 containing Oligo aCGH/ChIP-on-chip Wash Buffer 1.

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

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

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

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

   d Remove the microarray slide and place into slide rack in the slide-staining dish #2 containing Oligo aCGH/ChIP-on-chip Wash Buffer 1 at room temperature. Minimize exposure of the slide to air.

CAUTION Touch only the barcode portion of the microarray slide or its edges!

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

10 When all slides in the group are placed into the slide rack in slide-staining dish #2, stir using setting 4 (or 250 to 300 rpm for digital stir plates) for 5 minutes.

11 Transfer slide rack to slide-staining dish #3 containing Oligo aCGH/ChIP-on-chip Wash Buffer 2 at 31°C, and stir using setting 4 (or 250 to 300 rpm for digital stir plates) for 5 minutes.

12 Transfer slide rack to slide-staining dish #4 filled with Acetonitrile, and stir using setting 4 (250 to 300 rpm for digital stir plates) for 1 minute.

13 Immediately transfer the slide rack to slide-staining dish #5 containing Stabilization and Drying Solution, and stir using setting 4 (or 250 to 300 rpm for digital stir plates) for 30 seconds.

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

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

NOTE

The acetonitrile and the Stabilization and Drying Solution may be reused for washing of up to four groups of slides (that is, a total of 20 microarray slides). After each use, rinse the slide rack and the slide-staining dish that were in contact with the Stabilization and Drying Solution with acetonitrile followed by a rinse in Milli-Q water.

16 Repeat step 1 through step 15 for the next group of five slides using fresh Oligo aCGH/ChIP-on-chip Wash Buffer 1 and Oligo aCGH/ChIP-on-chip Wash Buffer 2 prewarmed to 31°C.

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

18 Dispose of acetonitrile and Stabilization and Drying Solution as a flammable solvents.

3
Scanning and Feature Extraction

This chapter describes the tasks for scanning the microarrays and analyzing with Feature Extraction Software.

Step 1. Scan the slides

Agilent Scanner Settings

1 Assemble the slides into an appropriate slide holder, either version B or A. Slides should be placed into slide holder such that when the holders containing the slide are oriented into the scanner carousel the numeric barcode is visible (*not* the "Agilent"-labeled barcode).

2 Place assembled slide holders into scanner carousel.

3 Verify scan settings for two-color scans.

Table 20 Scan Settings

	For 1x22K, 1x44K Formats	For 1x244K, 2x105K Formats	For 4x44K Formats
Scan region	Scan Area (61 x 21.6 mm)	Scan Area (61 x 21.6 mm)	Scan Area (61 x 21.6 mm)
Scan resolution (µm)	10	5	5
5µm scanning mode		Singe Pass	Singe Pass
eXtended Dynamic range			(selected)
Dye channel	Red&Green	Red&Green	Red&Green
Green PMT	100%	100%	XDR Hi 100% XDR Lo 10%
Red PMT	100%	100%	XDR Hi 100% XDR Lo 10%

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

4 Select settings for the automatic file naming

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

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

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

Gene Pix scanner settings

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

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

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

Step 2. Extract data using Agilent Feature Extraction Software

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

Feature Extraction (FE) 9.1 supports extraction of two-color tif images of Agilent microarrays scanned on Agilent Scanner or GenePix (Axon/Molecular Devices) scanner.

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

1 Open the Agilent Feature Extraction (FE) software version 9.1.

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

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

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

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

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

   - For auto assignment of the grid template, the image must be generated from an Agilent scanner or GenePix (4000A or 4000B) and have an Agilent barcode.

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

3 Set FE Project Properties.

   a Select the **Project Properties** tab.

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

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

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

⊟ General	
Operator	Unknown
⊟ Input	
Number of Extraction Sets Included	0
⊟ Output Folder	
Same As Image	True
Results Folder	
⊟ Outputs	
GEML	
⊞ MAGE	
JPEG	
TEXT	✔
Visual Results	✔
Grid	✔
QC Report	✔
⊟ Automatic Protocol Assignment	
Highest Priority Default Protocol	Grid Template Default
Project Default Protocol	
⊟ Automatic Grid Template Assignment	
Use Grid file if available	False
⊟ Other	
External DyeNorm List File	
Overwrite Previous Results	False
⊞ Send FTP Results	False

Figure 2 Default settings

4 Check the Extraction Set Configuration.

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

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

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

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

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

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

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

NOTE If scans are done with an Agilent scanner in XDR mode, the High and Low images are automatically combined when imported into the Feature Extraction software version 9.1. Images are not combined with non-Agilent scanned images.

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

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

7 Select **Project > Start Extracting**.

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

Refer to the application note on *Use of Agilent Feature Extraction Software (v8.1) QC Report to Evaluate Microarray Performance* (publication 5989-3056EN) for more details on quality assessment and troubleshooting with the Feature Extraction QC Report. This technical note can be downloaded from the Agilent Web site at www.agilent.com/chem/dnaapplications.

4
Reference

Reagents, Enzymes, and Buffers

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

Table 21 Cross-linking

Item	Size	Vendor	Catalog #	Stock Concentration
Formaldehyde	12 x 500 mL	J.T. Baker	2106-01	37%
Glycine	various	Sigma	G7126	n/a

Table 22 Immunoprecipitation

Item	Size	Vendor	Catalog #	Stock Concentration
Magnetic Beads	various	Dynal	Various	
Antibody		Various		
BSA, powder	50 g	Sigma	A7906-50G	0.5% (in PBS)
PBS	10X	Invitrogen	70013-032	1X

Table 23 Cell Sonication, Reverse Cross-linking and DNA Clean-up

Item	Size	Vendor	Catalog #	Stock Concentration
Protease Inhibitor (Complete Tablets)	20 tablets	Roche	1-697-498	50X
RNase A	10 mL	Invitrogen	12091-021	10 mg/mL
Proteinase K	5 mL	Invitrogen	25530-049	20 mg/mL
Sonicator machine	n/a	Various (see protocol)		n/a
Magnetic stand (MPC-S)	n/a	Dynal	120.20	n/a

Table 24 DNA Amplification (LM-PCR)

Item	Size	Vendor	Catalog #	Stock Concentration
T4 DNA Polymerase	150 or 750 units	NEB	M0203S or M0203L	3U/µL
T4 DNA Polymerase buffer		NEB	Supplied w/ enzyme	10X
BSA	25 mg	NEB	B9001S and supplied w/Pol	10 mg/mL
dNTP	4 x 100 mM	Amersham Biosciences	27-2035-01	10 mM and 2.5 mM
T4 DNA Ligase	20,000 or 100,000 units	NEB	M0202S or M0202L	400 U/µL
T4 DNA Ligase buffer	2 x 1 mL	Invitrogen	46300-018	5X
Linkers, see "To prepare linkers for LM-PCR" on page 27.				
Taq Polymerase	1000 units/tube	Applied Biosystems	N808-0156	5 U/µL
ThermoPol buffer	4 x 1.5 mL	NEB	B9004S	10X
oligo JW102, see "To prepare linkers for LM-PCR" on page 27				
oligo JW103, see "To prepare linkers for LM-PCR" on page 27				

Table 25 Cy Labeling, Hybridization and Washing

Item	Size	Vendor	Catalog #	Stock Concentration
CGH Labeling Kit	30 reactions	Invitrogen	18095-011	
Cy5-dUTP	~35 µL	Perkin Elmer	NEL579	1 mM
Cy3-dUTP	~35 µL	Perkin Elmer	NEL578	1 mM
Oligo aCGH/ChIP-on-chip Hybridization Kit	25 reactions	Agilent	5188-5220	
Human Cot-1 DNA	500 µg	Invitrogen	15279-011	
Oligo aCGH/ChIP-on-chip Wash Buffer 1	4 L	Agilent	5188-5221	
Oligo aCGH/ChIP-on-chip Wash Buffer 2	4 L	Agilent	5188-5222	
Acetonitrile	1 L or 4 L	J.T. Baker	9017-02 or 9017-03	n/a
Stabilization & Drying Solution	500 mL	Agilent	5185-5979	n/a

Table 26 General Use Buffers and Reagents

Item	Size	Vendor	Catalog #	Stock Concentration
Hepes-KOH, pH 7.5				1 M
NaCl	1 kg	Sigma	S7653	5 M
EDTA, pH 8.0	4 x 100 mL	Invitrogen	15575-038	0.5 M
EGTA, pH 8.0	100 g	Sigma	E3889	0.5 M
Glycerol	500 mL	Invitrogen	15514-011	50%
Nonidet P-40	100 mL	Sigma	I8896	10%
Triton X-100	250 mL	Sigma	T8787	10%
Tris-HCl, pH 8.0	1 L	Invitrogen	15568-025	1 M
Sodium Deoxycholate	100 g	Sigma	D6750	10%
N-Lauroylsarcosine	25 g	Sigma	61743(f)	20%
SDS	4 x 100 mL	Invitrogen	15553-027	10%
LiCl	500 g	Sigma	L4408	5 M
Glycogen	1 mL	Roche	901-393	20 mg/mL
Phenol/Chloroform/ Isoamyl Alcohol	100 mL	Fluka	77617	n/a
Heavy Phase Lock Gel Tubes	200 tubes	Eppendorf	955154045 (North America only), or 0032 005.152 (outside of North America)	2 mL size
Sodium Acetate	6 x 100 mL	Sigma	S7899	3 M
Ammonium Acetate	100 mL	Sigma	A2706	7.5 M

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
- 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 x 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 1X 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 run out on an agarose gel after cross-link reversal and DNA isolation (step 6). Fragments from the sonicated material should range from approximately 100 bp to approximately 1 kb.

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.

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 tested 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 when performing 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 Invitrogen's BioPrime Array CGH Genomic Labeling System and cyanine dyes with a slightly modified version of the Invitrogen protocol.
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 octamers to the DNA.
4. Extend octamers using high concentration Klenow enzyme and fluorescent-labeled nucleotides.
5. Purify labeled DNA using the purification columns supplied with the CGH labeling system.

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 10 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.

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 60C.
- 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 -   GAGAAAAGCGGAGGCAGAC              Tm = 63
Right -  TTGTCCTCGAACATCCCTTC             Tm = 60

Real-time PCR     (product = 65 bp)
Left -   GCAGACGGTGGATGACAACA             Tm = 59
Right -  CAACCACCTGCGCCAAA                Tm = 59
```

```
Promoter sequence chr17(-):35157700-35158000
.....CCAGCCAAGGGCCAAGGACAGGTCTTTCAGAATCTGAGGTACATC
TTCTTATCACATTTCCGGGGAGGGACTGCTAGGAGCTCCGGAGGAAAAAC
GGACTTTTTTTGAGGAGAAAAGCGGAGGCAGACGGTGGATGACAACACGT
CCCGCAGCTGCAGATTTTCGCGCGCtttggcgcAGGTGGTTGTGGGTAGC
GCGCCTGGGAGGGAGAAAGAAGTCGGGGGCCGTGGCGCGCAGCCCGCGGG
GCCTGAAGGGATGTTCGAGGACAAGCCCCACGCTGAGGGGGCGGCGGTGG
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 - CGCTTTCTTCTTCCCCTCTC Tm = 62

Right - CACCTCTTACCCAGGCTGTC Tm = 65

Real-time PCR (product = 72 bp)

Left - TCATTGGCCCAGCCTAGCT Tm = 59

Right - CAAACGGAATCCACCAATCAG Tm = 59

Promoter sequence chr3(-):48204760-48205135

.....TGACCTCTGCTCCCCCTCTCATTTTGATCCCCGCTCTTCTGCTC
TGGGCTCCGCCCCCTTCTGAGAGCCGATGACCTGGCAGAGTCCCGCGAGC
CGCTTTCTTCTTCCCCTCTCATTGGCCCAGCCTAGCTGCCATTCGGTTGA
GAGGAGGAGAAGTTGCTTACTGATTGGTGGATTCCG**tttg**gcgcCAACTA
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 - AGTGTGGTCCTGCGACTTCTAAG Tm = 59
Right - CCTGGGCTTGAGAGGTAGAGTGT Tm = 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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Version 9.0, August 2006

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